Centre-Eau Terre Environnement

STRATÉGIES DE FERMENTATION MICROBIENNE POUR LA PRODUCTION D'ENZYMES PAR LA VALORISATION DE MILIEUX RÉSIDUELS DESTINÉS AUX PRODUITS DE NETTOYAGE BIOSOURCÉS

MICROBIAL FERMENTATION STRATEGIES FOR ENZYME PRODUCTION VIA THE VALORIZATION OF RESIDUAL MEDIA FOR BIO-BASED CLEANING PRODUCTS

Par

Vu Mai Linh Nguyen

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Jury d'évaluation

Examinateur interne et Président de Jury	Eric Charles Peterson Institut National de la Recherche Scientifique (INRS)
Examinateur externe	Tagnon Missihoun Université du Québec à Trois-Rivières (UQTR)
Examinateur externe	Thi Thanh Ha Pham Université du Québec à Trois-Rivières (UQTR)
Directeur de recherche	Prof. Kokou Adjallé Institut National de la Recherche Scientifique (INRS)
Codirecteur de recherche	Prof. Jean-François Blais Institut National de la Recherche Scientifique (INRS)

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Vu-Mai-Linh Nguyen

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FOREWORD

This manuscript presents the research conducted in fulfillment of the requirements for the degree of Philosophiae Doctor (Ph.D.). The work falls within the domain of Biotechnology and is part of the doctoral program in Water Sciences at the National Institute of Scientific Research (INRS), Canada.

The core objective of this research was to develop cost-effective microbial fermentation strategies for enzyme production using various organic wastewaters as alternative substrates. These enzymes—protease, α-amylase, and lipase—were ultimately intended for use in bio-based detergent formulations, contributing to both waste valorization and sustainable product development.

This thesis is organized into seven chapters, four of which are presented in the form of scientific articles. The chapters are arranged to reflect the logical progression of the research and are interconnected to ensure narrative coherence.

- Chapter 1 provides a general introduction to the topic. It begins by outlining the background and current landscape of enzyme-based detergents and bioeconomy approaches. It then presents the research problem, underlying hypotheses, and specific objectives of the study.
- Chapter 2 describes the overarching methodology employed across all experimental stages. It details the strategies used for substrate screening, microbial selection, and process optimization.
- Chapter 3 (First article) offers a comprehensive review of *Bacillus* species and their growing role in enzyme and biosurfactant production for bio-detergent applications. These microbial agents, often regarded as "green chemicals," are central to advancing environmentally friendly cleaning solutions. The article emphasizes the advantages of *Bacillus* over other microbial genera, particularly in their robustness and enzyme yields, and discusses recent developments that enhance their utility in sustainable formulations.

- Chapter 4 (Second article) investigates the feasibility of using industrial wastewaters from Québec, Canada—including beverage wastewater (BW), food industry wastewater (FIW), starch industry wastewater (SIW), and pulp and paper mill sludge (PPMS)—as fermentation media for enzyme production. Commercial strains of *Bacillus licheniformis*, *B. amyloliquefaciens*, and *B. megaterium* were employed. The study identified SIW and PPMS as optimal for amylase and protease production, respectively. Following optimization via a Box–Behnken design, scale-up experiments in 5 L bioreactors confirmed high enzyme yields. These findings demonstrate the potential of wastewater valorization within a circular bioeconomy framework.
- Chapter 5 (Third article) focuses on the isolation and characterization of *Bacillus tropicus* P4 from PPMS, which showed exceptional protease production. The study optimized process conditions using minimal inputs and successfully scaled the fermentation to both 5 L and 150 L bioreactors. The use of Tween 80 as an inducer enhanced yield, and high productivity was maintained across scales, affirming the strain's industrial potential. This work underscores the practicality of low-cost, high-impact bioprocessing for enzyme production from waste.
- Chapter 6 (Fourth article) explores the use of *Acinetobacter tandoii* L3, isolated from PPMS, for lipase production. Using minimally treated secondary sludge as substrate, lipase activity was evaluated in shake flasks and scaled up to 150 L. Notably, enzyme production in the large-scale system was rapid and sustained over time, likely due to enhanced oxygen transfer. These results reveal the untapped potential of native strains for robust, scalable enzyme production in waste-based bioprocesses.
- Chapter 7 offers a general discussion that synthesizes the findings from all stages of the work. It evaluates the significance of the research in the context of industrial biotechnology and circular economy strategies and concludes with recommendations for future research directions.

RÉSUMÉ

Les enzymes hydrolytiques telles que les protéases, α-amylases et lipases d'origine microbienne représentent la plus grande part du marché mondial des enzymes, dont la majorité est consommée par l'industrie des détergents. Introduites pour la première fois dans les formulations de détergents il y a plus d'un siècle, les enzymes sont passées du statut d'additifs mineurs à celui de composants essentiels grâce à leur spécificité élevée et leur biodégradabilité. Toutefois, leur adoption à grande échelle, notamment dans les pays en développement, reste limitée en raison du coût élevé de production — principalement lié au prix des matières premières.

Afin de répondre à cette contrainte économique, la présente étude visait à développer des stratégies efficaces et économiques de production enzymatique en valorisant des résidus industriels fermentescibles à coût nul ou négatif. Les objectifs étaient doubles : (i) identifier des effluents industriels et agroalimentaires capables de soutenir la croissance microbienne et la biosynthèse enzymatique, et (ii) évaluer des souches microbiennes commerciales et indigènes pour leur potentiel de production d'enzymes d'intérêt pour la formulation de détergents.

Quatre effluents industriels ont été sélectionnés comme substrats modèles : les eaux usées de l'industrie de l'amidon (SIW), de l'industrie des boissons (BW), les boues de l'industrie papetière (PPMS) et les eaux usées de l'industrie alimentaire (FIW). Leur performance a été évaluée avec trois souches commerciales de *Bacillus* (*B. licheniformis*, *B. megaterium* et *B. amyloliquefaciens*). Le SIW et le PPMS se sont révélés les plus prometteurs, chacun soutenant la production enzymatique par des mécanismes nutritionnels distincts — richesse en sucres pour le SIW, richesse minérale pour le PPMS. À l'inverse, le BW a souffert d'une carence en azote, et le FIW s'est avéré inadapté en raison d'un déséquilibre nutritionnel extrême.

Pour affiner les conditions de production, une méthodologie de surface de réponse (RSM) a été appliquée pour optimiser la température, les solides totaux et la taille de l'inoculum. Chez *B. megaterium* cultivé sur PPMS, la production de protéase a été influencée positivement par les solides totaux et l'inoculum, tandis que la température n'a eu aucun effet significatif. L'ajout de 1 % de Tween 20 pour induire la lipase a modifié la réponse du modèle, rendant les effets des

variables négligeables — soulignant ainsi le rôle essentiel des surfactants dans l'activation sélective des voies métaboliques. En revanche, pour *B. amyloliquefaciens* cultivé sur SIW, les trois variables et leurs interactions ont significativement influencé la production d'α-amylase. Des essais complémentaires ont montré que le SIW pouvait tolérer une concentration en solides de 40 g/L, ce qui a permis d'augmenter le rendement enzymatique.

En complément des souches commerciales, l'étude a exploré le microbiote indigène du PPMS. Le criblage sur milieux sélectifs a permis d'isoler *Bacillus tropicus* P4 et *Acinetobacter tandoii* L3, présentant respectivement une activité protéasique et lipasique remarquables. Ces souches ont démontré une bonne tolérance aux conditions environnementales difficiles et un fort potentiel enzymatique, les positionnant comme des candidats prometteurs pour la formulation de détergents. Notamment, cette étude constitue la première mention de production de lipase par *A. tandoii*.

Afin d'évaluer la faisabilité à grande échelle, des essais en bioréacteur ont été menés à l'échelle de 5 L et 150 L. Chez *B. amyloliquefaciens*, la production d'α-amylase dans des bioréacteurs de 5 L a atteint 5,74 U/mL à 48 h dans des conditions optimisées, confirmant la robustesse du procédé. Chez *B. tropicus* P4, les titres de protéase ont dépassé 840 U/L dans le bioréacteur de 150 L et sont restés stables au-delà de la phase exponentielle — validant ainsi la possibilité d'un procédé de production enzymatique à grande échelle à partir d'eaux usées faiblement traitées.

En conclusion, cette étude démontre qu'une approche combinant la valorisation des substrats, la sélection de souches performantes et l'optimisation des procédés permet une production d'enzymes à faible coût, durable et adaptée à l'échelle industrielle. Le succès du passage à l'échelle confirme la pertinence industrielle de cette stratégie et son potentiel pour la formulation de détergents écologiques et d'autres applications biotechnologiques environnementales.

Mots-clés : enzymes, protéase, α-amylase, lipase, biodétergents, *Bacillus* sp., changement d'échelle

ABSTRACT

Hydrolytic enzymes such as proteases, α-amylases, and lipases derived from microbial sources constitute the largest segment of the global enzyme market, with the detergent industry accounting for the majority of their consumption. First introduced into detergent formulations over a century ago, enzymes have evolved from minor additives to essential components due to their high specificity and biodegradability. However, their widespread adoption in bio-detergents, particularly in developing countries, remains constrained by high production costs—primarily driven by the expense of raw materials.

To address this economic bottleneck, the present study sought to develop effective, low-cost enzyme production strategies by valorizing fermentable industrial residues with minimal or negative cost. The dual objectives were (i) to identify residual industrial and agri-food wastewaters capable of supporting microbial growth and enzyme biosynthesis and (ii) to evaluate both commercial and indigenous microbial strains for their enzyme-producing potential relevant to detergent applications.

Four industrial wastewaters were selected as model substrates: starch industry wastewater (SIW), beverage wastewater (BW), pulp and paper mill sludge (PPMS), and food industry wastewater (FIW). These were evaluated using three commercial *Bacillus* strains (*B. licheniformis*, *B. megaterium*, and *B. amyloliquefaciens*). Among these, SIW and PPMS emerged as the most promising, each supporting enzyme production through distinct nutrient mechanisms—carbohydrate availability in SIW and mineral enrichment in PPMS. In contrast, BW suffered from nitrogen limitation, and FIW proved unsuitable due to extreme nutrient imbalance.

To further refine production conditions, response surface methodology (RSM) was employed to optimize temperature, total solids, and inoculum size. For *B. megaterium* on PPMS, protease production was significantly influenced by total solids and inoculum size, while temperature had no notable effect. The introduction of 1% Tween 20 to induce lipase shifted this dynamic—rendering all parameters non-significant and underscoring the role of surfactants in selectively activating enzymatic pathways. In contrast, for *B. amyloliquefaciens* cultivated on SIW, all variables

and their interactions significantly impacted α-amylase production. Further trials showed that SIW tolerated increased solids loading up to 40 g/L, enhancing enzyme yield.

In addition to commercial strains, the study investigated the native microbial community within PPMS. Screening on selective media led to the isolation of *Bacillus tropicus* P4 and *Acinetobacter tandoii* L3, which exhibited exceptional protease and lipase activities, respectively. These strains demonstrated tolerance to harsh conditions and significant enzyme productivity, positioning them as promising candidates for detergent applications. Notably, *A. tandoii* L3 represents the first reported case of lipase production by this species.

To assess scalability, bioreactor trials were conducted at both 5 L and 150 L scales. For *B. amyloliquefaciens*, α-amylase production in 5 L bioreactors peaked at 5.74 U/mL at 48 h under optimized conditions, demonstrating high reproducibility and process robustness. For *B. tropicus* P4, protease titers exceeded 840 U/L in the 150 L reactor and remained stable beyond the exponential phase—highlighting the feasibility of large-scale enzyme production using indigenous strains and minimally treated wastewater.

In conclusion, this study demonstrates that a combined strategy of substrate valorization, microbial selection, and process optimization can enable low-cost and sustainable enzyme production. The successful scale-up of key enzyme processes underscores the industrial relevance of this approach and its potential contribution to environmentally friendly detergent formulations and broader biotechnological application.

Keywords: enzymes, protease, α-amylase, lipase, bio-detergents, *Bacillus* sp., scale-up

SOMMAIRE RÉCAPITULATIF

Ce manuscrit présente les travaux réalisés dans le cadre du doctorat en sciences de l'eau à l'Institut national de la recherche scientifique (INRS), Canada. Ces travaux s'inscrivent dans le domaine de la biotechnologie environnementale.

I. Introduction

L'essor des approches de bioéconomie circulaire et l'intérêt croissant pour l'exploitation des eaux usées industrielles ont entrainé une dynamique vers la production de bioproduits à valeur ajoutée notamment d'enzymes destinées aux détergents biologiques. Cet axe de recherche d'intérêt scientifique et économique nécessite une stratégie rigoureuse et structurée. Cette étude s'est basée sur une caractérisation physico-chimique exhaustive de différents résidus fermentescibles pouvant supporter des microorganismes de type Bacillus présents dans les banques de référence telles que ATCC. Ceci a permis une évaluation rapide et fiable de la capacité de différentes eaux usées à soutenir la croissance microbienne et la biosynthèse enzymatique. Dans les phases ultérieures, les résidus fermentescibles présentant le plus grand potentiel ont permis d'étudier la performance de production enzymatique des souches indigènes comparativement aux microorganismes présents dans les banques de référence.

II. Objectif du projet

Cette étude vise à évaluer le potentiel de différentes eaux usées industrielles pour la production d'enzymes notamment la protéase, l'α-amylase et la lipase en utilisant divers types d'eaux usées d'origine industrielle comme substrats économiques et durables. L'objectif général est de développer un procédé de production d'enzymes à formuler dans la production pilote de produits nettoyants de surface.

Le projet est divisé en deux étapes principales, chacune comportant des objectifs spécifiques:

❖ **Étape 1 : Criblage et optimisation à l'aide de souches commerciales**

- **Objectif 1 :** Sélectionner, sur la base d'une revue de la littérature, les types d'eaux usées industrielles pertinentes et des souches de *Bacillus* présentes dans les banques de référence comme candidats à la fermentation pour la production d'enzymes (protéase, amylase et lipase).

- **Objectif 2 :** Identifier les substrats d'eaux usées les plus prometteurs ainsi que les combinaisons souche–substrat potentielles en évaluant la croissance microbienne (densité cellulaire) et les activités enzymatiques extracellulaires des souches sélectionnées.
- **Objectif 3 :** Optimiser les principaux paramètres de fermentation (taille de l'inoculum, température, solides totaux) à l'échelle de laboratoire, puis évaluer la reproductibilité du procédé en bioréacteur de 5 L.

❖ **Étape 2 : Isolement de souches indigènes et fermentation à l'échelle pilote**

- **Objectif 4:** Isoler des souches indigène productrices de protéase à partir des eaux usées sélectionnées et réaliser des fermentations en bioréacteurs de 5 et 150 L.
- **Objectif 5 :** Isoler des souches indigènes productrices de lipase à partir des eaux usées sélectionnées et réaliser des fermentations en bioréacteurs de 5 et 150 L.

III. Chapitre 1. Introduction générale

Les détergents sont devenus essentiels pour l'hygiène personnelle et industrielle, et leur consommation mondiale augmente fortement, surtout depuis la pandémie de COVID-19. Au début, les détergents étaient des savons naturels, faits de graisses végétales ou animales. Ils étaient biodégradables et respectueux de l'environnement (Babajanzadeh et al., 2019; Mousavi & Khodadoost, 2019; Yangxin et al., 2008). Avec le temps, et pour répondre aux besoins de santé publique et de l'industrie, les détergents sont devenus plus complexes. Les détergents modernes contiennent un ou plusieurs agents nettoyants (tensioactifs) et de nombreux additifs pour mieux nettoyer. Ces additifs incluent des agents pour aider le nettoyage (phosphates, zéolites), des agents de blanchiment, des parfums, des enzymes, et des colorants. Ils sont fabriqués par différents procédés comme le mélange ou la production de liquides (IUPAC, 1997). La composition exacte de la plupart des détergents commerciaux est souvent secrète. Les détergents sont classés selon les agents qu'ils contiennent (avec ou sans phosphate) ou selon le type de tensioactif (anionique, cationique, non ionique, etc.) (Bajpai & Tyagi, 2007). Le LAS (sulfonate d'alkylbenzène linéaire), un tensioactif anionique, est le plus utilisé car il est efficace et peu coûteux (Yangxin et al., 2008). Même si chaque ingrédient aide à nettoyer, les tensioactifs et les agents de nettoyage synthétiques, surtout en grande quantité, posent de sérieux problèmes écologiques (Yangxin et al., 2008). Le lavage consomme beaucoup d'eau et produit des eaux usées

pleines de résidus de détergents. Ces substances finissent souvent dans l'eau ou le sol, causant des problèmes comme l'eutrophisation (trop d'algues), la mousse, le manque d'oxygène, la toxicité pour la vie aquatique et les plantes, et des problèmes pour le traitement des eaux usées (Mousavi & Khodadoost, 2019). Les détergents à base de phosphates sont connus pour causer l'eutrophisation, ce qui a mené à des lois restrictives dans de nombreux pays et au développement d'alternatives sans phosphate. Le LAS, malgré sa nature facilement biodégradable, peut nuire aux microbes importants pour le traitement des eaux usées (Stamatelatou et al., 2011). Ces problèmes montrent les limites des détergents classiques (Helmy et al., 2020). Historiquement, les préoccupations environnementales se concentraient sur les résidus de détergents. Cependant, on s'intéresse maintenant aussi à l'impact de la production des tensioactifs synthétiques. La plupart sont fabriqués à partir de pétrole, ce qui demande beaucoup d'énergie, émet des gaz à effet de serre et épuise les ressources. L'extraction et le raffinage du pétrole libèrent du CO_2 et peuvent causer des déversements. De plus, la fabrication de tensioactifs peut produire des substances dangereuses et dépend de ressources non renouvelables, ce qui soulève des questions de durabilité. Le marché mondial des détergents est en forte croissance (133-140 milliards USD en 2023, et plus de 180 milliards USD d'ici 2030), ce qui rend encore plus urgente la recherche de solutions durables (Giagnorio et al., 2017).

Les détergents contenant des enzymes ou d'origine biologique sont des alternatives prometteuses. Ils permettent un nettoyage efficace à des températures plus basses et avec moins de produit (Hasan et al., 2010). L'ajout d'enzymes dans les détergents améliore le lavage tout en réduisant la quantité de produit nécessaire et la consommation d'énergie (Hasan et al., 2010). De plus, cela diminue la dépendance aux phosphates et aux agents de blanchiment qui sont nocifs pour l'environnement (Bajpai & Tyagi, 2007). Les enzymes sont biodégradables et proviennent de sources microbiennes renouvelables, ce qui réduit leur impact environnemental. Elles aident aussi à protéger les tissus pendant le lavage (Gurkok, 2019). Malgré ces avantages, l'utilisation des enzymes dans les détergents commerciaux est limitée par leur coût de production élevé, leur stabilité parfois faible dans des conditions de lavage difficiles, et la nécessité qu'elles soient compatibles avec les autres ingrédients. Cependant, grâce aux progrès de la fermentation

microbienne et de l'ingénierie génétique, la production d'enzymes devient plus efficace et moins chère, favorisant ainsi leur utilisation plus large dans les détergents durables (Niyonzima & More, 2015; Vieira et al., 2021).

IV. Chapitre 2. Méthodologie

Ce projet de recherche a adopté une approche expérimentale en plusieurs étapes visant à évaluer la faisabilité de la production d'enzymes à partir de résidus industriels fermentescibles, en combinant des souches microbiennes issues de banque de référence et isolées dans les milieux résiduels (indigènes). La méthodologie a été structurée selon les cinq axes suivants :

1. **Sélection des substrats industriels**

Quatre effluents industriels générés au Québec ont été sélectionnés pour leur potentiel fermentescible et leur représentativité de secteurs industriels variés :

SIW : eaux usées de l'industrie de l'amidon,

BW : eaux usées de l'industrie des boissons,

FIW : eaux usées de l'industrie alimentaire,

PPMS : boues papetières secondaires.

Chaque effluent a été caractérisé physico-chimiquement avant utilisation, afin d'évaluer sa capacité à soutenir la croissance microbienne et la production enzymatique.

2. **Évaluation de souches commerciales**

Trois souches bactériennes du genre Bacillus, reconnues pour leur potentiel de production enzymatique, ont été utilisées dans un premier criblage :

Bacillus licheniformis (protéase),

Bacillus megaterium (lipase),

Bacillus amyloliquefaciens (amylase).

Ces souches ont été cultivées dans des milieux à base d'effluents industriels dilués. Des essais en flacons agités ont été réalisés pour mesurer l'activité enzymatique (U/mL), et déterminer les meilleures combinaisons souche-substrat.

3. **Optimisation des conditions de fermentation**

Pour maximiser la production enzymatique, les conditions de culture des souches commerciales ont été optimisées à l'aide de la méthodologie de surface de réponse (RSM). Les paramètres suivants ont été testés :

Concentration en solides totaux du milieu,

Température d'incubation,

Inoculum bactérien (% v/v).

Les expériences ont été menées selon un plan Box–Behnken. Les résultats ont permis d'identifier les conditions optimales de production pour chaque enzyme et substrat.

4. **Isolement et caractérisation de souches indigènes**

Des souches bactériennes indigènes ont été isolées directement à partir du PPMS, considéré comme le substrat le plus prometteur. Deux isolats ont été retenus pour leurs performances enzymatiques élevées :

Bacillus tropicus P4 : producteur de protéase,

Acinetobacter tandoii L3 : producteur de lipase.

Ces souches ont été purifiées, identifiées par séquençage 16S rRNA, et testées pour leur production enzymatique dans les mêmes conditions que les souches commerciales.

5. **Validation en bioréacteurs**

Les meilleures combinaisons souche-substrat identifiées en flacons ont été testées à plus grande échelle dans des bioréacteurs de 5 L, puis de 150 L, sous des conditions de culture contrôlées (pH, température, oxygénation). Les performances enzymatiques ont été suivies en continu pour évaluer la reproductibilité, la cinétique de production et la stabilité des enzymes**.**

V. Chapitre 3 Articles

(Article 1) : Revue de littérature portant sur les espèces de Bacillus et leur rôle dans les biodétergents

Dans un contexte où la demande en produits de nettoyage respectueux de l'environnement ne cesse de croître, les espèces du genre *Bacillus* se sont imposées comme des micro-organismes d'intérêt pour la production d'enzymes et de biosurfactants à usage industriel. Leur aptitude à produire un large éventail d'enzymes extracellulaires (protéases, amylases, lipases, cellulases)

ainsi que des biosurfactants de type lipopeptides leur confère une polyvalence importante dans les formulations de détergents biodégradables. Cette revue examine le potentiel de ces espèces, en soulignant leur capacité à fonctionner dans des conditions extrêmes de pH, température ou salinité, ce qui les rend compatibles avec de nombreuses applications industrielles. Ces attributs permettent non seulement d'améliorer l'efficacité du lavage, mais aussi de réduire l'usage de composés chimiques toxiques comme certains tensioactifs de synthèse. Toutefois, plusieurs défis subsistent, notamment en ce qui concerne le coût de production et la stabilité des enzymes dans les formulations. Pour y répondre, des pistes de recherche prometteuses sont discutées : l'optimisation des procédés de fermentation à partir de substrats à faible coût, le recours à des techniques de génie génétique et de modification des protéines, ainsi que la prospection de nouvelles souches aux propriétés enzymatiques originales (comme les enzymes actives à basse température ou les biosurfactants à faible toxicité). Ce chapitre montre que bien que l'adoption à grande échelle des détergents enzymatiques soit encore limitée, les progrès récents en biotechnologie microbienne, notamment autour des espèces *Bacillus*, offrent des perspectives intéressantes pour concevoir des détergents plus durables et performants sur les plans économique et environnemental.

(Article 2) : Évaluation des eaux usées industrielles comme ressources à faible coût pour une production durable d'enzymes par les espèces de *Bacillus*

Le premier article met en évidence le potentiel des espèces *Bacillus* dans la production d'enzymes et de biosurfactants pour les détergents biosourcés, soulignant leur capacité à utiliser des substrats de déchets à faible coût et leur maniabilité génétique pour améliorer l'efficacité des enzymes. Cela a naturellement conduit au second article, qui évalue expérimentalement l'utilisation des eaux usées industrielles comme milieu de fermentation pour la production d'enzymes, validant l'hypothèse selon laquelle les résidus industriels fermentescibles peuvent servir de ressources viables et à faible coût pour une production durable d'enzymes destinées aux détergents.

Cet article fait partie de l'étape 1 du projet, qui consiste à sélectionner les eaux usées prometteuses en utilisant des souches commerciales.

L'augmentation de la demande d'enzymes industrielles impose la mise en place de stratégies de production rentables et durables. Cette étude explore le potentiel des eaux usées industrielles

comme milieu alternatif de fermentation pour la synthèse d'enzymes, en cohérence avec les principes de l'économie circulaire de la bioéconomie. Quatre types d'eaux usées provenant du Québec, Canada, les eaux usées de boissons (BW), les boues secondaires de et papier (PPMS), les eaux usées de brasserie (FIW) et les eaux usées de l'industrie de l'amidon (SIW), ont été évaluées pour leur potentiel à soutenir la production de protéase, amylase et lipase à l'aide des souches *Bacillus licheniformis*, *Bacillus amyloliquefaciens* et *Bacillus megaterium*. Le criblage initial a identifié le SIW comme optimal pour la production d'amylase avec *B. amyloliquefaciens* et le PPMS pour la production de protéase avec *B. megaterium*. L'optimisation à l'aide du plan de conception de Box–Behnken a été réalisée, suivie par des expériences de mise à l'échelle dans des bioréacteurs de 5 L. *B. amyloliquefaciens* a atteint 5.73 ± 0,01 U/mL d'amylase à 48 h sous 40 g/L de solides totaux, à 30 °C et avec une taille d'inoculum de 2 % (v/v), tandis que *B. megaterium* a produit la plus haute protéase de 55.41 ± 3.54 U/mL à 24 h. La production de lipase est restée négligeable pour tous les milieux et souches. Ces résultats a démontré la faisabilité de la production d'enzymes à partir d'eaux usées, réduisant la dépendance aux substrats synthétiques coûteux, atténuant les charges environnementales et contribuant à la transition vers une bioéconomie circulaire.

(Article 3) : Valorisation des boues de l'industrie papetière pour la production de protéase par la souche indigène *Bacillus tropicus* P4

Cette étude explore le potentiel de l'utilisation des boues de papeterie (PPMS) comme substrat économique pour la production d'enzymes protéolytiques de haute valeur à l'aide d'une souche bactérienne indigène, *Bacillus tropicus* P4. Isolée directement à partir du PPMS, *B. tropicus* P4 a montré une capacité de production de protéase élevée, environ 134 U/mL après 48 heures, soit plus de trois fois le rendement de la souche de référence (*B. megaterium*). Parmi les divers additifs testés pour stimuler la production d'enzymes, le Tween 80 a émergé comme le plus efficace, augmentant l'activité enzymatique de plus de trois fois par rapport au témoin. Les expériences de mise à l'échelle dans des bioréacteurs de 5L et 150L ont confirmé que *B. tropicus* P4 maintient des rendements élevés de protéase dans des conditions de culture classiques avec des modifications minimales, en particulier l'ajout de Tween 80 (1%) et une concentration accrue de solides totaux (25 g/L). Dans le bioréacteur de 5L, la production d'enzymes a atteint un pic d'environ 755 U/mL en 24 heures, tandis que le bioréacteur de 150L a régulièrement atteint une activité enzymatique

élevée (~848 U/mL). Ces résultats soutiennent la faisabilité d'une approche simple et évolutive pour convertir les boues industrielles en enzymes protéolytiques de haute valeur, contribuant ainsi à la récupération des ressources et aux stratégies de bioéconomie circulaire.

(Article 4) : Production de lipase par l'isolat *Acinetobacter tandoii* pour la valorisation des boues de l'industrie papetière

Cette étude évalue le potentiel lipolytique d'*Acinetobacter tandoii* L3, une souche isolée des boues de papeterie, en utilisant des boues secondaires activées minimement traitées comme substrat de fermentation durable et à faible coût. La production de lipase a été surveillée dans des fioles agitées et mise à l'échelle dans des bioréacteurs de 5 et 150 L. Dans le bioréacteur de 150 L, l'activité lipasique a été détectée dès 3 h, et a considérablement augmenté à 6 h (0.52 U/mL), et a atteint un pic à 9 h (0.62 U/mL). Contrairement au bioréacteur de 5 L, où l'activité a chuté rapidement après le pic, le système de 150 L a maintenu des niveaux supérieurs à 0.53 U/mL jusqu'à 48 h. Cette activité soutenue suggère que l'amélioration du transfert d'oxygène et de l'agitation a renforcé la stabilité de l'enzyme. Ces résultats soulignent la capacité de production précoce et robuste de lipase de *A. tandoii* L3 et son potentiel dans la valorisation circulaire des boues de papeterie en bioproduits industriels pertinents.

VI. Chapitre 4 : Discussion

❖ Comparaison entre les souches de référence et les indigènes productrices de protéase

Les profils de production de protéase de la souche commerciale *Bacillus megaterium* et de l'isolat indigène *Bacillus tropicus* P4 révèlent des différences notables dans les rendements enzymatiques. Dans des expériences en fioles agitées, *B. megaterium* a produit 39.6 ± 3.53 U/mL après 48 heures sur PPMS avec 1 % de Tween 80, tandis que *B. tropicus* P4 a atteint environ 134 U/mL sans ajout de milieu enrichi, ce qui représente une augmentation de plus de trois fois l'activité de protéase. Cela met en évidence l'avantage de prélever des souches directement de leur environnement d'origine, où les micro-organismes indigènes sont mieux adaptés aux substrats complexes présents dans les milieux résiduels tels que les PPMS.

❖ Mise à l'échelle et cinétique de croissance

La transition des fioles agitées aux bioréacteurs de 5L et 150L a permis d'obtenir des informations précieuses sur la reproductibilité du procédé de production enzymatique et la cinétique de

croissance des microorganismes. Pour la production d'amylase par *B. amyloliquefaciens*, l'activité maximale s'est produite à 48 heures dans les deux séries de 5L. Lors de la production de protéase, *B. tropicus* P4 a surpassé *B. megaterium* à plus grande échelle. Dans les bioréacteurs de 150L, P4 a atteint 847,64 U/L à 24 heures, avec une légère baisse à 48 heures, ce qui indique une bonne stabilité de l'activité enzymatique cellulaire même durant la phase stationnaire de croissance de P4. Alors qu'*Acinetobacter tandoii* L3 a montré une plus haute activité atteignant 0,62 U/mL à l'échelle 150L. Ceci suggère que les eaux usées industrielles peuvent soutenir une production d'enzymes à échelle pilote lorsqu'elles sont associées à des souches compatibles dans des conditions optimisées. Il est à noter que les souches de référence comme *B. amyloliquefaciens* offrent une robustesse et une fiabilité sur des milieux moins complexe tels que les eaux usées d'amidon. Tandis que les souches indigènes comme *B. tropicus* P4 révèlent une capacité de production d'enzymes extracellulaires lorsque le substrat est complexe.

❖ **Conclusion**

Cette étude a démontré la faisabilité de produire des enzymes pertinentes pour les détergents, amylase, protéase et lipase, en utilisant des eaux usées industrielles comme milieux de fermentation, soutenues par des souches bactériennes commerciales et indigènes. Parmi les quatre eaux usées testées, les eaux usées de l'industrie de l'amidon (SIW) et les boues de papeterie (PPMS) ont émergé comme les substrats les plus prometteurs, chacun soutenant une forte croissance microbienne et une activité enzymatique sous un prétraitement minimal.
Les souches de référence comme *Bacillus amyloliquefaciens* et *Bacillus megaterium* se sont montrées efficaces sur des substrats comme SIW et PPMS, notamment lorsque les conditions de fermentation ont été optimisées par la méthodologie de surface de réponse. Notamment, *B. amyloliquefaciens* a atteint des rendements élevés d'amylase (5,73 ± 0,01 U/mL) dans des bioréacteurs de 5L en présence d'eaux usées industrielles d'amidon. En revanche, *B. megaterium* a produit des niveaux modérés de protéase sur PPMS, l'ajout de Tween 80 améliorant le rendement mais n'augmentant pas significativement la production de lipase.
Conscients des limitations des souches de référence dans les eaux usées complexes, les souches indigènes ont été criblées directement à partir du PPMS. Cette approche a conduit à l'isolation et à l'identification de *Bacillus tropicus* P4, qui a produit plus de trois fois l'activité de protéase de *B.*

megaterium en flacons, et *Acinetobacter tandoii* L3, qui a montré une production rapide et élevée de lipase en 9 heures. La performance supérieure de *B. tropicus* P4 a été confirmée dans une mise à l'échelle à un bioréacteur de 150L, où les titres de protéase ont dépassé 840 U/L et sont restés stables au-delà de la phase de croissance exponentielle, démontrant la robustesse et le potentiel industriel de la souche.

❖ **Recommandations**

1. **Développer des protocoles d'association substrat-souche** : Étant donné la diversité des profils chimiques des eaux usées industrielles, il est recommandé d'élaborer une approche structurée permettant d'associer chaque type d'effluent à des souches microbiennes adaptées (référence ou indigènes). Ce cadre devrait prendre en compte des critères tels que les bioproduits visés, la tolérance au pH, le profil nutritionnel, et la présence de composés inhibiteurs.
2. **Élargir les efforts de bioprospection dans les matrices de déchets complexes** : Le succès observé avec *Bacillus tropicus* P4 et *Acinetobacter tandoii* L3 met en lumière le potentiel sous-exploité des communautés microbiennes indigènes dans des substrats complexes comme le PPMS. Il serait pertinent de renforcer les campagnes de prospection en utilisant des outils tels que le criblage à haut débit ou la métagénomique pour identifier de nouvelles souches robustes possédant des activités enzymatiques d'intérêt industriel.
3. **Explorer le génie métabolique des souches indigènes** : Les souches indigènes comme *B. tropicus* P4, déjà performantes en termes de production enzymatique, pourraient être améliorées par des approches de génie génétique, telles que la technologie CRISPR. Cela permettrait d'augmenter la spécificité, la stabilité et la sécrétion des enzymes, tout en préservant la résilience naturelle de ces microorganismes
4. **Optimiser le moment de la fermentation et les stratégies de récolte** : Certaines enzymes, telles que les lipases produites par *A. tandoii* L3, présentent des temps d'activité restreintes. Il serait utile de développer des protocoles de fermentation intégrant un suivi en temps réel, ainsi que des stratégies d'alimentation ou de récolte adaptées, afin d'optimiser les rendements tout en limitant la dégradation des produits.
5. **Évaluer les systèmes de culture synergique en co-culture** : La co-culture de souches référence et indigènes pourrait permettre la production de plusieurs enzymes (par exemple,

amylase + protéase) ou une meilleure utilisation des substrats, en particulier sur des substrats mixtes.

6. **Réaliser des évaluations techno-économiques et de cycle de vie** : Afin d'assurer la transposition des résultats en contexte industriel, il est recommandé d'intégrer des évaluations coûts-bénéfices et des études d'impacts environnementaux. Ces analyses permettront d'identifier les configurations les plus viables pour la production d'enzymes destinées à des applications réelles comme les détergents.

7. **Étudier la formulation des enzymes et leur stabilité dans les systèmes de détergents** : Enfin, il est nécessaire de mener des essais en aval sur la compatibilité et la stabilité des enzymes produites avec les composants classiques des formulations de détergents (tensioactifs, agents de blanchiment, agents complexants, etc.), en simulant les conditions réelles d'utilisation (pH, température, dureté de l'eau).

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Beverage industry wastewater	BW
Deoxyribonucleic acid	DNA
Diisopropyl fluoro phosphate	DFP
Dinitrosalicylic	DNS
Dissolved oxygen	DO
Ethylene diamine tetraacetic acid	EDTA
Food industry wastewater	FIW
Indole-3-acetic acid	IAA
Linear alkylbenzene sulfonate	LAS
Phenylmethylsulfonyl fluoride	PMSF
Pulp and paper activated sludge	PPMS
Ribonucleic acid	RNA
Sodium dodecyl sulfate	SDS
Solid state fermentation	SSF
Starch industry wastewater	SIW
Submerged fermentation	SmF
Total solids	TS/ TSs
Total suspended solids	TSS/ TSSs
Total volatile solids	TVSs
Volatile suspended solids	VSSs

1 GENERAL INTRODUCTION

1.1 Context

Detergents have become vital products for both personal and industrial hygiene, with global consumption growing significantly—particularly in the wake of the COVID-19 pandemic (Figure 1.1). The earliest detergent was natural soap, composed of biodegradable fatty acid salts derived from plant or animal sources. These early formulations were environmentally benign and intended primarily for basic cleaning purposes (Babajanzadeh et al., 2019; Mousavi & Khodadoost, 2019; Yangxin et al., 2008). However, as illustrated in Figure 1.1, rising public health demands and industrial growth have significantly driven the evolution of detergent compositions in terms of both quality and quantity over time. This evolution has led to the development of increasingly complex formulations designed to meet the needs of different markets and applications (Scheibel, 2004).

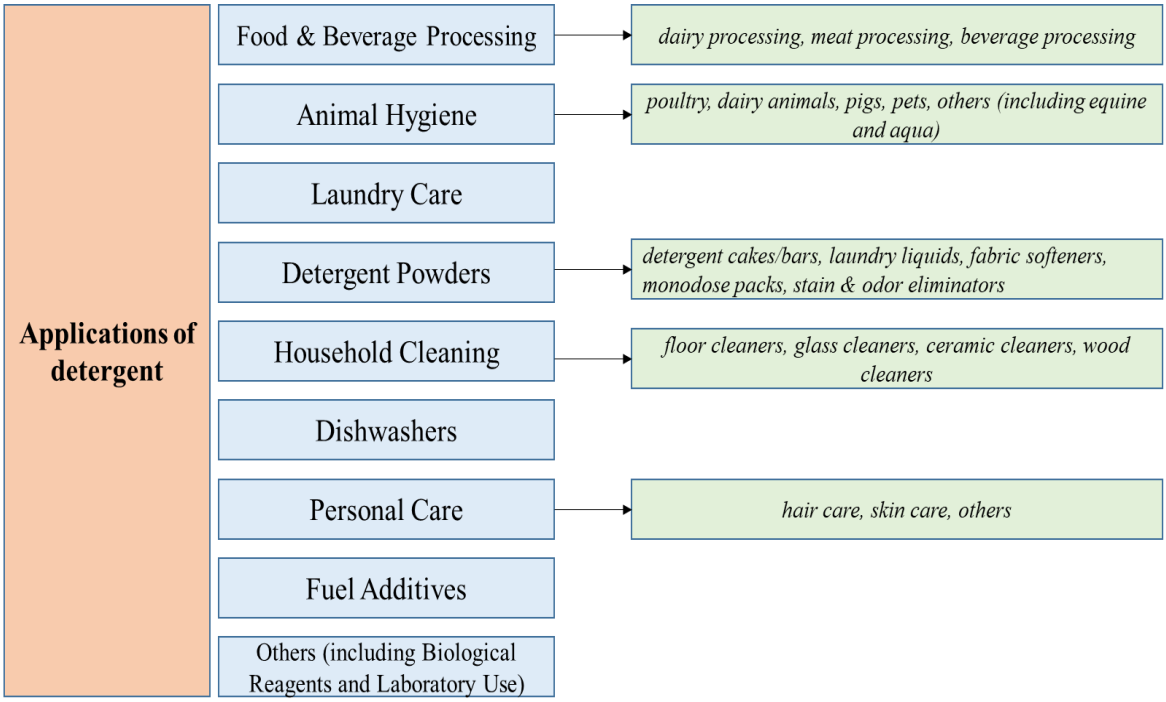


Figure 1.1 Applications of detergent in global markets

(https://www.transparencymarketresearch.com/detergents-market.html)

Modern detergents are typically composed of one or more surfactants (15-40%), along with a wide array of functional additives tailored to enhance cleaning performance. These additives include builders (such as phosphates, zeolites, and polycarboxylic acids), bleaching agents (e.g., chlorine-releasing compounds), fillers, foam stabilizers, perfumes, soil-suspending agents, enzymes, dyes, optical brighteners, and biocidal agents—most commonly in the form of quaternary ammonium compounds. Commercial detergents are produced through four primary manufacturing processes: blending, agglomeration, slurry production (primarily for powders), and liquid formulations (IUPAC, 1997). Despite their widespread use, the exact formulations of most commercial detergents are proprietary and not publicly disclosed.

In terms of classification, detergents are generally divided into two types based on the builders used: phosphate detergents and phosphate-free detergents (Bajpai & Tyagi, 2007). They may also be categorized according to the nature of the surfactants, including anionic, cationic, nonionic, zwitterionic (ampholytic), and bio-based detergents (Transparency Market Research). Among these, linear alkylbenzene sulfonate (LAS)—a partially biodegradable anionic surfactant—remains the most widely used due to its cost-effectiveness and strong detergency (Yangxin et al., 2008).

Although each detergent ingredient contributes to overall cleaning efficiency, research has shown that synthetic surfactants and builders—especially those used in large volumes—pose significant ecological risks (Yangxin et al., 2008). Figure 1.2 illustrates the environmental pathways resulting from detergent use. As washing processes consume substantial volumes of freshwater, they consequently generate large amounts of wastewater containing residual detergent components. These substances are often discharged into aquatic environments or end up in soil, where they have been associated with a range of environmental impacts, including eutrophication, excessive foaming, reduced oxygen transfer, toxicity to aquatic organisms and plants, interference with water coagulation, and diminished efficiency of wastewater treatment systems (Mousavi & Khodadoost, 2019). Phosphate-based detergents have long been associated with eutrophication—a process that results in excessive algal growth, oxygen depletion, and degradation of aquatic

ecosystems. Public concerns over these impacts led to regulatory restrictions in many countries beginning in the mid-1960s and prompted a shift toward phosphate-free alternatives. Similarly, LAS, despite its high biodegradability (95–99%), has been found to exhibit antimicrobial properties that inhibit the microbial communities essential for biological wastewater treatment (Stamatelatou et al., 2011). These ecological concerns highlight the limitations of traditional detergent chemistry (Helmy et al., 2020).

While the primary environmental concerns historically focused on residual detergent discharge—which contributes to eutrophication, excessive foaming, oxygen depletion, aquatic toxicity, and interference with wastewater treatment—recent attention has also turned upstream to the environmental burden of synthetic surfactant production. Most synthetic surfactants, including LAS, are derived from petroleum-based feedstocks. Their manufacture involves energy-intensive processes that contribute to greenhouse gas emissions, air pollution, and resource depletion. Petroleum extraction and refining not only release significant amounts of CO_2 but also pose risks of oil spills and ecosystem degradation. Moreover, the synthesis of surfactants can yield hazardous by-products and depends on finite, non-renewable inputs, raising further concerns regarding sustainability.

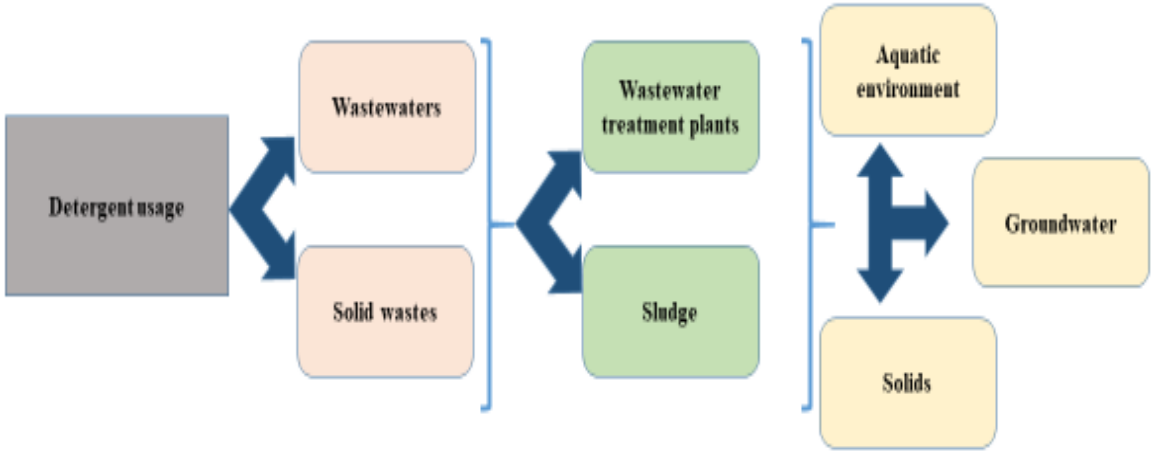


Figure 1.2 Fate of detergent usage ending up in the environment

As detergent usage continues to rise globally, its environmental footprint has become increasingly significant. According to recent estimates, the global detergent market was valued at approximately USD 133–140 billion in 2023 and is projected to exceed USD 180 billion by 2030,

reflecting increased consumption across both domestic and industrial sectors. Household laundry products constitute the largest segment of this market, followed by dishwashing and surface-cleaning agents (Giagnorio et al., 2017), further emphasizing the need for sustainable innovation. Enzyme-based and bio-derived detergents offer promising alternatives by facilitating effective cleaning at lower temperatures and reduced product dosages (Hasan et al., 2010).

By integrating enzymes, the washing efficiency can be boosted when applying smaller quantities of products even at lower temperatures when compared with synthetic detergents. Thus, these detergents minimise energy consumption, especially in the context of the energy crisis (Hasan et al., 2010). Additionally, the adoption of enzymes in detergents reduces the reliance on phosphates and oxidative bleaching agents, which are associated with eutrophication and aquatic toxicity (Bajpai & Tyagi, 2007). Being biodegradable and derived from renewable microbial sources, enzymes contribute to a lower environmental burden. In addition, they help preserve fabric quality by minimizing mechanical damage and color fading during washing (Gurkok, 2019). With advancements in microbial fermentation and genetic engineering, enzyme production has become increasingly efficient and cost-effective, supporting their broader application in sustainable detergent technologies (Niyonzima & More, 2015; Vieira et al., 2021).

1.2 Enzymes: structure, function, and classification

Enzymes are biologically produced catalysts that accelerate chemical reactions by lowering the activation energy required for those reactions to occur. Unlike chemical catalysts, enzymes function with remarkable specificity and efficiency, typically acting on a single type of substrate or reaction pathway (Hopmann & Himo, 2010). Most enzymes are composed of proteins and are synthesized by living organisms to regulate and sustain metabolic processes. However, some non-protein enzymes also exist—such as ribozymes (catalytic RNA molecules) and abzymes (catalytic antibodies)—though these are beyond the scope of the present discussion (Palmer & Bonner, 2011).

Structurally, enzymes exhibit four levels of organization. The primary structure refers to the linear sequence of amino acids. This folds into secondary structures such as α-helices and β-sheets

through hydrogen bonding along the peptide backbone. Further folding driven by side-chain (R-group) interactions results in the tertiary structure, determining the enzyme's overall three-dimensional shape. Enzymes composed of multiple subunits have a quaternary structure, which describes the spatial arrangement of these units (Palmer & Bonner, 2011). The enzyme's active site—often a cleft or groove on its surface—is formed during this folding process and is crucial for substrate binding and catalysis (Figure 1.3 and Figure 1.4) (Pelley, 2012). Although the active site represents a small fraction of the enzyme, it determines substrate specificity through complementary interactions. In the native (unbound) state, water molecules help stabilize the active site conformation (NCBI Bookshelf, 2024).

Some enzymes require a non-protein component, called a cofactor, to become catalytically active. The protein portion alone, known as the apoenzyme, becomes a fully functional holoenzyme when associated with its cofactor. Cofactors can be inorganic metal ions (e.g., Ca^{2+}, Fe^{2+}, Zn^{2+}) or organic molecules known as coenzymes, which often act as transient carriers of specific atoms or groups during catalysis (Palmer & Bonner, 2011).

A standardized system for enzyme nomenclature was first introduced by the Enzyme Commission (EC) in 1961 to address the growing complexity and inconsistency in enzyme naming. This system was designed to classify enzymes based on the specific chemical reactions they catalyze. By the sixth edition, published in 1992, the EC system had catalogued nearly 3,200 enzymes, and with annual updates, the number has since expanded to over 5,000 entries (https://wou.edu/chemistry/courses/online-chemistry-textbooks/ch450-and-ch451-biochemistry-defining-life-at-the-molecular-level/chapter-7-enzyme-kinetics/).

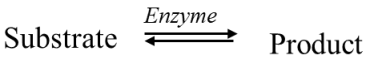
Each enzyme in this system is assigned a unique four-part EC number, which provides a hierarchical classification. The first digit designates the broad class of reaction catalyzed (e.g., oxidation–reduction, hydrolysis), while the second, third, and fourth digits offer increasingly specific information regarding the type of substrate, bond, or group involved in the reaction (Table 1.1).

Enzymes possess several fundamental properties that underpin their widespread biological and industrial relevance (Robinson, 2015):

Catalytic activity – accelerating reaction rates by several orders of magnitude.

Specificity – acting selectively on particular substrates to produce defined products.

Reversibility – enabling reactions to proceed in either direction under suitable conditions, often establishing equilibrium.



High turnover number (kcat) **-** a single enzyme molecule can process many substrate molecules per second — directly explaining why only small amounts are needed.

Reusability and non-consumption - unlike stoichiometric reagents, enzymes aren't consumed in the reaction, making them efficient over time.

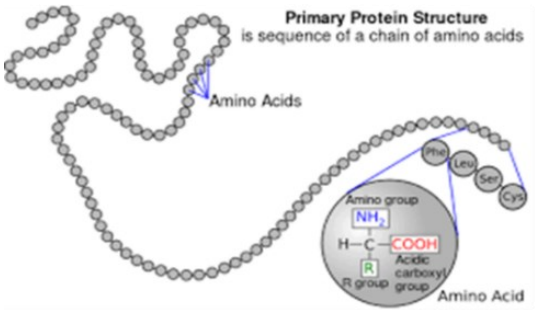
Biodegradability and non-toxic nature **-** particularly important in detergents and food applications — enzymes are safe and environmentally friendly.

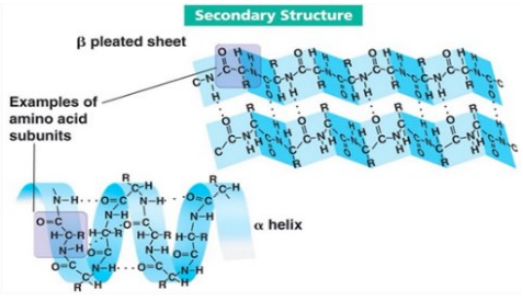
Stability under process conditions - in industry, enzymes need to remain active under varying pH, temperature, ionic strength, or in the presence of surfactants or oxidants.

Among the six main classes of enzymes, hydrolases (EC 3) are particularly significant in industrial and household applications, especially in the formulation of enzyme-based detergents. These enzymes catalyze the hydrolysis of various complex molecules by cleaving covalent bonds in the presence of water. In detergent systems, hydrolytic enzymes contribute directly to the breakdown of stain components—such as proteins, fats, and carbohydrates—on fabrics and surfaces, thus enhancing the overall cleaning efficiency.

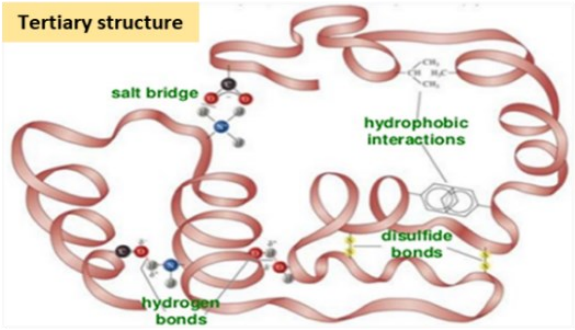
Table 1.1 Enzyme classification: Main classes of enzymes in EC system

First EC digit	Enzyme class	Reaction type
1.	Oxidoreductases	Oxidation/reduction
2.	Transferases	Atom/group transfer (excluding other classes)
3.	Hydrolases	Hydrolysis
4.	Lyases	Group removal (excluding 3).
5.	Isomerases	Isomerisation
6.	Ligases	Joining of molecules linked to the breakage of a pyrophosphate bond
7	Translocases	Catalyzing the transfer of a specific functional group from one molecule (the donor) to another molecule (the acceptor)

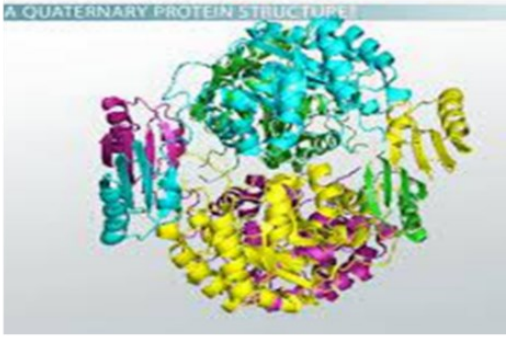




Hydrogen bounding of peptide backbone form folded structures in repeating pattern



Side chain interactions determine the tertiary structure of enzyme



When proteins contain more than one peptide chain

Figure 1.3 Protein structure

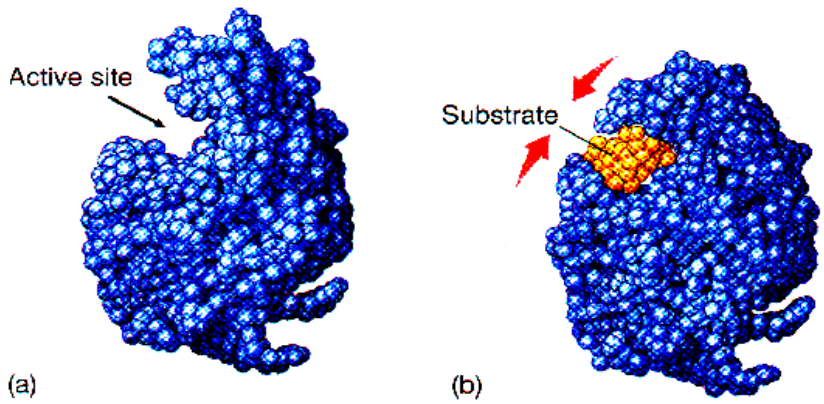


Figure 1.4 Structure of enzymes

(https://sites.lps.org/sputnam/Biology/U4Metabolism/Metabolism.htm)

1.3 Compatibility and stability of enzymes in detergents

Enzymes have become indispensable components of modern detergent formulations due to their high catalytic efficiency, substrate specificity, and environmental safety. Active and stable under washing conditions yet nontoxic upon discharge, enzymes are widely accepted across various detergent types, including powder and liquid household products, laundry pre-spotters, stain removers, automatic dishwashing detergents, and institutional or industrial cleaners (Valls et al., 2011). They complement the action of surfactants by targeting and breaking down persistent organic stains—particularly proteins, starches, and lipids—which constitute the majority of difficult-to-remove soils (Bajpai & Tyagi, 2007; Hellmuth & Dreja, 2016).

Enzymes were first introduced into detergent production more than a century ago (Gurkok, 2019), and today, the detergent industry remains the largest consumer of industrial enzymes, accounting for approximately 25–30% of total enzyme sales (Enzymes Market Report, 2019). The global enzyme market was valued at USD 9.9 billion in 2019, rising to USD 10.6 billion in 2020, with projections reaching USD 14.9 billion by 2027. Despite their widespread adoption, enzyme cost remains a limiting factor in detergent formulations (Benvegnu et al., 2008; Scheibel, 2004).

Microorganisms are the primary source of industrial enzymes, contributing approximately 85% of global enzyme production, with 50% derived from fungi and yeasts and 35% from bacteria (Liu

& Kokare, 2017). Their dominance in the enzyme market is largely due to their suitability for large-scale fermentation, genetic tractability, and biochemical versatility. In detergent formulations, microbial enzymes play a central role due to their stability under alkaline and thermally variable conditions. These properties are critical for leaning performance during washing. Unlike plant or animal-derived enzymes, microbial enzymes can be produced efficiently in large quantities and tailored through strain improvement or protein engineering to meet the specific performance requirements of detergent applications. Moreover, microbial diversity enables the exploitation of waste-derived substrates for enzyme production and supports the discovery of novel enzymes through metagenomics, genome mining, and extremophile screening. Despite the identification of over 4,000 microbial enzymes, only about 200 are commercially available, and fewer than 20 are produced at industrial scale (Liu & Kokare, 2017), presenting significant potential for innovation in sustainable detergent development.

Several classes of enzymes are now standard in detergent products. Alkaline proteases, lipases, α-amylases, and cellulases are commonly used to target stains from food, body fluids, and fabric residues. Recent developments have extended interest to enzymes like mannanases and pectinases for heavy-duty laundry and dishwashing applications (Jayasekara & Ratnayake, 2019).

Despite their functional advantages, enzymes in detergents must withstand various destabilizing conditions. During storage and washing, they are exposed to alkaline pH, elevated temperatures, surfactants, bleaching agents, and oxidizing compounds, all of which can compromise structural integrity and reduce catalytic activity (Robinson, 2015; Vitolo, 2020). Alkaline environments (pH 8–10.5) may alter the ionization of amino acid residues at the active site, disrupting substrate binding. High temperatures accelerate reaction rates but can also lead to irreversible denaturation of tertiary or quaternary protein structures. Surfactants interfere with hydrophobic interactions that maintain enzyme folding, while oxidants like hydrogen peroxide and perborates can chemically modify sensitive residues such as methionine or cysteine, leading to inactivation (Hasan et al., 2010; Helmy et al., 2020). Builders and chelating agents (e.g., EDTA) may further reduce activity by sequestering essential metal cofactors (Vitolo, 2020).

To address these challenges, the selection of robust enzymes is critical. Screening enzymes from extremophilic microorganisms—such as thermophiles, alkaliphiles, or psychrophiles—offers a route to naturally stable biocatalysts adapted to high pH, variable temperatures, and oxidative environments (Hasan et al., 2010). Additionally, protein engineering approaches like site-directed mutagenesis and directed evolution have enabled the creation of enzyme variants with improved resistance to denaturing agents and broader working ranges for temperature and pH (Helmy et al., 2020; Palmer & Bonner, 2011). These engineered enzymes often outperform their wild-type counterparts in commercial detergent settings.

Beyond selection, maintaining enzyme stability in the formulation is essential. Additives such as calcium ions, polyols, and sugars (e.g., trehalose) are commonly used to stabilize enzyme conformation and activity (Vitolo, 2020). Advanced encapsulation methods—such as microencapsulation or coating with water-insoluble or pH-sensitive polymers—can shield enzymes from oxidants, surfactants, and moisture until optimal conditions are reached during the wash (Hasan et al., 2010). Granulation techniques, such as spray-drying or layered coating, not only protect enzymes during storage and processing but also enhance handling and reduce airborne dust. Finally, detergent formulations must be designed to avoid incompatibilities; for instance, removing bleach activators or minimizing surfactant concentration may be necessary to maintain enzyme longevity and activity (Giagnorio et al., 2017; Helmy et al., 2020).

In summary, enzymes in detergents must remain active, stable, and compatible with a range of formulation components. They should function across varying temperatures—from cold washes involving synthetic fibres to high-temperature industrial settings—and tolerate strongly alkaline conditions where most soils are efficiently hydrolysed and dispersed. The ability to maintain enzyme activity under these conditions is critical to achieving optimal cleaning performance and supports the development of greener, more energy-efficient detergent products.

1.3.1 Serine proteases

Alkaline proteases represent the largest segment of the global enzyme market and are among the most commercially significant enzymes, particularly due to their extensive use as detergent

additives (K. M. Sharma et al., 2017). These enzymes catalyze the hydrolysis of peptide bonds in proteins, rapidly converting them into smaller peptides or free amino acids. This action is especially valuable in laundry applications where proteases efficiently degrade proteinaceous stains such as blood, milk, eggs, grass, and human sweat, which tend to bind strongly to fabrics (Gurkok, 2019; Matkawala et al., 2021). Proteases were the first enzymes introduced into detergent formulations, initially as supplementary components, but they have since become indispensable in modern detergent products due to their stain-removal efficiency and contribution to sustainable washing practices (Vojcic et al., 2015).

The incorporation of proteases into detergents offers both environmental and economic benefits. By enabling effective stain removal at lower washing temperatures and shorter wash cycles, protease-containing detergents reduce energy and water consumption, supporting eco-friendly household and industrial laundering. Commercial alkaline proteases are typically used at low concentrations (0.4–0.8%) and are compatible with a variety of detergent components, including oxidizing agents (e.g., perborates, percarbonates), surfactants, and sequestering agents such as EDTA. These enzymes are valued for their high catalytic efficiency, broad activity range (typically pH 7.0–11.0), thermal stability, and prolonged shelf life (Kumar & Bhalla, 2004; Vojcic et al., 2015). Additionally, their biodegradability and phosphate-free formulations align with growing regulatory and consumer demands for greener cleaning products.

Proteases are broadly classified into mechanistic groups based on their catalytic residues and reaction mechanisms: aspartate, cysteine, glutamate, metallo, serine, threonine proteases, and asparagine peptide lyases. Among them, serine proteases, particularly alkaline serine endopeptidases, dominate detergent applications due to their superior activity under non-physiological conditions, including high alkalinity, elevated temperatures, and the presence of calcium chelators and surfactants (Klein et al., 2018). These enzymes cleave peptide bonds via a catalytic triad typically composed of serine, histidine, and aspartate residues, making them highly efficient and adaptable to detergent matrices.

Serine peptidases constitute the largest class of proteases, with numerous variants differing in substrate specificity and structural stability. They are predominantly derived from microbial

sources, as microbes offer several advantages over plant or animal systems, including faster growth rates, easier cultivation, and genetic tractability. A wide range of bacterial and fungal strains have been reported as prolific producers of serine proteases, including *Bacillus*, *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Psychrobacter*, *Halobacillus*, *Microbulbifer*, *Shewanella*, *Aspergillus tamarii*, and *Alternaria solani* (Banerjee & and Ray, 2017).

Historically, enzymatic detergents trace back to the early 20th century, when Otto Röhm first proposed using crude pancreatic proteases for stain removal in 1913. However, large-scale commercial adoption did not occur until the 1960s, following advances in microbial enzyme production. The advent of microbial proteases from *Bacillus* spp. marked a turning point in detergent formulation, offering scalable, cost-effective, and more robust enzyme solutions (Singh & Bajaj, 2017). Today, all detergent-grade proteases in commercial use are serine proteases produced by *Bacillus* strains. A milestone in this field was the introduction of BIOTEX by Novo Industry A/S in 1960, containing subtilisin Carlsberg derived from *Bacillus licheniformis*—a breakthrough that revolutionized enzymatic detergents and set the stage for future innovations in enzyme biotechnology (Razzaq et al., 2019).

Several commercial protease products are widely utilized in detergent formulations, predominantly produced by *Bacillus* species and engineered for performance under harsh washing conditions. For example, Alcalase®, a serine endopeptidase primarily composed of subtilisin A, is derived from *Bacillus licheniformis* and functions effectively between 50–75 °C (Al-Ghanayem & Joseph, 2020; Sarmiento et al., 2015). Other serine protease-based products from Novozymes include Durazym®, Everlase™, and Savinase®, all derived from mutant *Bacillus* strains expressing subtilisin A. Esperase®, also based on subtilisin A, originates from *B. halodurans*, while Neutrase®, a metalloprotease, is obtained from *B. amyloliquefaciens*. Additionally, Protamex™, a general protease blend, and Purafect® Prime, a subtilisin enzyme from *B. lentus* produced by Genencor International, are tailored to operate in a variety of detergent conditions, including lower temperature ranges of 20–40 °C for Purafect® Prime (Gurkok, 2019).

1.3.2 α-amylases

Among the amylase family, α-amylase (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1) has received significantly greater attention than β- and γ-amylases due to its broad industrial applicability. It plays a vital role in sectors including food and beverage processing, fermentation, textiles, pharmaceuticals, pulp and paper, and household care (Saini et al., 2017; Souza, 2010). α-amylase functions as an extracellular endoamylase, in contrast to exoamylases that work from the ends of the polymer, makes α-amylase faster and more effective for applications requiring rapid starch breakdown.

α-amylases are classified based on their action patterns into liquefying enzymes, which typically hydrolyze 30–40% of glycosidic linkages to reduce starch viscosity, and saccharifying enzymes, which can cleave 50–60% of the linkages to produce higher amounts of fermentable sugars. Acting on both amylose and amylopectin, they randomly cleaves internal α-1,4-glycosidic bonds in starch molecules, producing short-chain carbohydrates such as oligosaccharides, maltose, glucose, and dextrins. In detergents, α-amylases—primarily of the liquefying type—are employed to target starchy food residues, generating soluble fragments that lower viscosity and thereby facilitate effective stain removal (Tiwari et al., 2015).

In the detergent industry, α-amylase is the second most widely used enzyme, accounting for approximately 25% of enzymatic detergent formulations (Mitidieri et al., 2006). It was the second enzyme introduced after proteases and is now incorporated in nearly 90% of modern liquid detergents (Lahmar et al., 2017; Souza, 2010). It facilitates the removal of starchy stains from food residues such as pasta, potatoes, gravy, custard, and chocolate by breaking them into water-soluble fragments, enhancing their release from fabrics. Furthermore, α-amylase contributes to the anti-redeposition of soils, preventing reattachment of solubilized particles onto fabric surfaces (Gurkok, 2019).

α-amylases are produced from plants, animals, and microbes, but microbial α-amylases—particularly from *Bacillus* and *Aspergillus* species—are favored for detergent applications due to their alkaline and thermostable properties (Gupta et al., 2003; Mojsov, 2016). Key bacterial sources

include *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, and *B. amyloliquefaciens*, while fungal strains like *Aspergillus oryzae* and *A. niger* are also widely used in enzyme industries (Tiwari et al., 2015).

A notable biochemical feature of most α-amylases is their dependence on calcium ions (Ca^{2+}) as structural cofactors. Crystallographic analyses reveal that Ca^{2+} binds to two of the three enzyme domains, stabilizing the tertiary structure and ensuring optimal catalytic activity (Saini et al., 2017; Souza, 2010). However, detergent formulations often include builders and chelating agents such as EDTA or phosphonates, which sequester calcium ions to soften water, potentially inhibiting Ca-dependent amylases. This has spurred efforts to discover or engineer Ca-independent α-amylases that retain activity in the presence of chelators, thereby maintaining performance in detergent systems (Roy et al., 2012).

Several commercial α-amylases have been developed and optimized for detergent use, primarily by *Novozymes*, *Solvay*, and *Gist-brocades*. These enzymes differ in origin, stability, and operational pH or temperature range to meet the varying demands of detergent formulations.

1.3.3 Lipases

Lipolytic enzymes, particularly lipases (triacylglycerol hydrolases; EC 3.1.1.3), are gaining significant attention due to their vast biotechnological potential. Ranking third among industrial enzymes after proteases and carbohydrases, lipases are widely used in both household and industrial detergents (Guncheva & Zhiryakova, 2011). These enzymes catalyze the hydrolysis of water-insoluble triacylglycerols into glycerol, mono-/diacylglycerols, and free fatty acids, enabling the efficient breakdown of greasy stains such as butter, margarine, oils, sauces, soups, cosmetics, and human sebum (Gurkok, 2019).

The catalytic activity of lipases is mediated by a conserved triad—serine, histidine, and either aspartic or glutamic acid—located in the enzyme's active site. Most lipases also contain a structural feature known as a "lid", a polypeptide chain that covers the active site and regulates substrate accessibility based on interfacial activation (Guncheva & Zhiryakova, 2011).

Lipases are sourced from plants, animals, and microorganisms, but microbial lipases dominate industrial use due to their high stability, diversity, and ease of production. Key bacterial genera include *Bacillus*, *Pseudomonas*, *Burkholderia*, and *Chromobacterium*; yeast sources include *Candida rugosa*, *Yarrowia lipolytica*, and *Candida antarctica*; and mold-derived enzymes are obtained from *Aspergillus*, *Trichoderma*, and *Penicillium* species (Gupta et al., 2004; Hasan et al., 2006; P. Sharma et al., 2017).

Commercial lipase products used in detergents are largely derived from fungal and bacterial sources, each offering distinct advantages in terms of stability and performance. Among the fungal-derived enzymes, *Humicola lanuginosa* has been particularly influential. It was the original source of Lipolase®, the first lipase ever incorporated into detergent formulations, introduced by Novozymes. This breakthrough was followed by improved, protein-engineered variants including Lipolase® Ultra and Lipo Prime®, both designed for enhanced activity and resistance to harsh washing conditions. Another fungal source, *Thermomyces lanuginosus*, gave rise to Lipex®, also marketed by Novozymes, which is prized for its thermostability and compatibility with modern detergent systems (Hasan et al., 2010).

On the bacterial side, Genencor International developed Lumafast®, featuring lipase from *Pseudomonas mendocina*, known for its strong stain-removal capabilities. Similarly, Lipomax®, produced by Gist Brocades, is derived from *Pseudomonas alcaligenes* and has proven effective against a wide range of fatty soils. These products collectively highlight the strategic harnessing of microbial biodiversity and enzyme engineering to meet the evolving demands of the detergent industry (Gurkok, 2019; Hasan et al., 2010).

1.4 Enzyme production

1.4.1 Fermentation methods

The enzyme industry has experienced exponential growth in recent years. To meet increasing global demand, fermentation has emerged as a practical and efficient method for large-scale enzyme production (Renge et al., 2012).

Two primary cultivation techniques are commonly employed for microbial enzyme production: submerged fermentation (SmF) and solid-state fermentation (SSF).

- SmF in an oxygen-rich liquid nutrient medium and is considered a conventional, well-established method.
- SSF, by contrast, is conducted on solid substrates without the presence of free liquid and is more recent in development (Krishna, 2005).

Although debates persist regarding the superior method, each offers distinct advantages depending on the target enzyme and process requirements (Kapoor et al., 2016).

In SmF, the liquid medium facilitates uniform nutrient distribution and allows for easier monitoring and control of environmental parameters such as pH, temperature, and dissolved oxygen. Additionally, the aqueous environment simplifies the recovery and purification of target products, making SmF particularly suitable for large-scale enzyme production due to its relatively straightforward downstream processing. There are two main operational modes of SmF:

- Batch fermentation, which involves filling a closed bioreactor with sterilized medium and inoculum. After fermentation completes, the entire content is removed for downstream processing, and the reactor is cleaned and reused for subsequent batches. This approach, however, typically yields lower productivity due to feedback inhibition and downtime between batches.
- Continuous fermentation, where fresh medium is continuously fed into the reactor while culture fluid, including microbial biomass and products, is simultaneously removed. This system supports stable microbial growth under optimal conditions and can significantly enhance productivity by maintaining uninterrupted operations (Al-Maqtari et al., 2019).

SSF has garnered renewed interest over the past decade, particularly because certain microbial strains, including engineered mutants, demonstrate enhanced growth and productivity under SSF conditions. SSF offers several advantages, including:

(1) high volumetric productivity,

(2) elevated product concentrations,

(3) reduced effluent generation, and

(4) simpler equipment requirements (Renge et al., 2012).

However, SSF is not without challenges. It often involves low moisture levels, limiting the range of microorganisms that can be employed. Fungi, particularly filamentous species, are generally more suited for SSF due to their ability to thrive in low-water environments (Liu & Kokare, 2017).

1.4.2 Effects of different factors on fermentative production of microbial enzyme

While enzymes are not classified as secondary metabolites, they are often considered primary metabolic products when produced during the exponential phase of microbial growth. However, in industrial fermentation, many commercially important enzymes—such as proteases, lipases, and some amylases—are non-growth-associated. Their synthesis typically occurs during the late exponential or stationary phase under nutrient-limited conditions, reflecting a regulatory pattern similar to that of secondary metabolism. The efficiency of enzyme production is critically influenced by various factors, including nutrient composition, pH, temperature, inoculum preparation, and aeration.

1.4.2.1 Production media

As enzymes are typically primary metabolites or growth-associated products, the choice of media compositions can affect production. While the basic formulation includes carbon and nitrogen sources along with essential minerals, strategic modifications are often required to maximize productivity. Notably, production media often mirror the growth media but are optimized for enzyme biosynthesis rather than cell proliferation (Masuko et al., 2005).

In real-world applications, complex media rich in undefined nutrients tend to enhance enzyme yields due to the presence of natural inducers (S. Sood et al., 2011). Carbon sources play a pivotal role in providing primary energy source and carbon skeleton for biomass and metabolite synthesis. Quantity and type influence whether cells prioritize growth or enzyme production. The industrial enzymes are generally produced by microorganisms with low carbon source concentration. This makes the production cost effective used. In some cases, carbon sources can

serve as inducers (e.g., starch for amylase, casein for protease, oil for lipase) stimulate enzyme gene expression (Niyonzima, 2019). However, when excessive readily metabolizable carbon (e.g., glucose) presented often causes catabolite repression, thereby inhibiting enzyme synthesis due to mechanisms like the lac operon and cAMP-CRP system (Wang et al., 2019). This phenomenon, known as *diauxie*, reflects microbial preference for simple sugars, with enzyme production typically initiated only after these are depleted. Hence, complex carbon sources such as polysaccharides (e.g., starch), oligosaccharides (e.g., lactose), and oils (e.g., soybean oil) are generally more favorable for enzyme induction (Marinelli & Marcone, 2011).

Nitrogen availability significantly influences enzyme synthesis, as it provides essential components for the biosynthesis of cell wall components, amino acids, peptides, proteins, nucleotides, and coenzymes. While inorganic nitrogen sources such as ammonium salts can support rapid microbial growth, they may also suppress enzyme production through catabolite repression mechanisms. In contrast, slowly metabolized organic nitrogen sources—including protein hydrolysates, peptones, and specific amino acids—are often more favorable for enzyme biosynthesis, as they sustain balanced growth while promoting enzyme expression (Niyonzima, 2019).

Similarly, minerals and trace elements serve as vital cofactors or structural stabilizers for both microbial metabolism and enzyme function. Imbalances—whether deficiency or excess—of key ions such as magnesium, calcium, iron, or zinc can adversely affect microbial growth and enzyme integrity. Certain ions, such as calcium (Ca^{2+}), not only support microbial growth but also enhance the activity and thermostability of enzymes like α-amylase. Moreover, trace metals such as copper (Cu^{2+}) can regulate the expression of specific enzymes, as demonstrated in the case of laccase induction in fungal systems (Myszograj et al., 2018).

1.4.2.2 Temperature

Temperature control during fermentation is critical for microbial metabolism, enzyme productivity and protein denaturation. Each group of microorganisms exhibits distinct optimal growth temperatures, leading to production of enzymes with varying thermal profiles (Al-

Ghanayem & Joseph, 2020). While mesophilic enzymes currently dominate the commercial market, cold-active enzymes—especially those derived from Antarctic strains—are gaining traction for low-temperature applications, particularly in the detergent industry (Sharma & Rampelotto, 2016). For instant, *Bacillus* species, widely used in industrial processes, span various thermal classes: psychrophilic, mesophilic, and thermophilic (Harirchi et al., 2022).

1.4.2.3 pH

pH significantly influences both microbial growth and extracellular enzyme activity. The pH of the culture medium influences all enzymatic reactions and the transport of various solutes across the microbial cell membrane. Although the molecular mechanisms underlying the effects of pH on bacterial metabolism in broth culture remain not fully understood, it is known that pH can alter the proton motive force involved in chemiosmosis. Consequently, metabolic efficiency is likely enhanced within an optimal pH range, where membrane potential and energy transduction systems function most effectively. Therefore, pH is a critical parameter that must be optimized in fermentation processes (K. M. Sharma et al., 2017). Each microorganism has a narrow pH range for optimal performance (Jin & Kirk, 2018). For example, *Aspergillus niger* produced maximum cellulase activity at pH 4.0 (Sulyman et al., 2020), while *Bacillus subtilis* protease activity peaked at pH 6-9.0, highlighting species-specific pH optima (Danilova & Sharipova, 2020).

pH can be regulated directly through the addition of buffering agents such as phosphates, calcium carbonate, ammonia, or acids. It can also be controlled indirectly through nutrient formulation: carbohydrate metabolism often leads to acidification, while nitrate assimilation tends to raise pH (S. Sood et al., 2011). In SmF systems, maintaining a stable pH before and during cultivation is a widely adopted strategy to ensure consistent enzyme yields. However, allowing pH to "float" after initial adjustment can sometimes be beneficial, particularly when the natural pH drift promotes enzyme synthesis. Depending on the microbial strain and substrate composition, pH may either increase or decrease during fermentation. Thus, pH variations during fermentation can serve as a kinetic indicator of enzyme production phases. Monitoring these shifts provides a simple, non-invasive means of tracking the timing and progression of protease biosynthesis (Kumar & Takagi, 1999).

1.4.2.4 Inoculum volume and age

Inoculum preparation is a crucial step in scaling up fermentation processes determining how rapidly the production phase is reached and how synchronized the culture is. Seed cultures should be harvested during the mid-logarithmic phase to ensure vigorous microbial activity. Subsequent inoculum transfers are conducted under conditions that simulate large-scale fermentation until the desired volume is reached. An optimal inoculum size correlates with enhanced microbial biomass and product formation, highlighting its importance in achieving efficient fermentation outcomes (Kapoor et al., 2016; S. Sood et al., 2011).

In bacterial enzyme production processes, inoculum concentrations in the range of 0.6% to 4% (v/v) have been reported as optimal for achieving high yields. Inoculum levels below this range often result in delayed growth and extended lag phases, while higher concentrations can lead to rapid nutrient depletion and decreased enzyme output due to biomass overload and oxygen limitation (Kapoor et al., 2016; Niyonzima, 2019).

1.4.2.5 Aeration and agitation

Adequate aeration is essential for the growth of aerobic microorganisms and, consequently, for optimal enzyme production. In submerged fermentation (SmF), oxygen supply is more easily controlled due to the presence of a liquid medium, typically achieved through flask shaking or via spargers and impellers in stirred-tank bioreactors. In contrast, oxygen diffusion in solid-state fermentation (SSF) is more challenging, owing to the absence of free-flowing liquid, which limits gas exchange. During scale-up, oxygen transfer and heat dissipation become critical bottlenecks, necessitating close monitoring and engineering interventions to maintain favorable fermentation conditions (Kapoor et al., 2016).

Aeration not only supplies the oxygen required for microbial respiration but also helps remove metabolic exhaust gases produced during fermentation. However, high aeration rates can reduce the working volume of the fermentation broth and may increase foam formation. Additionally, while sufficient dissolved oxygen is crucial, excessive oxygen concentrations can lead to oxygen toxicity in certain microorganisms (Zhou et al., 2018).

Dissolved oxygen profiles can be modulated by: (i) adjusting the aeration rate, (ii) varying the agitation speed, or (iii) using customized gas mixtures, such as air-oxygen or air-nitrogen blends (Kumar & Takagi, 1999). Agitation primarily serves to mix the broth, enhance gas–liquid contact, and distribute heat and nutrients uniformly. Efficient agitation also helps break large air bubbles into smaller ones, increasing the gas–liquid interfacial area and preventing mycelial clumping, which improves oxygen absorption. However, overly vigorous agitation can introduce high shear forces and non-uniform mixing zones, potentially damaging shear-sensitive organisms and negatively impacting enzyme yields. Conversely, insufficient agitation may cause increased broth viscosity and poor mass transfer efficiency (Zhou et al., 2018).

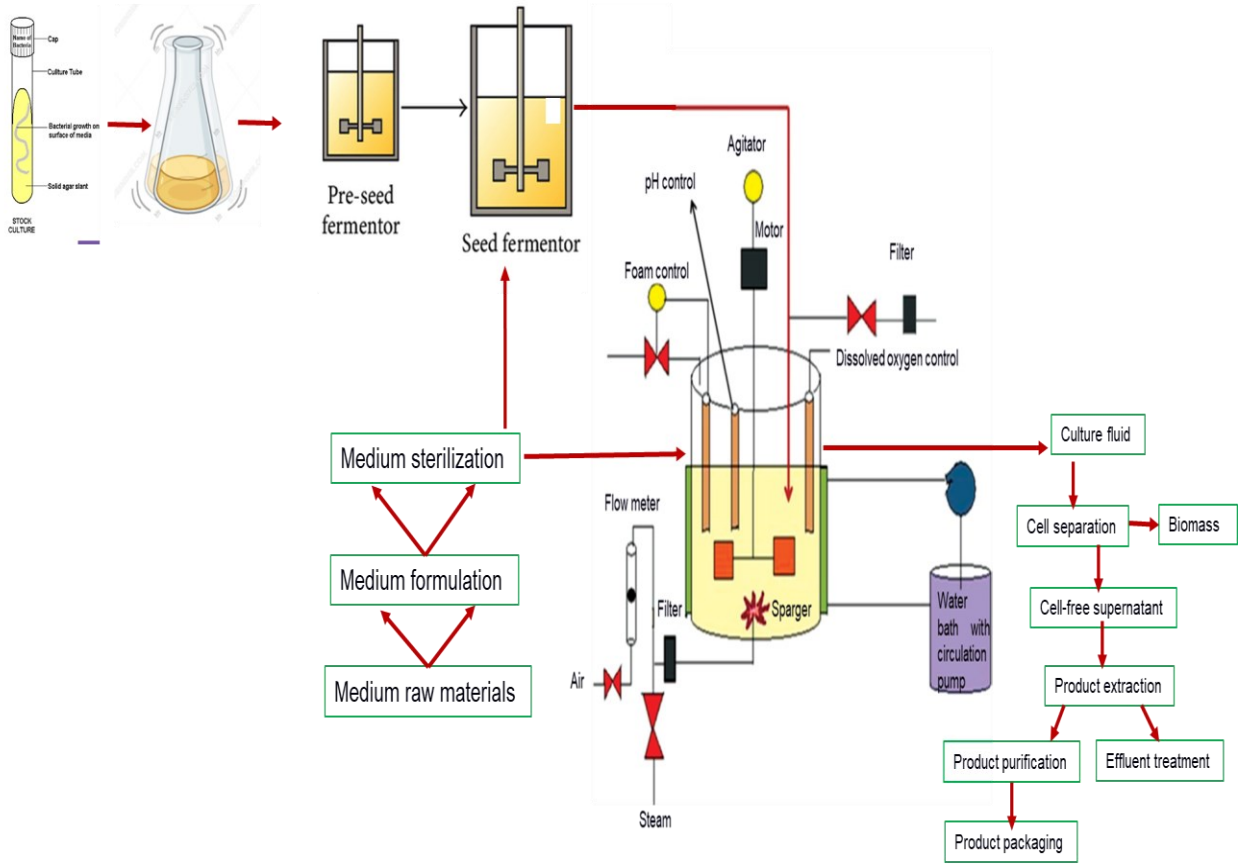


Figure 1.5 Design of a large-scale fermenter with some modifications, ranging from starter culture to products in association with the control of several important factors during fermentation

(S Sood et al., 2011)

1.5 Challenges in scale-up fermentation for producing enzymes

Fermentation lies at the core of industrial enzyme production and broader biotechnological applications. However, scaling up fermentation from laboratory to industrial scales presents substantial challenges. Despite extensive optimization at the laboratory level, scale-up often results in reduced biomass accumulation, lower substrate conversion, and diminished product titers (Schmidt, 2005).

Among the available strategies, solid-state fermentation (SSF) offers several advantages, including higher enzyme yields, efficient substrate utilization, and minimal water requirements. However, despite these benefits, SSF faces major challenges that hinder its adoption at industrial scale. These include difficulties in maintaining process uniformity, controlling temperature and aeration, and ensuring reproducibility. As a result, large-scale SSF operations often rely on multiple small units—such as parallel tray systems or rotating drum fermenters—instead of a single large bioreactor (Al-Maqtari et al., 2019; Niyonzima, 2019). One of the primary concerns is heat accumulation within the substrate matrix, which can raise temperatures to levels that denature the enzymes produced. While forced aeration has been proposed to mitigate this issue, it may exacerbate water loss through evaporation, complicating moisture regulation and further affecting microbial activity and enzyme stability. The inability to standardize SSF processes across scales remains a significant limitation for commercial enzyme production (Ravindran & Jaiswal, 2016).

In contrast, submerged fermentation (SmF) remains the most widely adopted method for large-scale enzyme production, primarily due to its superior control over process parameters and compatibility with automation. SmF enables the secretion of large amounts of extracellular enzymes directly into the liquid medium, thereby facilitating downstream recovery and purification. Parameters such as temperature, pH, aeration, and agitation can be accurately monitored and adjusted in real time. Nonetheless, discrepancies often arise during scale-up due to variations in mixing efficiency, oxygen transfer, shear forces, and heat distribution between small and large reactors. While these challenges are better studied and more manageable in SmF, they still require careful engineering considerations (Crater & Lievense, 2018; Takors, 2012)..

Furthermore, SmF processes are capital-intensive. A major portion of annual operating costs arises from the use of expensive synthetic media, as well as equipment, energy, and installation expenses. These factors ultimately influence the overall cost of the enzyme and, indirectly, the final product in which the enzyme is used. Compounding the issue, economic projections are often based on laboratory data, which may not reflect the full complexity of large-scale operations (Ravindran & Jaiswal, 2016).

Therefore, the development of robust, scalable fermentation strategies—alongside the search for low-cost substrates—is essential to ensure consistent enzyme yield, quality, and process reproducibility. Addressing these scale-up and cost-related challenges remains a central focus in industrial biotechnology.

1.6 Bioconversion as a strategy for developing bio-based detergents

Despite the increasing global demand for enzymes, large-scale production remains economically challenging (Emran et al., 2020). This financial barrier has limited the widespread adoption of bio-based detergents, particularly in economically developed countries. Therefore, the development of enzyme-based detergents necessitates well-planned strategies to bridge the gap between financial investment and industrial scalability. A key approach to reducing production costs involves optimizing fermentation processes, especially by minimizing the expense of raw materials, which often constitute the bulk of total costs. One promising solution is the use of renewable resources and industrial waste streams as alternative fermentation substrates (Sahay & Chouhan, 2018).

1.6.1 Starch wastewater

Starch is widely used as a raw material in both food industries—such as in bakery products, confectioneries, canned goods, caramel, and monosodium glutamate—and non-food sectors including chemical, textile, pharmaceutical, paper, mining, construction, and consumer goods industries (Industrial Starch Market Size, Share & Trends Analysis Report, 2020–2028). However, the extensive industrial use of starch generates large volumes of wastewater: the production of 1 ton of starch typically results in 10–20 m^3 of wastewater. This effluent poses a significant

environmental burden due to its high concentrations of chemical oxygen demand (COD: 5,000–50,000 mg/L), biological oxygen demand (BOD: 3,000–30,000 mg/L), and suspended solids (SS: 1,000–5,000 mg/L) (Cai et al., 2019).

Although starch wastewater is non-toxic and primarily composed of dissolved starch, it also contains small amounts of proteins, organic acids, dust, minerals, and residual fats and oils (Adjalle et al., 2009). Its high organic content makes it susceptible to natural fermentation, causing the water to turn black and malodorous. When discharged into aquatic systems, the high oxygen demand for biodegradation leads to eutrophication, threatening aquatic ecosystems, animal populations, and human health (Cai et al., 2019).

Various treatment strategies have been developed to remediate starch wastewater, aiming primarily to remove carbon and nitrogen content and produce water clean enough for reuse following biological treatment (Shubhaneel et al., 2018). However, due to its acidic nature, pH adjustment—through dilution or chemical neutralization—is often necessary before conventional treatment. Among biological methods, the activated sludge process is commonly employed, yet it suffers from several drawbacks including high energy demands, greenhouse gas emissions, low sludge degradability, and disposal challenges (Li et al., 2019). These issues are increasingly criticized in light of climate change and the need for sustainable, low-emission technologies (Zhang et al., 2021).

Consequently, there is a growing emphasis on resource recovery, cyclic utilization, and the integration of circular economy principles. This includes recovering valuable compounds from starch wastewater and adopting combined treatment technologies (Cai et al., 2019). Numerous studies have explored the valorization of starch wastewater for the production of polyhydroxyalkanoates (PHA), high-value animal feed, biosurfactants, amino acids, organic acids, antibiotics, *Bacillus thuringiensis* (Bt) biopesticides, and industrial enzymes (Brar et al., 2005; Hamilton et al., 1998; Li et al., 2019).

1.6.2 Pulp and paper mill activated sludge (PPMS)

The pulp and paper industry is a key sector of the North American economy, ranking fifth in the U.S. and contributing significantly to Canada's GDP in terms of production value and employment (Pokhrel & Viraraghavan, 2004). Globally, annual production of paper and paperboard reached 413 million tonnes (FAOSTAT, 2017), and demand continues to grow due to economic expansion and the increasing need for renewable resources. However, this industry is also among the largest consumers of freshwater, requiring approximately 20,000 to 60,000 gallons per ton of product, which leads to the generation of large volumes of wastewater (Pokhrel & Viraraghavan, 2004). Conventional treatment processes, such as activated sludge and aerated lagoon systems, are commonly employed.

Sludge generated in pulp and paper mills can be categorized into four types: (1) primary sludge (PS), derived from virgin wood fiber processing; (2) de-inking paper sludge (DPS), from recycled paper de-inking; (3) secondary sludge (SS), originating from secondary (biological) wastewater treatment; and (4) combined sludge. In Canada, the solid sludge composition consists of 42% PS, 12% DPS, 26% SS, and 18% combined sludge. While PS has been utilized in applications such as hardboard production, and DPS in building materials, SS—due to its high biological content—has often been excluded from such uses (Geng et al., 2007). On average, each Canadian pulp mill produces around 40 oven-dry tons of secondary sludge daily, which is typically dewatered and disposed of via landfilling or incineration. Across the country, more than half a million tons of sludge are produced annually from various sources, including secondary treatment plants, power boilers, and sawmills (https://watershedsentinel.ca/article/the-pulp-pollution-primer/).

PPMS, the organic residual from the biological treatment of wastewater, consists primarily of microbial biomass along with cellulose, hemicellulose, and lignin (Kaur et al., 2020). The biological process reduces levels of dissolved organic matter, chemical oxygen demand (COD), and biochemical oxygen demand (BOD) in the effluent (Kamali & Khodaparast, 2015). However, the management of PPMS remains a significant challenge due to its potential to cause environmental pollution if not properly handled.

PPMS is typically rich in organic carbon and relatively poor in nutrients such as sodium, potassium, calcium, magnesium, manganese, iron, and chloride. Due to its complex composition, PPMS can be subjected to physical, biological, or thermo-chemical pretreatments to release fermentable sugars, which can be used for the production of value-added products such as biofuels, bioplastics, and enzymes (Zhang et al., 2017).

Moreover, the microbial diversity within PPMS makes it a promising reservoir of resilient and adapted bacteria, which may serve as robust candidates for industrial enzyme production. Several studies have explored the microbial communities inhabiting paper mill sludge, revealing that it acts as an incubator for a wide range of enzymatically active microorganisms (Karn et al., 2013; Maki et al., 2011). For example, Ghribi et al. (2016) isolated several *Bacillus* species—including *B. amyloliquefaciens*, *B. subtilis*, *B. tequilensis*, *B. thuringiensis*, and *B. cereus*—from PPMS samples collected in Trois-Rivières, Québec. These strains demonstrated the capacity to secrete a broad spectrum of extracellular hydrolytic enzymes, including esterase, lipase, amylase, cellulase, xylanase, protease, and ligninolytic enzymes. This finding highlights the potential of PPMS as a valuable substrate and microbial source for enzyme production, particularly using *Bacillus* species.

PPMS has gained attention as a promising substrate for bioconversion into various value-added products, contributing to waste reduction and resource recovery. Enzyme production is of important application, where Bacillus strains isolated from PPMS have been shown to secrete cellulase, xylanase, and other hydrolytic enzymes (Ghribi et al., 2016; Kaur et al., 2020). Collectively, that exemplifies the potential of PPMS as a sustainable input for integrated biorefinery applications.

1.6.3 Food and beverage wastewater

There has been growing interest in the valorization of food and beverage (F&B) waste through biorefinery approaches, which aim to convert organic residues into value-added products. Globally, food waste exceeds 1.3 billion metric tons annually, with a significant portion discarded even before consumption due to inadequate purchase planning and strict aesthetic standards.

Among these waste streams, residues from beverage production—including by-products and processing waste—represent a substantial and underutilized flow of materials (Kusch-Brandt et al., 2019; Kwan et al., 2019).

Traditionally, F&B waste is managed through methods such as composting, anaerobic digestion, or animal feed supplementation. However, biorefineries offer a more integrated and sustainable solution by converting diverse food industry residues into high-value biochemicals, enzymes, biofuels, and functional ingredients (Kwan et al., 2019).

Extensive studies have evaluated the complex composition, seasonal variability, and conversion potential of various F&B waste streams. These include oil cakes, meat and fish waste, dairy waste, and fruit and vegetable residues. While many waste types are rich in non-starch carbohydrates and lignin, grain-based wastes also contain considerable amounts of starch, proteins, lipids, and glucans (Ravindran et al., 2018).

The beverage sector generates substantial volumes of bioresidues that can serve as renewable biorefinery feedstocks. These fall into three main categories: (1) pomaces from fruit and vegetable processing (e.g., skins, seeds, stalks), (2) residues from coffee, cocoa, or tea drink production, and (3) waste from wineries, breweries, and distilleries. Apple pomace and similar by-products are additionally valuable as sources of polyphenols, dietary fiber, and bioactive compounds that can enhance microbial growth in fermentation. Valorizing these materials contributes to the development of a circular economy and supports the transition toward renewable resource-based production systems, unlocking synergies between the food and bioenergy sectors (Kusch-Brandt et al., 2019).

F&B industry wastewaters have been widely explored as alternative substrates for microbial bioconversion into high-value products, owing to their rich organic content, including sugars, proteins, fats, and micronutrients. For example, cheese whey—a byproduct of dairy processing rich in lactose, proteins, lactic acid, and minerals—has been successfully used for enzyme production. *Bacillus subtilis* was shown to produce nattokinase, a fibrinolytic enzyme, using cheese whey as the sole fermentation medium (Sahoo et al., 2020). Similarly, *Bacillus sp.* isolated

from coffee beans synthesized alkaline protease in a medium containing nutrient broth and cheese whey, demonstrating the utility of this waste stream for industrial enzyme production (Dias et al., 2008). Olive oil mill wastewater (OOMW), known for its high organic load and phenolic compounds, supports the growth of selected microbes such as *Candida cylindracea*, which produced extracellular lipase when cultivated in undiluted OOMW supplemented with NH_4Cl (D'Annibale et al., 2006). In the beverage sector, brewery wastewater has been used as a fermentation medium for *Bacillus subtilis*, resulting in the simultaneous production of amylase and protease, thanks to its content of sugars, ethanol, and soluble starches (Blanco et al., 2016). Winery wastewater, another carbohydrate-rich effluent, has served as a feedstock for mixed microbial cultures to produce polyhydroxyalkanoates (PHA), biodegradable plastics with broad industrial applications (Fang et al., 2019). Dairy wastewaters have also been utilized for biosurfactant production; for instance, *Lactobacillus pentosus* was cultivated in dairy effluent to yield glycolipid biosurfactants with emulsifying properties (Gudiña et al., 2015). These examples underscore the versatility of food and beverage wastewater as a low-cost, nutrient-rich resource for microbial bioconversion, aligning with circular economy strategies and offering sustainable solutions for waste valorization.

1.7 Selection of bacteria

In the category of microbial enzymes, bacteria, particularly *Bacillus* species, are gained much attention. *Bacillus* spp. bacteria occur widely in nature. They can be found in niche environments but are predominantly found in soil, thus, they must produce a variety of precious substances for their metabolism. With few exceptions, *Bacillus* species are saprophytes and the majority, with the notable exceptions *B. cereus*, *B. anthracis*, and *B. thuringiensis*, are on the Food and Drug Administration's GRAS list which is generally recognised as safe for use (Schallmey et al., 2004).

Members of the genus *Bacillus* are industrial microorganisms of choice since their growth conditions are relatively simple (Schallmey et al., 2004) and they produce diverse, highly valued products. *Bacillus* spp. vary their colony morphology considerably from species to species. It is studied that Bacillus spp. exhibit optimal growth on peptone or nutrient agar plates, at pH 7 and between 30 and 45°C, but at 65°C for thermophilic strains (Grutsch et al., 2018). All *Bacillus* spp.

can utilise organic substrates, which help them spread out in most natural environments. Their typical metabolic mechanisms, which are mainly categorised into aerobic respiration, cellular respiration, and fermentation, convert an abundance of substances, namely sugars, organic acids, and amino acids. Through these processes, *Bacillus* spp. are well known for their ability to secret an array of both primary and secondary metabolites, such as antibiotics, probiotics, enzymes, vitamins, the plant hormone IAA, biosurfactants, etc. which are in high demand in agriculture, biopharmaceuticals, and industries (Grutsch et al., 2018).

Bacillus species are also major workhorses among the industrial microorganisms applied in many sectors. *Bacillus* spp. can concomitantly produce copious substances in a short cycle time but with their higher extracellular protein yields. In addition, *Bacillus* genome has been well studied. This makes it an ideal subject for large-scale production. A key approach for future development and advanced exploitation of these bacteria in industrial processes will involve genomic sequences as they shed the light on the relationship between biochemistry, physiology, and genetics. Since the successful sequencing of *B. subtilis* as the first gram-positive bacterium whose genome was obtained, the genomes of several more *Bacillus* species have been sequenced (Sharma & Satyanarayana, 2013). This genome analysis provides cutting-edge methods for manipulating *Bacillus* species into producing diverse enzymes and chemicals, thus, enhancing their competitiveness as production hosts for genetic engineering (Dong & Zhang, 2014).

1.7.1 *Bacillus amyloliquefaciens*

The first *Bacillus amyloliquefaciens* strain was isolated from soil and named by the Japanese scientist Juichiro Fukumoto in 1943. The species name reflects its enzymatic function: "amylo" refers to starch, "lique" to liquefaction, and "faciens" to production—indicating its ability to produce a liquefying α-amylase (Ngalimat et al., 2021). However, *B. amyloliquefaciens* was not included in the Approved Lists of Bacterial Names due to its close phylogenetic and phenotypic similarity to *B. subtilis*, *B. licheniformis*, and *B. pumilus*, which together form the *B. subtilis* species complex. Based solely on classical phenotypic tests, *B. amyloliquefaciens* is difficult to distinguish from *B. subtilis*, and as a result, it was not recognized as an independent species in the Approved Lists published on 1 January 1980 (Nannan et al., 2018).

More recently, genome-based and gene-derived phylogenetic analyses have provided strong evidence to revive the species name *B. amyloliquefaciens*, as it shares less than 25%, 13%, and 5% DNA–DNA homology with *B. subtilis*, *B. licheniformis*, and *B. pumilus*, respectively, under optimal hybridization conditions (60–65 °C) (Nannan et al., 2018). Taxonomically, *B. amyloliquefaciens* is classified within the *B. subtilis* species complex, family Bacillaceae, class Bacilli, and phylum Firmicutes (Ngalimat et al., 2021).

This species is commonly isolated from soil and industrial amylase fermentation environments (Nannan et al., 2018), and is widely exploited for the commercial production of α-amylase and protease (Ngalimat et al., 2021). Due to its high secretion capacity, non-pathogenic status, and robustness under extreme processing conditions, *B. amyloliquefaciens* is considered a key industrial workhorse in enzyme biotechnology. Its thermostable and alkaline-tolerant enzymes are particularly valuable in the detergent industry (Hasan et al., 2010; Schallmey et al., 2004). For example, Nour et al. (2024) reported the production of a keratinolytic protease from *B. amyloliquefaciens* EGY3 with high activity and stability in the presence of commercial detergent components, supporting its potential as a sustainable additive in laundry formulations. Similarly, the patent DE19824705A1 (1998) describes the successful incorporation of α-amylase derived from *B. amyloliquefaciens* into detergent formulations, improving the removal of starchy stains when used alongside proteases (https://patents.google.com/patent/DE19824705A1/en). These examples demonstrate the versatility and industrial relevance of *B. amyloliquefaciens*-based enzymes in modern detergent systems.

General characteristics of *Bacillus amyloliquefaciens* sp. nov., nom. rev., introduced by Priest et al. (1987) are summarized in Table 1.2.

Table 1.2 General information about *Bacillus amyloliquefaciens* sp. nov., nom. Rev

A single cell	• Rod-shape. • Size: 0.7 to 0.9 by 1.8 to 3.0 μm. • Cells can form chains and mobile
Spores	• Oval shape. • Size: 0.6 to 0.8 by 1.0 to 1.4 p,m. • Spores are at the central or paracentral in sporangia which are not swollen

Temperature for growth	• Optimal temperature: 30-40°C. • No growth: <15°C and >50°C.
Substrates	• Degradable substrates: casein, elastin, gelatin, starch, tributyrin, Tween 20, Tween 40, and Tween 60. • Non-degradable substrates: adenine, cellulose, guanine, hypoxanthine, pectin, testosterone, tyrosine, and xanthine.
Salinity tolerance	• 10% NaCl for most strains

1.7.2 *Bacillus megaterium*

Bacillus megaterium was first described in 1884 by De Bary and named for its notably large cell and spore size—the name meaning "big beast" (Vary, 1994). Its vegetative cells are approximately 100 times larger than those of *Escherichia coli*, which has long intrigued microbiologists interested in its morphology, physiology, and its potential to produce industrially significant enzymes (Vary, 1994). Officially adopted in industrial biotechnology over 50 years ago, *B. megaterium* has since generated considerable economic value.

Taxonomically, 16S rRNA sequence analysis places *B. megaterium* within the *B. subtilis* group, although it is less closely related to *B. subtilis* than to *B. licheniformis*, *B. cereus*, *B. anthracis*, or *B. pumilus* (Vary, 1994). Initially regarded as a soil bacterium, *B. megaterium* has been isolated from diverse environments, including rice paddies, seawater, dried foods, sediments, fish, bee honey, and even herbicide-contaminated sites. It can metabolize over 62 carbon sources, including all tricarboxylic acid cycle intermediates, formate, and acetate, reflecting its impressive metabolic flexibility.

This physiological versatility supports the production of a broad array of industrially valuable compounds, including penicillin amidase, steroid hydrolases, vitamin B12, oxetanocin, antimicrobial agents, and numerous extracellular enzymes. B. *megaterium* is a robust producer of enzymes highly relevant to detergent applications—such as α-amylase, β-amylase, lipase, and both neutral and alkaline proteases.

Several strains of *B. megaterium* have demonstrated exceptional enzyme performance under detergent-relevant conditions. For example, *B. megaterium* TK1, isolated from seawater, produces a thermostable extracellular serine protease that remains active across a broad pH range (7.0–11.0) and at elevated temperatures (20–70 °C). This enzyme retained significant activity after a one-hour incubation in commercial laundry detergents such as Surf Excel and Tide, and even exhibited dehairing potential on cowhide, highlighting its dual relevance to both detergent and leather processing industries (Manavalan et al., 2020). Similarly, an alkaline protease from *B. megaterium* RRM2 showed strong activity in detergent-compatible conditions and improved fabric cleaning efficiency (Rajkumar et al., 2011).

Lipase production has also been reported from *B. megaterium* AKG-1 issolated from soil, which yielded a thermostable enzyme in submerged fermentation (Sekhon et al., 2006). This lipase maintained stability at alkaline pH and elevated temperatures, supporting its utility for breaking down lipid-based stains in detergents. Another notable strain, *B. megaterium* F25—isolated from the gut of the aquatic insect *Rhantus suturalis*—produced a lipase with applications not only in the food and biodiesel sectors but also in detergents due to its effective lipid hydrolysis under industrial conditions (Karaman et al., 2024). Festus and Phebe (2017) reported the ability to produce lipase of *B. megaterium* in submerged fermentation of palm oil press fibres and effluent.

In terms of starch degradation, *B. megaterium* has been identified as a producer of stable α-amylases with strong activity toward carbohydrate-rich stains. Takasaki (Vary, 1994) reported an α-amylase with unusual cleavage specificity.

1.7.3 *Bacillus licheniformis*

Bacillus licheniformis is a member of the *Bacillus subtilis* group and has gained considerable industrial relevance due to its capacity to produce carbohydrases and proteases. The species was officially listed in the Third Edition of the Food Chemicals Codex in 1981, reflecting its recognition as a safe and effective producer of enzymes for large-scale food processing (de Boer et al., 1994). Although there have been isolated reports associating *B. licheniformis* with food-borne diarrheal illnesses, toxin production, infant mortality, and bovine abortion, the species is widely accepted

as non-pathogenic due to its lack of invasive characteristics and virulence factors (de Boer et al., 1994).

B. licheniformis can be isolated from various natural sources, including soil, plant material, and marine environments. It possesses significant industrial potential as a robust host for the production of both native and recombinant exoenzymes. These include allantoinase, cellulase, chitinase, cycloglucosyltransferase, dextranase, endo-arabinase (ABNase), β-glucosidase, β-glucanase, β-galactosidase, levanase, lipase, mannanase, pectate lyase, penicillinase, pentosanase, xylanase, and various proteases. Among its proteolytic arsenal are clostripain-like proteases, serine proteases, zinc-dependent proteases, glutamyl endopeptidase, metalloproteases, as well as multiple endo- and exopeptidases. In addition, *B. licheniformis* is a prolific producer of industrially important amylases (Muras et al., 2021).

Notably, *B. licheniformis* has been extensively studied for its production of detergent-compatible enzymes. For instance, the strain VSG1, isolated from tannery effluent, was found to concomitantly produce protease and lipase on a common medium. Both enzymes were purified and characterized, revealing optimal activity at alkaline pH and elevated temperatures, making them suitable for detergent formulations (Sangeetha et al., 2010). Similarly, *B. licheniformis* NH1 was reported to co-produce multiple extracellular proteases and a thermostable α-amylase, both exhibiting stability and activity under conditions relevant to detergent applications (Hmidet et al., 2009).

Furthermore, the alkaline protease from *B. licheniformis* RP1 demonstrated excellent stability and compatibility with a wide range of commercial solid detergents at temperatures from 40 to 50 °C, highlighting its potential as a detergent additive (Sellami-Kamoun et al., 2008).

These examples underscore the versatility and industrial significance of *B. licheniformis* in producing enzymes that enhance detergent performance by targeting a broad spectrum of stains, including proteins, fats, and carbohydrates.

1.8 Problems, hypothesis, objectives and originality

1.8.1 Problems

Based on the literature provided above, remarkable problems are still existing as follows:

- **Industrial wastewaters as abundant economic substrates for enzyme production**

Feasibility studies of newly developed bioprocesses must always include an economic evaluation that considers the balance between monetary investment, material consumption, and manpower requirements against the value of the final product. The total production cost of a biologically derived product, such as enzymes, typically comprises several components: (a) the cost of raw materials—including nutrients, additives, control chemicals, and packaging; (b) capital investment in equipment such as fermenters, separation units, air sterilization systems, recovery and purification apparatus, waste disposal systems, and instrumentation and control systems; (c) operating costs, which cover labor, repairs and maintenance, and overhead expenses; (d) utility or service costs, such as cooling water, refrigeration, electricity, steam generation, chilled water, sterile air, inert gases, and effluent treatment; and (e) research and development expenditures (Jackson, 1985). Among these, raw materials account for a substantial proportion of total production costs (Sahay & Chouhan, 2018). Conventional biomass sources—such as crops and agricultural residues—are commonly used but can be costly and may compete with food production, raising concerns about long-term sustainability. To address this, the valorization of industrial by-products has emerged as a cost-effective and sustainable strategy. Industrial sludge and wastewater offer a promising alternative feedstock, providing a continuous and renewable supply—an essential factor for the viability of bioprocesses in a biomass-based economy. This approach not only reduces dependency on conventional, high-cost feedstocks but also contributes to the principles of the bioeconomy by promoting waste minimization, resource efficiency, and the conversion of low-value residues into high-value bioproducts such as industrial enzymes, supporting the principles of the circular bioeconomy.

Pulp and paper mill sludge (PPMS), for example, is generated in large volumes and contains valuable components such as cellulose, hemicellulose, and microbial biomass. Yet, its disposal

remains a significant challenge, often involving environmentally and economically costly options like landfilling or energy recovery. Likewise, the starch industry produces wastewater rich in starch, proteins, and organic acids, but traditional treatment processes are energy-intensive and generate excess sludge. The food and beverage industry also contributes to the burden of organic wastewater, producing effluents containing carbohydrates, fats, proteins, and additives—components that are well-suited for microbial fermentation.

- **Efficiency of submerged fermentation for enzyme production at large-scale**

While SSF is often favored for its low energy and water requirements as well as its relatively simple infrastructure (Balakrishnan et al., 2021; Kapoor et al., 2016), SmF remains the dominant method for enzyme production—particularly when liquid substrates are used (Singh & Gupta, 2014). In SmF systems, in addition to controllable fermentation parameters—such as the type and concentration of carbon and nitrogen sources, pH, temperature, aeration, inoculum size, and inoculum age—technical challenges associated with scale-up are essential for in determining overall process efficiency. A primary goal of scale-up is to meet industrial and commercial demands by producing large volumes of enzymes with consistent quality. However, transitioning from laboratory-scale to industrial-scale fermentation is far from straightforward. Large bioreactors often exhibit non-homogeneous conditions, which can unpredictably impact microbial metabolism and enzyme synthesis. Therefore, an incremental scale-up approach using progressively larger bioreactors at a controlled dissolved oxygen level of 30–50% is recommended to balance oxygen transfer efficiency and microbial productivity during scale-up.

In conclusion, this project aims to develop a pilot-scale fermentation strategy that transforms abundant, underutilized residual media into a cost-effective substrate, thereby mitigating the economic burden of enzyme production.

1.8.2 Hypothesis

1.8.2.1 Industrial wastewaters are low-cost and fermentable residues

Organic wastewater from various industrial sources—such as the starch industry, pulp and paper mills, and the food and beverage sector—is generated in large volumes and can serve as a low-cost substrate for microbial fermentation. These wastewaters have been shown to contain essential nutrients for bacterial growth, including carbon, nitrogen, and minerals, and can support the production of enzymes such as protease, α-amylase, and lipase enzymatique (Choubane et al., 2015; Mazhar et al., 2016; Sánchez Blanco et al., 2016). Selection of a suitable agro-industrial residue for enzyme production must consider factors such as availability, transportation cost, and the need to minimize pretreatment and refined raw material supplementation (Domínguez Rivera et al., 2019). Therefore, with appropriate modifications or mild pretreatment to improve nutrient availability, wastewater can be converted into a viable fermentation medium.

1.8.2.2 *Bacillus spp.* are potential cultures for the conversion of organic waste into enzymes

Bacillus species are known for their ability to grow on a wide range of substrates and to produce multiple extracellular enzymes simultaneously. Their enzymatic products possess desirable characteristics—such as thermostability, alkalinity, and substrate specificity—that are especially valuable in detergent applications. According to existing literature, various *Bacillus* strains (e.g., *B. amyloliquefaciens, B. licheniformis, B. megaterium*) can adapt to the nutrient profiles of industrial wastewaters and are capable of producing targeted enzymes under such conditions (Muras et al., 2021; Nannan et al., 2018; Sekhon et al., 2006).

1.8.2.3 Isolating and screening native microorganisms from wastewater can reveal robust enzyme producers better suited for fermentation on complex waste substrates

A strategic approach involving the isolation of bacteria from specific environments—such as wastewater—can lead to the discovery of robust strains with high metabolic efficiency and adaptability (Masi et al., 2023). These native microorganisms may outperform commercial strains by producing higher enzyme titers when cultured on complex waste substrates. Hence, microbial

screening remains one of the most effective methods for identifying novel candidates suitable for enzyme production from agro-industrial waste streams.

1.8.2.4 Scale-up studies can aid the production of enzyme in large quantities

Successful translation from lab-scale experiments to industrial applications requires stepwise scale-up strategies. Shake flask studies alone are insufficient for technology transfer, as they do not reflect the heterogeneous conditions encountered in larger bioreactors. Optimization in bench-scale stirred tank reactors (STRs) is essential to address factors such as oxygen transfer, mixing, and shear stress. Among these, aeration and agitation rates are particularly critical, given the oxygen-dependent nature of enzyme secretion in aerobic organisms. The outcomes from optimization in 5 L bioreactors will serve as a basis for further scaling to 15 L and 150 L fermenters (Ndao et al., 2017). By maintaining dissolved oxygen (DO) levels between 30–50% throughout the scale-up, it is expected that high enzyme yields can be achieved under controlled fermentation conditions.

1.8.3 Objectives

This study aims to evaluate the potential wastewaters for producing enzymes—specifically protease, α-amylase, and lipase—using various types of organic wastewater as cost-effective and sustainable substrates.

The overall goal is to develop a pilot-scale enzyme production process, with the resulting enzymes being incorporated into bio-detergent formulations alongside other functional components. The project is divided into two main stages, each with specific objectives:

❖ ***Stage 1: Screening and optimization using commercial strains***

Objective 1: To select relevant types of industrial wastewaters and commercial *Bacillus* strains as fermentation candidates—based on literature review—for the production of detergent-relevant enzymes (protease, amylase, and lipase).

Objective 2: To identify the most promising wastewater substrates and strain–substrate combinations by evaluating microbial growth (cell density) and extracellular enzyme activities of the selected commercial strains.

Objective 3: To optimize key fermentation parameters (e.g., inoculum size, temperature, total solids) at the flask scale using the selected wastewater, and to assess reproducibility in 5 L bioreactors.

❖ ***Stage 2: Native strain isolation and pilot-scale fermentation***

Objective 4: To isolate protease-producing strains from the selected wastewater and conduct scale-up fermentation studies using the native isolates in 5 L and 150 L bioreactors.

Objective 5: To isolate lipase-producing strains from the selected wastewater and conduct scale-up fermentation studies using the native isolates in 5 L and 150 L bioreactors.

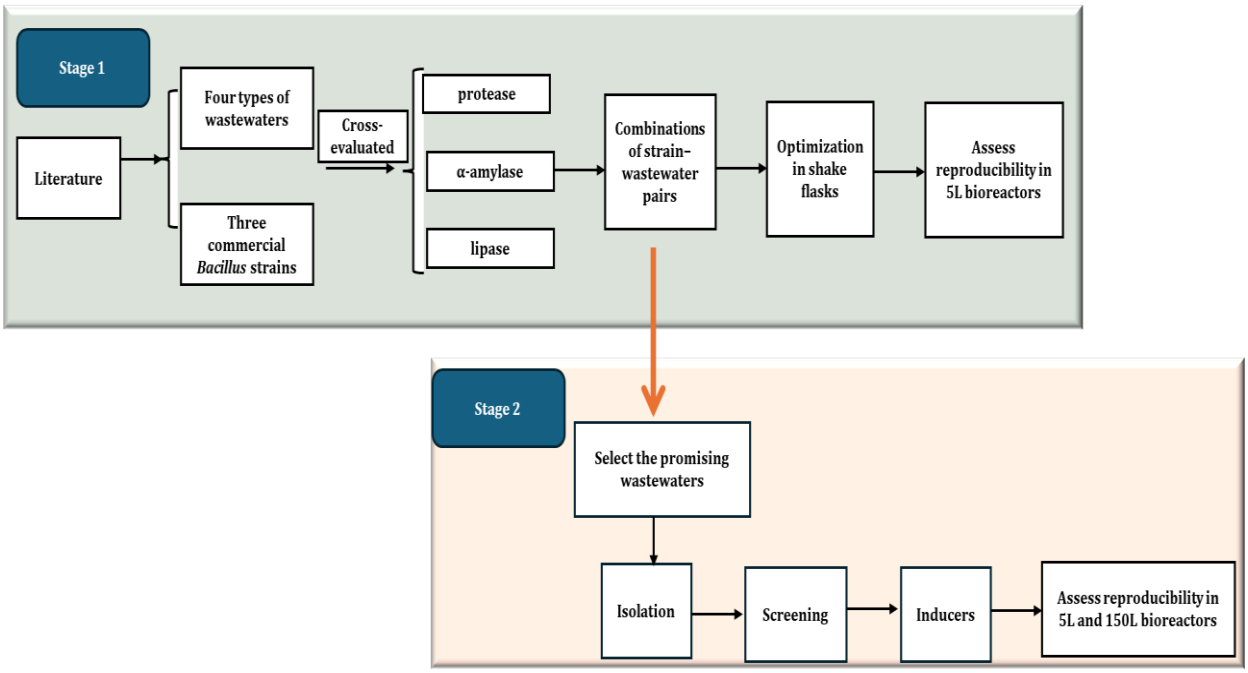


Figure 1.6 Process flow of enzyme production from industrial wastewaters

1.8.4 Novelty of the research

The originality of this study lies in its integrated approach to developing a pilot-scale fermentation strategy for detergent-compatible enzyme production using locally sourced industrial wastewaters. Unlike previous studies focused solely on commercial strains or lab-scale work, this project first identified promising waste streams through fermentation with commercial *Bacillus*

species, and subsequently isolated and characterized native microbial strains—specifically *Bacillus tropicus* and *Acinetobacter tandoii*—from the selected substrates.

This two-phase strategy allowed for the tailored selection of microbial candidates adapted to real wastewater conditions. Submerged fermentation using both commercial and native strains enabled the production of extracellular proteases, amylases, and lipases. Pilot-scale validation in a 150 L bioreactor demonstrated the process's scalability and industrial potential.

Moreover, this approach addresses two critical challenges simultaneously: reducing production costs through waste valorization and supporting environmental sustainability by converting low-value residues into high-value bio-based enzymes. The dual impact aligns with the principles of the circular bioeconomy and supports the development of affordable, eco-friendly detergent products.

2 METHODOLOGY

2.1 Selecting wastewater media by using commercial bacteria

Selecting proper wastewater media: Four types of wastewaters was collected locally in Québec, Canada. These media were selected according to their carbon/nitrogen/phosphorus compositions and their potential to support microbial growth, as well as the results of previous work carried out in our laboratory. Table 2.1 presents these four media, the different characterization parameters, as well as the analysis methods used.

Table 2.1 Characterization and analysis methods of wastewater media

Media	Sources	Storage	Analysis of total organic carbon (TOC) and total nitrogen (TN)	Analysis of mineral content	Analysis of different types of sugar
Starch industry wastewater (SIW)	ADM-Ogilvie (Candiac, Québec, Canada)	All types of wastewaters were stored in a cold room at 4˚C for 2 months	Instrumentation used: Shimadzu VCPH	Instrumentation used: ICP-5110 Dual View	Different sugars were investigated, including Glucose, Fructose, Lactose, Sucrose, Galactose, Xylose, Trehalose
Beverage wastewater (BW)	Brasserie rurale, (Cookshire-Eaton, Québec, Canada)				
Kruger pulp and paper mill activated sludge (PPMS)	Kruger Inc (Montreal, Québec, Canada)				
Food industry wastewater (FIW)	Diana Food Canada Inc (Champlain, Québec, Canada)				

***Screening of potential bacteria**:* according to the literature and some quick experiments carried out in a shake flask, three main strains were the subject of this project. The features of these bacteria are shown as follows (Table 2.2):

Table 2.2 Features of studied *Bacillus* strains

Strains	Conditions of growth	Enzymes
Bacillus licheniformis ATCC 14580	Temperature: 30-37°C	amylase, protease, lipase
Bacillus amyloliquefaciens ATCC 23842	pH 7	amylase, protease
Bacillus megaterium	Media: nutrient broth	protease, lipase

For the experiment, a volume of 2% of preculture of each strain were transferred into four media containing wastewater and nutrient broth (as control) after sterilization. Samples were collected at 24 and 48 h to check the growth of bacteria by using the Standard Plate Count technique and the measure of the activity of different enzyme production. The different analysis methods used are described in section 2.5.

2.2 Optimization of process parameters at shake flask scale

One of the primary objectives in enzyme production is to achieve maximum productivity; hence, process optimization is a critical component of research in this area. Preliminary optimization studies are commonly conducted in shaking flasks to identify favorable conditions, which serve as the foundation for subsequent scale-up investigations. Among various statistical tools, the Box–Behnken design (BBD) offers an efficient method for exploring the response surface with fewer experimental runs compared to traditional full factorial designs. This design strategically places experimental points at the midpoints of the edges of the experimental domain, typically coded as −1, 0, and +1, enabling robust modeling of interaction and quadratic effects.

In this study, the BBD was employed to investigate the influence of three independent variables—total solids (TS), inoculum size, and temperature—on enzyme activity. Enzyme activity, measured in U/mL, served as the response variable. The experimental design and data analysis were performed using Design-Expert® software (Version 7.0.0). The relationship between the independent variables and enzyme yield was modeled using a second-order polynomial equation:

$$Y = \beta 0 + \sum_{i-1}^{k} \beta iXi + \sum_{i-1}^{k} \beta iiXiXi + \sum_{i-1}^{k-1} \sum_{j-2}^{k} BijXiXj$$

where, Y is the predicted yield (U/mL), β0 is the intercept (a constant) and βi, βii, βij are regression coefficients for linear effects, regression coefficients for squared effects, regression coefficients for interaction effects. Xj and_Xj are the coded values of the independent variables. This model facilitates the identification of optimal conditions by examining the individual and combined effects of the selected factors on enzyme production.

2.3 Isolation and screening microorganisms from wastewater

2.3.1 Isolation

A total volume of 0.5 mL of PPMS was aseptically transferred into 4.5 mL of sterile distilled water to obtain a 10^{-1} dilution. Serial dilutions were then performed up to 10^{-5} by transferring 0.5 mL from each dilution into 4.5 mL of sterile distilled water in subsequent tubes. From the 10^{-3}, 10^{-4}, and 10^{-5} dilutions, 100 μL of each sample was inoculated onto nutrient agar plates (10 g/L peptone, 3 g/L beef extract, 5 g/L NaCl; pH 7.0) supplemented with 1% casein (w/v)/ 1% Tween 20 (v/v) using the spread plate technique. The plates were then incubated at 30 °C for 48 hours to allow for microbial growth and colony formation.

2.3.2 Screening

The screening of protease-producing isolates was conducted in two successive stages. Initially, a qualitative assessment was performed using spot inoculation on selective agar plates, followed by quantitative evaluation through cultivation on sludge medium.

For the qualitative screening of protease-producing isolates, spot inoculation was performed on selective agar plates containing (per liter): K_2HPO_4, 2 g; glucose, 1 g; peptone, 5 g; and casein, 10 g. The plates were incubated at 30 °C for 48 hours. Following incubation, the plates were flooded with 25% (w/v) trichloroacetic acid (TCA) and maintained at 45 °C for 15 minutes to precipitate unhydrolyzed proteins, thereby enhancing the visibility of proteolytic zones. The formation of clear zones surrounding the colonies was considered indicative of proteolytic activity.

Isolates exhibiting lipolytic activity on spotted agar were selected for further screening in liquid culture. Each isolate was inoculated into PPMS-based medium supplemented with 1% (v/v) Tween 20 and incubated at 30 °C, pH 7.0, for 48 hours under continuous agitation at 180 rpm using an orbital shaker. Following incubation, lipase activity in the culture supernatants was measured spectrophotometrically to identify the most efficient producers for further characterization.

For quantitative screening, isolates demonstrating clear zones were cultured in liquid media under the following conditions: incubation at 30 °C, pH 7.0, for 48 hours in an orbital shaker set at 180 rpm. Following fermentation, protease production was assessed to identify the most promising isolates for further characterization

2.4 Scale-up studies using 5, 150 L bioreactors

The optimal conditions obtained from previous work at the flask level are used as the baseline for further optimization on larger scale. Scale-up processing for enzyme production using selected wastewater was carried out with 5 L and 150 L bioreactors.

Fermentation experiments were conducted in a 5-liter glass bioreactor with a working volume of 3 liters, containing sludge with total solids of 25 g/L, unsupplemented or supplemented with 1% (v/v) Tween 80. The bioreactor was equipped with a programmable logic control (PLC) system to regulate and monitor key parameters, including dissolved oxygen (DO), pH, temperature, agitation speed, aeration rate, and antifoam addition. To ensure sterility, the PPMS medium was autoclaved at 121 °C and 15 psi for 30 minutes. Following sterilization and cooling down of the PPMS medium, 2% (v/v) of the pre-incubated inoculum—grown at 30 °C for 16 hours—was aseptically transferred into the medium. DO levels were maintained within a range of 30–50% by adjusting aeration and agitation. Samples were collected every 12 hours to track fermentation progress, with the goal of reaching cell concentrations of 10^8 CFU/mL and maximizing protease production.

Scale-up studies were conducted in a 150-liter stirred-tank reactor (STR) with a working volume of 90 liters, inoculated with 2% (v/v) pre-cultured inoculum. Cultivation was carried out at 30 °C, following the conditions optimized in the 5-liter bioreactor experiments. The sole PPMS medium

or added Tween 80, prepared and sterilized separately as previously described, were employed as the fermentation substrates. Dissolved oxygen (DO) levels were maintained between 30% and 50% by regulating aeration and agitation to support optimal microbial performance. Samples were withdrawn every 12 hours throughout the fermentation process for protease activity assays, enabling monitoring of enzyme production dynamics at the pilot scale.

2.5 Analysis Parameters

After fermentation in media, bacterial growth and the production of proteases, α-amylases, and lipases were tested to select the potential strains.

2.5.1 Colony forming units (CFU)

The samples for CFU were serially diluted in saline solution (0.8% w/v) followed by spread plating on nutrient agar plates (Harley, 2004). All the analysis was carried out in duplicates. The plates were incubated at 37°C for 24 h and CFU was determined as:

CFU/mL= (Colony count dilution factor)/ volume of sample taken for spread plating (mL)

2.5.2 Protease assay

Protease activity was measured using a casein hydrolysis method. A reaction was set up by mixing 1 mL of diluted enzyme with 1 mL of 1% casein in 50 mM Tris HCl buffer (pH 7) and incubating it at 50°C for 10 minutes. To stop the reaction, 2 mL of 15% trichloroacetic acid was added, and the mixture was centrifuged for 10 minutes at 10,000 rpm at 4°C. After centrifugation, 0.5 mL of the supernatant was taken and mixed with 2.5 mL of 2% sodium carbonate and 0.25 mL of 1 N Folin's reagent. The mixture was incubated at room temperature for 30 minutes. Absorbance was measured at 660 nm using a spectrophotometer. One unit of protease activity is the amount of enzyme that releases 1 μg of tyrosine under these conditions (Ramkumar et al., 2018).

2.5.3 Lipase assay

Lipase activity was determined using *p*-nitrophenyl palmitate (pNPP) as the substrate. The assay buffer was prepared by mixing 25 mL of 100 mM Tris-HCl (pH X), 5 mL of 100 mM $CaCl_2 \cdot 2H_2O$, 0.15 mL of Triton X-100, and 19.85 mL of distilled water. To prepare the substrate solution, 1 mL of 20 mM pNPP was dissolved in 19 mL of the assay buffer. For the assay, 2.76 mL of the substrate solution was mixed with 0.24 mL of enzyme solution. The reaction was carried out at 30 °C for 30 minutes, and the release of *p*-nitrophenol was monitored by measuring the absorbance at 410 nm. One unit (U) of lipase activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute under the assay conditions (Shart & Elkhalil, 2020).

2.5.4 Amylase assay

Amylase activity was determined via the DNS method. The reaction mixture, containing 0.1 mL of appropriately diluted enzyme and 0.9 mL of 1.0% (w/v) corn starch in 50 mM Tris HCl buffer (pH 7.0), was incubated at 50°C for 10 minutes. The amount of reducing sugars liberated was determined using the dinitrosalicylic (DNS) acid method. After the reaction, 1.5 mL of DNS reagent was added, and the mixture was boiled for 10 minutes before being diluted with 5 mL of distilled water. The absorbance was then measured at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per minute. A standard curve was plotted using D-glucose. The α-amylase activity is represented by the mean value of two determinations, each performed in duplicate (Kherouf et al., 2021).

3 *BACILLUS* SPECIES: EVOLVING ROLES IN BIO-BASED DETERGENTS

Review

Espèces de *Bacillus*: rôles évolutifs dans les détergents biosourcés

Vu-Mai-Linh Nguyen[1], Adama Ndao[1], Eric Charles Peterson[1], Jean-François Blais[1], Kokou Adjallé[1,]

[1] Centre Eau Terre Environnement (ETE), Institut National de la Recherche Scientifique (INRS), 490 Rue de la Couronne, Québec City, QC G1K 9A9, Canada

Kokou Adjallé. Email: kokou.Adjalle@inrs.ca (Corresponding author)

Vu_mai_linh.nguyen@inrs.ca

Adama Ndao. Email: adama.ndao@inrs.ca

Eric.peterson@inrs.ca

Jean-françois.blais@inrs.ca

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3.1 Abstract

Enzymes and biosurfactants, often referred to as "green chemicals," play pivotal roles in enhancing the washing performance of bio-based detergents—a growing trend driven by environmentally conscious consumers. However, the widespread adoption of such bio-based detergents faces challenges, including high costs, limited efficiency, and the need for ongoing innovations. *Bacillus* species have long been universally acknowledged and exploited for industrial applications, and *Bacillus* spp. are largely differentiated from other microorganisms for their enzymatic applications, particularly in detergent production. Recent developments in bio-surfactant production by *Bacillus* sp. supports the adoption of green detergents and these bacterial biosurfactants are a promising source for detergent manufacturing. This article provides an overview of the current understanding of promising *Bacillus* species and their potential to advance and accelerate the production of bio-based detergents.

Keywords: bio-based detergents; proteases; α-amylase; cellulases; lipases; biosurfactants; *Bacillus* species; low-cost substrates; cold enzymes; directed evolution; CRISPR-based genetic tools; Cas9.

Les enzymes et les biosurfactants, souvent qualifiés de « produits chimiques verts », jouent un rôle essentiel dans l'amélioration des performances de lavage des détergents biosourcés—une tendance croissante stimulée par les consommateurs soucieux de l'environnement. Cependant, l'adoption généralisée de ces détergents bio-sourcés se heurte à plusieurs défis, notamment des coûts élevés, une efficacité limitée et un besoin constant d'innovations. Les espèces de *Bacillus* sont depuis longtemps reconnues et exploitées dans les applications industrielles, et se distinguent particulièrement des autres micro-organismes par leur potentiel enzymatique, notamment dans la production de détergents. Les avancées récentes dans la production de biosurfactants par des souches de *Bacillus* soutiennent l'essor des détergents écologiques, ces biosurfactants bactériens représentant une source prometteuse pour la fabrication de détergents. Cet article propose un aperçu des connaissances actuelles sur les espèces de *Bacillus* les plus prometteuses et de leur potentiel pour faire progresser et accélérer la production de détergents biosourcés.

Mots-clés : détergents biosourcés ; protéases ; α-amylase ; cellulases ; lipases ; biosurfactants ; espèces de *Bacillus* ; substrats à faible coût ; enzymes actives à froid ; évolution dirigée ; outils génétiques basés sur CRISPR ; Cas9

3.2 Introduction

Detergents have become an indispensable commodity in modern society, driven by factors such as population growth, increased urbanization, and industrialization, particularly in the wake of the COVID-19 pandemic. However, the growth of detergent market risks increased adverse environmental impact through use of non-biodegradable detergent, due to the bioaccumulation of synthetic surfactants, low biodegradability, and higher solid content, putting greater pressure on ecosystems (Giagnorio et al., 2017).

Detergents have changed significantly over history, with incremental improvements to their ingredients for various reasons, yet surfactants remain the central component (Cheng et al., 2020; IUPAC, 1997). Traditional detergents, derived from soaps produced by saponifying fats or oils with lye (sodium or potassium) were the first natural and eco-friendly surfactants used for fabric washing (Babajanzadeh et al., 2019; Mousavi & Khodadoost, 2019; Yangxin et al., 2008). These soaps remained the sole detergent source until 1916. During World War I, the scarcity of fats, combined with the petrochemical boom in World War II, accelerated the shift to synthetic surfactants (Yangxin et al., 2008). The advantages of synthetic surfactants such as their easy availability, low cost, and expanded application areas have made them increasingly popular. However, synthetic surfactants mainly derived from petroleum have been the primary culprit behind environmental concerns, from petrochemical processing to the discharge of washing wastewater (Farias et al., 2021; Giagnorio et al., 2017; Warne & Schifko, 1999). This has raised public awareness about the acute toxicity of detergents and their harmful effects on freshwater organisms, among other negative environmental impacts. A 2009 survey revealed that 40-64% of consumers across Germany, France, the UK, and USA preferred clean and green detergents (HouseholdandPersonalCareToday, 2009). This consumer pressure and changes to environmental laws have become the key drivers behind modern detergent innovations. (Scheibel, 2004).

To meet growing consumer demand and comply with environmental regulations, detergent companies have adopted various approaches. Some focus on sourcing renewable ingredients, while others prioritize reducing environmental impact through compact packaging, lower wash temperatures, and minimal water consumption. Consumer response to the former has been positive; a survey found that around 75% of respondents expressed concern about detergent

ingredients, favoring biodegradable options to reduce chemical and water pollution (HouseholdandPersonalCareToday, 2009). Enzymes, once considered minor additives, have gained increasing significance in recent years, cementing their role in sustainable detergent formulations (HouseholdandPersonalCareToday, 2009). While bio-based surfactants have garnered consumer support for their ecological benefits and diverse substrate availability, the market is still in its early stages, facing challenges such as technical limitations, higher costs compared to synthetic alternatives, and a shortage of skilled labor (MarketsandMarkets, 2019). Enzymes and biosurfactants are key to bio-based laundry detergents, offering benefits like superior cleaning at lower temperatures, reduced energy use, and decreased fossil fuel dependence (Farias et al., 2021; HouseholdandPersonalCareToday, 2009). However, high enzyme production costs limit their availability to mature markets like Europe, the USA, and Japan (Grbavčić et al., 2015). As a result, consumers are demanding more affordable, higher-performance products (Benvegnu et al., 2008; Scheibel, 2004).

Compared to plant- and animal-based sources, microbes have garnered significant attention in the enzyme and biosurfactant industries due to their scalability and cost-effectiveness. Microbial production offers advantages such as rapid growth, high yields, controlled fermentation, production efficiency, scalability, raw material availability, and genetic engineering (Gurkok, 2019). Despite these benefits, there is still room for improvement. To further lower costs, studies have explored the valorization of agro-industrial organic waste as low-cost substrates, as they represent 30-50% of the end product value (Nazareth et al., 2021; Özbek Yazıcı & Özmen, 2020). Coproducing enzymes in a single fermentation batch using microorganisms, while optimizing enzyme proportions, offers a cost-effective solution to improve production efficiency (A. Singh et al., 2021). Genetic manipulation enables the creation of microbial enzymes with enhanced properties (Song et al., 2022), which could contribute to the invention of the next generation of smart bio-based detergents. Much research provides evidence that *Bacillus* species, in particular offer significant potential, as they can meet both performance and cost demands, paving the way for more sustainable and effective detergent formulations (Niyonzima & More, 2015; Vieira et al., 2021).

The genus *Bacillus* has also become the dominant microbial group used in recent microbial-based cleaning products. These products, which incorporate live microbial strains as active ingredients, are increasingly adopted across various countries and regulated under legal frameworks in regions such as Europe, the United States, and Canada. Notably, several *Bacillus* species including *B. subtilis*, *B. megaterium*, and *B. pumilus* have been officially approved for use in cleaning formulations and evaluated for safety under Canada's *Canadian Environmental Protection Act, 1999 (CEPA 1999)*. These strains are also classified as Risk Group 1 by the U.S. Centers for Disease Control and Prevention (CDC), indicating a low risk to human health and the environment, and many hold GRAS (Generally Recognized as Safe) status by the U.S. Food and Drug Administration (FDA). Regulatory acceptance of microbial levels up to 10^4 CFU/m^3, which may be released during product application, has further supported their commercialization. As a result, *Bacillus*-based strains have been successfully incorporated into a wide variety of cleaning products—including hard surface cleaners, odor control formulations, degreasers, and septic system treatments—offering environmentally friendly alternatives to conventional chemical-based solutions (Berg et al., 2018; OECD, 2015).

However, while *Bacillus* species themselves are well-accepted, the use of genetically modified microorganisms (GMMs) as live agents in microbial cleaning products remains highly restricted. Barriers such as regulatory complexity, consumer skepticism, and the prohibitive costs and lengthy timelines associated with GMM approval have limited their commercial deployment in direct-use cleaning formulations. Nonetheless, using GMMs in upstream fermentation processes strictly for metabolite production has emerged as a viable and legally permissible approach. Provided the final product is free of viable GM cells and residual recombinant DNA, and meets established safety, purity, and labeling standards, GMM-derived enzymes and biosurfactants can be incorporated into detergent formulations. This strategy enables the harnessing of cutting-edge bioengineering while maintaining regulatory compliance and consumer safety, thus supporting the development of next-generation, high-performance cleaning products (GmbH, 2020; OECD, 2015).

While existing literature addresses the industrial applications of *Bacillus* sp., this review delves deeper into their specific role in the detergent industry. Focusing on *Bacillus* species, it explores

their potential in producing enzymes and biosurfactants tailored for bio-based detergent formulations.

3.3 Biosurfactants of *Bacillus* species in detergents

Biosurfactants are surface-active compounds that have microbial origins, such as bacteria and yeast. According to chemical structures, there are different types of microbial biosurfactants produced by a wide range of microorganisms, but the most popular compounds are glycolipids (Jimoh & Lin, 2019; Li et al., 2016), followed by lipopeptides and phospholipids (Karlapudi et al., 2018). Lipopeptides are one of the most interesting and potent classes of biosurfactants produced chiefly by *Bacillus* sp. and *Pseudomonas* sp. (Kumar & Ngueagni, 2021; Sarubbo et al., 2022). Biosurfactants are superior to their synthetic counterparts in numerous areas, such as higher biodegradation, low toxicity, biocompatibility, and extremophilic tolerance (pH, temperature, salt concentration) as well as a reduced carbon footprint relative to that of synthetic surfactants (Li et al., 2016; Sarubbo et al., 2022).

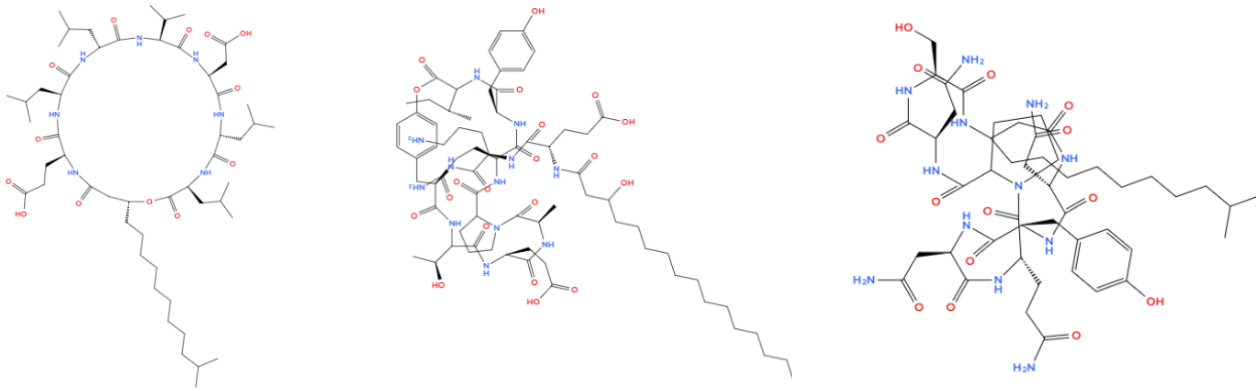
Detergents represent the largest application segment for both synthetic surfactants and biosurfactants (Vieira et al., 2021), as surfactants constitute 15–40% of modern detergent formulations, serving as their most crucial component (Hellmuth & Dreja, 2016; Sarubbo et al., 2022; Smulders & Sung, 2000; Yangxin et al., 2008). In 2022, the global value of surfactants was worth 41.9 billion US dollars (USD) and is expected to rise to 60.0 billion USD in 2030 (https://www.vantagemarketresearch.com/industry-report/surfactants-market-1671), with household detergents accounting for 46% of total consumption (https://www.spglobal.com/commodityinsights/en/ci/research-analysis/global-surfactants-industry.html). In contrast to the mature synthetic surfactant market, the global biosurfactants market is still developmental, as the first biosurfactants were only discovered between 1948 and 1949, during research on the production of antibiotics and hemolysin by bacteria (Vieira et al., 2021). In 2023, the biosurfactant market was estimated at 4.4 billion US dollars, and is expected to reach 4.7 billion USD in 2024 to USD 6.71 billion by 2032 (https://www.fortunebusinessinsights.com/biosurfactants-market-102761). Despite facing intense cost competition with synthetic surfactants, the exploitation and commercial competition of

biosurfactants in detergents are forecasted to increase fuelled by environmental concern and higher quality (Sarubbo et al., 2022; Vieira et al., 2021).

Linear alkylbenzene sulphonates (LAS) are among the most widely used synthetic anionic surfactants, primarily employed in household detergents such as laundry powders and liquids, dishwashing products, and all-purpose cleaners. Commercial LAS products are complex mixtures, typically containing homologues with alkyl chains ranging from C10 to C14. In 2005, LAS consumption in European detergent applications covered by the Human and Environmental Risk Assessment (HERA) reached approximately 350 kt representing over 80% of the total LAS usage in Europe, estimated at 430 kt. After use and disposal, LAS can enter the environment through direct discharge or via sewage treatment plant effluents (Jiang et al., 2005). Although LAS is considered readily biodegradable, its high volume of use can result in bioaccumulation, posing ecological risks. In contrast, biosurfactants offer a sustainable alternative due to their superior environmental profiles. They are biodegradable, exhibit low toxicity, and maintain functionality under extreme conditions such as low temperatures, high salinity, and extreme pH. Furthermore, biosurfactants typically possess significantly lower critical micelle concentrations (CMC), often 10 to 40 times lower than those of synthetic surfactants (Domínguez Rivera et al., 2019), allowing them to reduce surface tension at much lower concentrations. These properties make them promising substitutes for synthetic surfactants in detergent formulations (Bouassida et al., 2018; Romero Vega & Gallo Stampino, 2025).

In detergents, biosurfactants can mimic traditional surfactants but with higher efficiency. (Bouassida et al., 2018). Alongside essential surface-active properties such as lowering surface tension and forming stable emulsions—key characteristics shared with conventional surfactants (Romero Vega & Gallo Stampino, 2025)—biosurfactants exhibit a significantly lower critical micelle concentration (CMC, the minimum concentration required for micelle formation). In general, biosurfactants exhibit CMC values 10 to 40 times lower than those of synthetic surfactants (Domínguez Rivera et al., 2019), meaning they require significantly lower concentrations to achieve the same surface tension reduction.

Among the three major lipopeptide biosurfactants produced by *Bacillus* strains, surfactin, fengycin and iturin are the most widely studied. However, fengycin and iturin have higher CMC values due to their rigid cyclic peptide rings (Figure 3.1), which hinder micelle formation. Fengycin forms micelles at 15–18 mg/L (Sivapathasekaran et al., 2009) while iturin requires ≥25 mg/L (Hamley, 2015; Jauregi et al., 2013), indicating weaker self-assembly into micelles compared to surfactin. The superior efficiency of surfactin is attributed to its cyclic structure, formed by a β-hydroxy fatty acid linked to a loop of seven amino acids: L-asparagine (Asn), L-leucine (Leu), glutamic acid (Glu), L-leucine (Leu), L-valine (Val), and two D-leucine residues (Sarubbo et al., 2022). These amino acids are connected via a lactone linkage, which is more flexible than the amide bonds found in fengycin and iturin. This structural flexibility makes surfactin highly dynamic, enhancing micelle formation and surface tension reduction efficiency. Combined with its low molecular weight, this property underscores surfactin's superior emulsifying and solubilizing capabilities for hydrophobic stains (Kumar & Ngueagni, 2021), making it an ideal ingredient in detergent formulations. Surfactin forms micelles at and above 10 mg/L (Figure 3.2) (Jauregi et al., 2013) and is the most well-known lipopeptide compatible with commercial detergents (Bouassida et al., 2018; Janek et al., 2021). Even at concentrations below its CMC (7.5–10 mM, depending on buffer conditions), detergent-like permeabilization effects were observed, while complete solubilization and mixed micelle formation occurred at the CMC (Hamley, 2015). Additionally, structural diversity within the surfactin family—with more than 30 known variants—results from differences in amino acid and fatty acid residues. However, identical surfactin molecules are observed depending on their chiral sequence, further influencing their functional properties (Kumar & Ngueagni, 2021).



A B C

Figure 3.1 Chemical structure of biosurfactants (A) surfactin, (B) fengycin and (C) iturin A

(https://app.molview.com/)

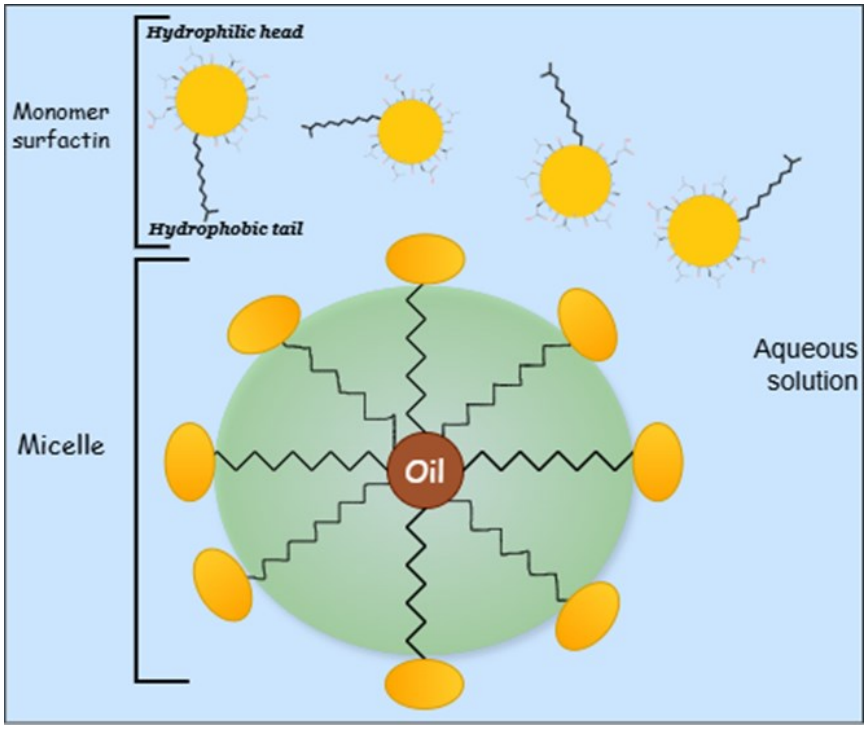


Figure 3.2 Micelles of surfactin.

The surface-active properties of *Bacillus* biosurfactants as detergent ingredients have been demonstrated through several publications. *Bacillus subtilis* strain SPB1, for instance, produces a

lipopeptide biosurfactant that reduces water surface tension by 34 mN/m and effectively removes hydrophobic stains, such as coffee and turmeric. When combined with a commercial detergent, it improves oil (45%) and tea (65%) removal efficiency, compared to 34% and 58% with the detergent alone (Bouassida et al., 2018). Similarly, thermophilic *Bacillus subtilis* strains DM-03 and DM-04 produce biosurfactants that remain stable at 80°C for 60 minutes across a pH range of 7.0–12.0. While DM-03 primarily secretes iturins and DM-04 is rich in surfactins, leading to different wash performances, the latter showed better emulsification with oils when combined with laundry detergents. However, the overall oil and blood stain removal efficiency of both strains was still lower than that of detergent alone (Mukherjee, 2007).

Simultaneously with biosurfactant secretion, the co-production of stable enzymes by *Bacillus* species not only facilitates detergent formulations but also improves their economic viability in detergent applications. For example, *Bacillus subtilis* PF1 simultaneously produces proteases, amylase, and biosurfactants when grown on agro-industrial by-products, with the resulting biosurfactant maintaining stability at alkaline pH (10–11) and temperatures between 30–60°C. More importantly, its combination with hydrolytic enzymes improves stain removal from cotton fabrics, outperforming SDS-based treatments (Bhange et al., 2016). Similarly, Kavuthodi and Sebastian (2018) studied the simultaneous production of pectinase and biosurfactant by *B. subtilis* BKDS1 using pineapple stem extract in a 1L fermenter, confirming its potential for scale-up.

Despite its superior performance at low concentrations—surpassing synthetic surfactants like LAS and common biosurfactants such as rhamnolipids and sophorolipids, surfactin's broader adoption in detergents remains limited, with its use largely confined to high-value sectors such as biomedicine and cosmetics. The major barrier to commercial viability lies in high production costs and low yields from wild-type *Bacillus* strains (0.1–1 g/L) (Table 3.1).

Downstream processing, accounting for 60–80% of total cost, is the major economic barrier to biosurfactant production, alongside costly substrates (~50%) (Chong & Li, 2017). This high cost is influenced by various factors such as the biosurfactant's solubility, ionic nature, and cellular localization (intracellular, extracellular, or membrane-bound). Liquid–liquid solvent extraction

commonly using organic solvents like chloroform-methanol or ethyl acetate is widely reported, but it is environmentally unfriendly and cost-intensive due to the large volumes of solvents required (Pardhi et al., 2022). To address this, environmentally benign alternatives are being explored, including adsorption onto activated carbon or resins, centrifugation, ion exchange chromatography, ultrafiltration, and foam fractionation. These methods enable the recovery of highly pure biosurfactants at lower cost with reusable materials. Among them, foam fractionation is especially attractive due to its solvent-free nature. This approach enables simultaneous production and recovery of biosurfactants by continuously removing surface-active molecules adsorbed on air bubbles, which can also prevent product accumulation that inhibits biomass growth (Sarubbo et al., 2022). Still, the overall production cost of biosurfactants is 10–12 times higher than that of synthetic surfactants (Dhanarajan & Sen, 2014). Equipment costs for downstream processing can comprise up to 76% of total capital investment, compared to just 21% for upstream processes, with the remainder being facility-dependent. As no single downstream method is sufficient, multi-step recovery strategies are required to obtain biosurfactants with various purity levels. While crude biosurfactants may suffice for environmental remediation and detergents, high-purity products for industrial applications necessitate more sophisticated separation and purification steps (Pardhi et al., 2022).

Additionally, low biosurfactant yields of wild-type *Bacillus* strains remain a major obstacle to commercializing in bio-based detergents, but recent advances in genetic engineering have significantly improved biosurfactant production in *Bacillus* species. The *srfA* operon, which encodes the mega-enzyme surfactin synthetase, was revealed as a crucial player in biosurfactant synthesis, significantly enhancing detergent potential (Kumar & Ngueagni, 2021). Building on this finding, the *srfA* gene from *Bacillus* sp. SK320—originally isolated from endosulfan-contaminated cashew plantation soil—was cloned into *E. coli*, resulting in substantially higher biosurfactant production compared to the wild-type *Bacillus* strain (Sarubbo et al., 2022). In a separate study, a non-producing strain, *Bacillus subtilis* 168, was subjected to extensive metabolic engineering. This included the integration of a complete *sfp* gene, reduction of competing metabolic pathways, enhancement of cellular tolerance to surfactin, increased supply of branched-chain fatty acid precursors, and redirection of acetyl-CoA flux toward surfactin biosynthesis by upregulating *srfA*

transcription. These combined interventions elevated surfactin production to 12.8 g/L (Q. Wu et al., 2019). Moreover, genome shuffling applied to *Bacillus amyloliquefaciens* led to recombinant strains with up to a 15.7-fold increase in surfactin production compared to the wild type (Zhao et al., 2012).

By contrast, rhamnolipids and sophorolipids offer a more practical and balanced profile for detergent use. Both exhibit strong surface activity (CMC ranges of 10–200 mg/L and 40–100 mg/L, respectively), along with high biodegradability and low eco-toxicity. Sophorolipids are particularly advantageous due to their high production yields, often exceeding 200 g/L and reaching over 400 g/L at commercial scale (Dhanarajan & Sen, 2014). Their relatively low production cost (approximately $3/kg) and favorable properties have facilitated their incorporation into sanitizer and detergent formulations, especially in mild detergents and personal care products. Rhamnolipids, although promising in terms of performance, face significant challenges in large-scale production, particularly excessive foaming during fermentation and regulatory scrutiny, owing to their microbial origin from *Pseudomonas aeruginosa*, an opportunistic human pathogen (Soberón-Chávez et al., 2021).

Regardless of these challenges, *Bacillus* biosurfactants are gaining industrial recognition. A notable example is the French company Lipofabrik SAS (Lesquin, France), which has developed and commercialized lipopeptide-based formulations obtained from *B. subtilis* fermentation using renewable resources (Vieira et al., 2021). These advancements indicate a growing trend toward integrating biosurfactants into commercial detergent formulations. Given their exceptional stability, synergy with enzymes, and enhanced cleaning efficacy, *Bacillus* biosurfactants hold great potential as sustainable alternatives to synthetic surfactants in the detergent industry.

Table 3.1. Comparative profile of synthetic and biosurfactants used in detergents

Category	LAS	Rhamnolipids	Sophorolipids	Surfactin	Refs
Typical Source	Chemical synthesis	*Pseudomonas aeruginosa*	*Starmerella bombicola*	*Bacillus subtilis*	(Romero Vega & Gallo Stampino, 2025) (Qiao et al., 2024)
CMC (ppm)	C10-14 433-650 C12LAS ~ 360 C13LAS ~ 150	10-200	40-100	~10–20	(Jauregi et al., 2013; Jiang et al., 2005; Romero Vega & Gallo Stampino, 2025; Yangxin et al., 2008)
Surface Tension (mN/m)	Commercial BIO-SOFT® S-101 C11.3 ~35	~30–35	~30–40	~27	(Romero Vega & Gallo Stampino, 2025; Sałek et al., 2022)
Biodegra-dability	97-99% (Aerobic)	High	High	High	(Sarubbo et al., 2022; Scott & Jones, 2000)
Eco-toxicity	EC_{50}=3.5 ppm *Dunaliella* sp.	Low	Low	Low	(Badmus et al., 2021; Sarubbo et al., 2022)
Cost	~2$ highly scalable	~223$/100g 90% pure	~3$/kg	~22.3$/mg ≥ 98% pure	(Dhanarajan & Sen, 2014; Sarubbo et al., 2022)
Yield (g/L)	Industrial scale	39-112	> 200	0.1–1 WT *Bacillus* sp.	(Henkel et al., 2017; Zhen et al., 2023)
Scaling	Fully commercial	Limited commercial	Fully commercial	Pre-commercial	(Dierickx et al., 2022; Henkel et al., 2017)
Use	Household and industrial detergents	Ecodetergents, bioremediation, cosmetics	Detergents, cosmetics, skincare	Pharma., cosmetics, skincare	(Romero Vega & Gallo Stampino, 2025)

3.4 Enzymes from *Bacillus* species in detergents

Since Otto Röhm's patent in 1913 described the application of enzymes in the detergent industry over a century ago, steady adoption of this technique has progressed (Razzaq et al., 2019). Enzymes are natural catalysts produced by living organisms that are active and stable during the

washing processes but nontoxic in discharge. Thus, they are well-accepted ingredients in a variety of existent detergent types, ranging from powder and liquid household detergents, laundry pre-spotters and stain removers, automatic dishwashing detergents, and industrial and institutional cleaners (Valls et al., 2011). As mentioned above, surfactants are able to disperse, solubilize and remove stains, effectively with particular small dirt and liquid fatty soils. However, organic soiling arising from long polymer chains or solid fat can attach strongly to surface textiles. In such cases, the synergistic action between surfactants and enzymes enhances soil removal, enabling more efficient degradation and detachment of these stubborn residues (Hellmuth & Dreja, 2016). Most recently, large numbers of alkaline enzymes, such as proteases, lipases, α-amylase, and cellulases have been introduced in heavy-duty laundry and automatic dishwashing detergents (Jayasekara & Ratnayake, 2019). Although the total enzyme content is low (0.2-2%) in detergent formulations, depending on whether solid or liquid forms of detergents, these highly effective multi-enzyme systems can facilitate the transition towards compact detergents, which bring the subsequent significant environmental savings (American cleaning Institue et al.).

Enzymes used in detergents must meet specific requirements. They must remain stable and efficient at a wide range of alkaline pH levels and a variety of temperatures (from low temperatures with synthetic fibres to high temperatures with cotton ones) throughout washing processes. Detergents are generally alkaline as most of the difficult-to-remove soils are more easily hydrolysed (saponified), chelated and dispersed at alkaline pH levels. Moreover, the enzymes must be strictly compatible with the remaining detergent chemicals like surfactants, builders, bleaching agents, and different detergent enzymes, etc. The stability and compatibility of enzymes within a detergent formulation are important to determining the cleaning efficiency of the enzymes (Gurkok & Ozdal, 2021).

The detergent market is the single biggest consumer of enzymes, with around 25–30% of total industrial enzyme sales by 2014 (Gurkok & Ozdal, 2021; Ramnani et al., 2005). The global enzyme market was estimated to be worth 14.0 billion USD in 2024, and revenue is projected to be 20.3 billion USD in 2030 *(Enzymes Market Size, Share & Trends Analysis Report, 2024).* Within this landscape, *Bacillus*-based products alone accounted for at least USD 18 billion in 2020, considering only the applications discussed in the current context. Notably, *B. subtilis*, *B. amyloliquefaciens*, and

B. licheniformis are estimated to collectively contribute to around 50% of global industrial enzyme production, underscoring their technical and commercial significance. This dominant market share suggests that enzymes derived from *Bacillus* species are both high-performing and cost-effective compared to those produced by other microbial genera. Moreover, the consistent submission of over 650 patent documents annually since 2017 highlights ongoing innovation and substantial industrial investment in this genus (Herrmann et al., 2024; Vojnovic et al., 2024).

3.4.1 Proteases from *Bacillus* species

Proteases are the largest enzyme group across the global market and are extremely popular in many industrial sectors, including food and feed industry, waste management, leather industry, chemical industry, medical field and detergent industry (Solanki et al., 2021). The half-life of peptide bonds at neutral pH and 25°C is over 100 years, and stubborn protein-based stains can become permanent when subjected to bleaching and drying processes due to oxidation and denaturation. Through proteolysis, proteases can catalyze these peptide stains in milliseconds. Thus, these enzymes serve to remove proteinaceous stains like eggs, milk, grass, blood, human sweat, etc. that strongly adhere to fabrics. Proteases were the first enzymes incorporated into detergents (Razzaq et al., 2019; Solanki et al., 2021). Initially having included as an add-on, they progressively became common ingredients in various types of detergents (Vojcic et al., 2015).

Although the very first enzymatic detergent proposed by Otto Röhm in 1913 included crude pancreatic proteases, the efficacy of enzymes was not widely recognized until the 1960s, with the advent of microbial proteases extracted from *Bacillus* spp. (Singh & Bajaj, 2017). Microbes became the chief protease producers and *Bacillus* spp. represented the most important strains for alkaline protease production (A. Singh et al., 2021). In 1960, Novo industry A/S, one of the most well-known enzyme manufacturers, marketed a trade product called BIOTEX related to subtilisin Carlsberg from *B. licheniformis* (Razzaq et al., 2019).

Based on the structure of active sites and proteolytic mechanism, proteases were classified into seven groups: the serine- (EC 3.4.21), cysteine- (EC 3.4.22), aspartic-(EC3.4.23), metallo- (EC 3.4.24), threonine peptidases (EC 3.4.25), glutamic peptidases (currently included in EC 3.4.23)

and asparagine peptide lyases (EC 4.3.2). Among those, while metalloproteases become inactive because of the loss of their metal cofactors by chelating agents, and thiol (or cysteine) proteases can be oxidised by the bleaching agents, alkaline serine endopeptidases are the most suitable in detergents because of their high stability in alkaline conditions and resistance to oxidizing chemicals (Gurkok, 2019; Klein et al., 2018).

Many studies have looked at the suitability of alkaline proteases produced by *Bacillus* spp. as detergent ingredients. *Bacillus clausii* KSM-K16 and *Bacillus sp. strain* KSM-KP43 were successfully used to produce alkaline proteases in bulk, which was then introduced into laundry cleansers (Saeki et al., 2007). Nadeem et al. (2013) purified serine proteases produced by mutant *B. licheniformis* UV-9 to homogeneity and characterised it to elucidate this additive's precise properties. The serine proteases could also maintain a high level of its relative activity regardless of the addition of common inhibitors, metal ions, surfactants and oxidants, but was found to be sensitive PMSF, DFP, SDS (Nadeem et al., 2013). The essential characteristics of proteases produced by *Bacillus licheniformis* NH1 have also been investigated. The enzyme suffers little from non-ionic surfactants, like Tween 20 and Triton X-100 (Hmidet et al., 2009). A greater effect was observed with the addition of oxidants and bleaching agents. This study hypothesised that Ca^{2+} ions help to maintain the enzyme's structural configuration, thus, enabling it to remain stable at high temperatures (over 65˚C) (El Hadj-Ali et al., 2007). Recently, *Bacillus pumilus* MP 27, isolated from marine water, has emerged as a promising candidate. This strain produced a thermophilic protease that is stable over a broad range of temperatures, from 10 to 70˚C, tolerating pH values as high as 11. The enzyme's stability was measured after adding Triton X-100 as a surfactant, and Tide as a commercial detergent (Baweja et al., 2016). Protease from *Bacillus* sp. APR-4 was active at pH 9.0 and tolerant to temperatures up to 80°C. The enzyme showed high resistance towards bleaching and oxidising agents (sodium hypochlorite) and commercial detergents (Fena®, Farishta®) (Kumar & Bhalla, 2004). Finally, *Bacillus cereus* BM1, *Bacillus clausii* Sm3, *Bacillus licheniformis* ALW1 were examined as potential candidates for the detergent industry (Emran et al., 2020; Mienda & Huyop, 2013). In the current market, several *Bacillus* proteases are already being used in detergents, as shown in the Table 3.2 (Al-Ghanayem & Joseph, 2020; Sarmiento et al., 2015).

Table 3.2. Commercial protease products used in detergents

Commercial name	Specificity	Producer	Origin	Working temperature
Alcalase®	Serine endopeptidase (Subtilisin A)	Novozymes	*Bacillus licheniformis*	Between 50 - 75 ˚C
Durazym®	Subtilisin	Novozymes	mutant *Bacillus* sp.	
Everlase™	Subtilisin A	Novozymes	mutant *Bacillus* sp.	
Savinase®	Serine endopeptidase (Subtilisin A)	Novozymes	mutant *Bacillus* sp.	
Esperase®	Serine endopeptidase (Subtilisin A)	Novozymes	*B. halodurans*	
Neutrase®	Metalloprotease	Novozymes	*B. amyloliquefaciens*	
Protamex™	Protease	Novozymes	*Bacillus* sp.	
Purafect® Prime	Subtilisin	Genencor Intl	*Bacillus lentus*	Between 20-40°C
Properase®	Protease	Genencor Intl	*Bacillus clausii*	

3.4.2 α-amylases from *Bacillus* species

With amylase enzymatic system, α-amylase has gained greater attention than β-amylase and γ-amylase due to its potential applications, especially in industrial sectors, ranging from the food and beverage, fermentation, paper, textile and pharmaceutical industries (Saini et al., 2017; Souza, 2010). The enzyme, α-amylase (1,4-α-d-glucan glucanohydrolase [E.C. 3.2.1.1]), is an extracellular enzyme that breaks down 1,4-α-d-glycosidic starch linkages at random to release short-chain carbohydrates. α-amylase is the second most significant position in the global enzyme market (accounting for 25–33% of the total market value), second only to proteases (Azad et al., 2009), of which, the global value in 2022 was 1.84 billion US dollars (https://www.persistencemarketresearch.com/market-research/alpha-amylase-market.asp) preceded by 2 billion US dollars of proteases (https://www.futuremarketinsights.com/reports/protease-market). In addition to its substantial market share, α-amylase is also highly demanded across industries, particularly in detergents, where it is historically the second most utilized enzyme after serine proteases (Mitidieri et al., 2006) and is present in about 90% of modern liquid detergents (Lahmar et al., 2017; Souza, 2010).

α-amylase converts starchy foods such as spaghetti, pasta, potatoes, gravy, custards, chocolate, etc. into water-soluble products (oligosaccharides and dextrins) for easy removal. Moreover, α-amylase is also responsible for the anti-adhesion of suspended soils (Gurkok, 2019). Microbial α-amylase possess characteristics that are well suited to detergents and both *Bacillus* species and *Aspergillus* species are the chief suppliers for alkaline and thermostable α-amylase (Mojsov, 2016; Souza, 2010).

Several findings have indicated that adding Ca^{2+} stimulates most bacterial amylases because Ca^{2+} plays an important role as an essential cofactor for these enzymes (Saini et al., 2017), hence, amylase is generally considered a metalloenzyme. Analysis of α-amylase's three-dimensional structure reveals that Ca^{2+} is bonded to two of its three domains and plays the role in remaining the solid tertiary structure of the enzyme, resulting in stable amylolytic activity (Souza, 2010). However, the stability of enzymes and the availability of Ca^{2+} could be threatened when combined with builders as calcium-chelating agents. These chemicals act to soften hard water and enhance the performance of liquid detergents. Therefore, the search for Ca-independent α-amylase to boost the quality of detergent formulations is ongoing (Roy et al., 2012). In addition, like other metalloenzymes, amylase is inhibited by the presence of EDTA, which induces a need to find novel amylase-producing strains.

Bacillus α-amylase has been investigated by many authors. Remarkably, the *Bacillus subtilis* strain AS-S01a was isolated from a soil sample and produced an alkaline α-amylase which does not require Ca^{2+} for allosteric activation. This purified enzyme was most active at 55°C and pH 9, and the existing activity remained when treated with EDTA (2 mM) (a chelating agent), 1% Triton X-100, Tween 20, and Tween 80 (non-ionic surfactants). The α-amylase from strain AS-S01a also exhibited 69-100% stability at 30°C and 37°C towards commercial detergents, such as Surf excel® and Wheel®, Tide® and Ariel®, Henko®, Fena Ultra®, Safed® and Ujala® (Roy et al., 2012). A partially purified α-amylase obtained from *Bacillus* sp. strain TSCVKK remained stable at the pH range of 6-9.5, and enzymatic hydrolysis was enhanced by mixing 1% soluble starch plus 5 mM $CaCl_2$ at 55°C, while inhibition was observed by 8M urea, and 5 mM EDTA. Other factors tested, such as SDS (an anionic surfactant), Triton X-100, Tween 20, Tween 40, and Tween 80 had only a slight effect on the original activity (Kanthi Kiran & Chandra, 2008). *Bacillus cereus* strain GA6 was

able to synthesise cold-active α-amylase which was stable at the lower temperature of 4-37°C, pH ranging from 7 to 11. However, the cold-active amylase was denatured by Fe^{2+}, Zn, $CuSO_4$ and H_2O_2. Unlike amylase secreted by other strains which were stimulated by Ca^{2+}, the amylase from *Bacillus cereus* strain GA6 still showed good quality in the presence of EDTA as well as Urea and SDS (Kuddus & Ahmad, 2012). The enzyme was compatible with commercial detergents (e.g. Tide and Ghari detergents), thus, proving its potential application in this field (Niyonzima & More, 2014).

Numerous commercial *Bacillus*-derived amylases are used in detergents, namely BAN® (*Bacillus amyloliquefaciens)*, Stainzyme® (mutant *Bacillus licheniformis)*, Duramyl, Maxamyl (*Bacillus* sp.), Solvay amylase (Gurkok, 2019), Termamyl® and Takaterm (*B. licheniformis*) (Offen et al., 2015; Rojo et al., 2007).

3.4.3 Lipases from *Bacillus* species

In terms of general commercial consumption and detergent preparations, lipases (triacylglycerol hydrolases, E.C.3.1.1.3) are the third most important biocatalysts, after proteases and carbohydrases (Guncheva & Zhiryakova, 2011). Lipases added to household and industrial cleansers digest fatty stains and greasy soils, including butter, margarine, fats, fat-based sauces, salad oils, soups, human sebum or certain cosmetics (Hasan et al., 2010). The addition of lipase is beneficial as this innovation can help replace harsh chlorine bleach, and indirectly mitigate environmental pollution from laundry effluents (Chandra et al., 2020). Lipase acts by attacking ester bonds in triacylglycerols to liberate diacylglycerols, monoacylglycerols, long-chain fatty acids and glycerol under an aqueous solution at the lipid-water interface. In 1958, Sandra and Denuelle described the catalytic mechanism in kinetics terms as "interfacial activation," hence, the reaction does not follow Michaelis–Menten kinetics. This unique phenomenon was not found in true esterases (EC 3.1.1.1, carboxyl ester hydrolases), which act on substrates soluble in water (Guncheva & Zhiryakova, 2011; Verger, 1997). Because lipases are members of the alpha/beta-hydrolase fold family, their secondary structure, with a central β-sheet surrounded by α-helices, eclipses the active site by establishing a lid. This causes the enzyme to become inactive in homogeneous aqueous environments until an oil-water interface is introduced. The catalytic

center containing the triad Ser-Asp (or Glu)-His has to undergo a conformational rearrangement to allow substrates to access the active site (Guncheva & Zhiryakova, 2011).

The vast majority of lipases comes from bacteria (Gupta et al., 2004; Hasan et al., 2006; P. Sharma et al., 2017). Various brands have successfully launched a number of lipase-based products (Hasan et al., 2010). The first commercial detergent containing fungal lipases, named Lipolase, was developed in 1988 by Novo Nordisk, and manufacturers have continued to develop upgraded versions using fungi (*Humicola lanuginose*, *Aspergillus oryzae,* e.g.). So far, bacterial lipases used in two laundry detergents, Lumafast (Genencor Inter-national) and Lipomax (Gist Brocades), were isolated from *Pseudomonas mendocina* and *Pseudomonas glumae*, respectively (Hasan et al., 2010). Despite the fact that *Bacillus* is not the dominant lipase producer for detergents, its potential has been recognised with great interest and the ability of an array *Bacillus* sp., e.g. *Bacillus subtilis* JPBW-9, *Bacillus licheniformis*, *Bacillus licheniformis* VSG1, *Bacillus pumilus* SG2, *Bacillus flexus* XJU-1, to synthesise detergent-compatible lipases has been studied (Chandra et al., 2020; Verma et al., 2021).

Bacillus lipases possess characteristics that are valued for detergent applications (Chandra et al., 2020). Mostly, the enzymes' activity is stable in neutral to slightly alkaline media (pH = 7-9), their optimal temperature is around 45-50°C, and they tolerate high levels of Ca^{2+}, surfactants, bleaching agents, organic solvents, and proteases. A variety of *Bacillus* species have been reported to produce alkaliphilic lipases, such as *Bacillus subtilis* DR8806, *Bacillus licheniformis*, *Bacillus* sp. RSJ-1, *Bacillus* sp. LBN2 (Verma et al., 2021). The lipase from *Bacillus methylotrophicus* PS3 is thermostable with optimal conditions of 55°C and pH 7.0. The stability of the enzyme is stimulated by Mg^{2+}, Triton X-100 and organic solvents (particularly methanol) (P. Sharma et al., 2017). *Bacillus cereus* C7 produces lipase which preserves its activity when in combination with commercial detergents, hydrogen peroxide, sodium hypochlorite and trypsin (Dutta & Ray, 2013). *Bacillus* sp. DH4, *Bacillus* sp. RSJ-1 lipases show significant tolerance to surfactants and laundry detergents. *B. sphaericus* 205y lipase retains high activity with proteolysis (Guncheva & Zhiryakova, 2011).

3.4.4 Cellulases from *Bacillus* species

Cellulases belong to the glycoside hydrolase family that hydrolyse β-1,4-glycoside linkages of cellulose polymers. Cellulases are a complex system with three single enzymes: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). While endoglucanases randomly cleave internal bonds in amorphous cellulose to generate new shorter chain ends, exoglucanases cleave the non-reducing and reducing ends of cellulose to produce cellobiose as major products, and β-glucosidase hydrolyzes the cellobiose into glucose (Jayasekara & Ratnayake, 2019). Current detergent preparations usually include a cellulose cocktail. In laundry, they protect colour and maintain fabric's smoothness (Niyonzima, 2019). In contrast to other enzymes in detergent formulations, cellulase does not react directly with soils on the fabric's surfaces. Instead, it reacts with cellulose chains in the amorphous region of the fibres. The effect is not only to remove stains but also to eliminate the microfibrils and fuzzes of well-worn cotton clothing which gives rise to the fabric of grayish or dull appearance (Ito, 1997).

Although fungal cellulases have been studied extensively, they are either acid or neutral enzymes and cannot be compatible with detergent's alkaline medium. Thus, bacteria have been considered in manufacturing because they are an abundant source of alkaline enzymes (Niyonzima, 2019). Among microbes, *Bacillus* genus is the most promising candidate. Cellulases from *Bacillus* sp. are usually alkalophilic and generally compatible with other detergent ingredients (Niyonzima, 2019; Özbek Yazıcı & Özmen, 2020). In 1987, Kao developed the first detergent cellulase produced by *Bacillus* sp., which demonstrated significantly improved washing performance. Since 1998, Genecor International has marketed an endo-cellulase product named Puradax that is extracted from alkaliphilic *Bacillus* and used in detergents (Gurkok, 2019).

Bacillus cellulases share common characteristics which confer tolerance to specific conditions of detergents. In 1988, (1997)studied the enzymatic properties and genetics of *Bacillus* sp. KSM-635 and extended the use of this species to heavy detergents. This was the first application of *Bacillus* cellulase in a detergent. *Bacillus* cellulases usually remain stable at a pH of 8-10, a broad range of temperatures from 40 to 120°C, and are slightly inhibited by EDTA (Ito, 1997). Recent studies demonstrating the considerable properties of *Bacillus* sp. SMIA-2 has made it another potential

strain for use. This bacterium can simultaneously produce an array of enzymes, including protease, amylase and cellulase. Thus, cellulase from this strain could withstand proteases. Cellulase from *Bacillus* sp. SMIA-2 was stable in SDS, non-ionic surfactant and RENEX 95, Ultra Biz® detergents but not in Triton X-100, H_2O_2 and Ariel® (Ladeira et al., 2015). In contrast, cellulase produced by *Bacillus licheniformis* AMF-07 maintained its activity in the presence of Triton X-100 but was inhibited by H_2O_2. In terms of interactions with commercial detergents, the enzyme was stimulated by Dioxigene (122%), Shooma (116%); and slightly inhibited by Barf (90%), Kaf (85%), Taj and Darya (33%) (Niyonzima, 2019).

3.5 Recent innovations in harnessing *Bacillus* species in bio-based detergents

3.5.1 Affordable green detergents via low-cost substrate utilization

Despite the growing demand for enzymes and biosurfactants, the large-scale production of these organic molecules continues to pose a challenge in terms of process economics (Emran et al., 2020). A careful design is required to narrow the gap between the necessary financial investment and industrial production because profit is always of significant concern when developing at industrial scale (Jackson, 1985). Various options have been considered to reduce the price of fermentation operations, and in many cases, raw materials account for the majority of production costs of industrial enzymes (Özbek Yazıcı & Özmen, 2020). The same difficulty has been witnessed in production of microbial biosurfactants, which increased the price of microbial biosurfactants up to $ 34 per kilogram compared to $ 1-4 per kilogram of the average price of synthetic surfactants and decelerated the growth rate of biosurfactant market. The substrates in fermentation necessary to generate biosurfactants occupy more than 50% of total cost (Farias et al., 2021). One practical option is to use agro-industrial residues and by-products as media for fermentation (Barcelos et al., 2020). Using these low-cost substrates also offer a variety of additional benefits, such as minimizing pollution, increasing the availability of a diverse spectrum of substrates, and being nontoxic to microorganisms. The volume of organic waste generated is surging in parallel to the mounting consumption of global population explosion, which leads to a heavy burden on the environment and financial responsibility to waste management, unless

valorization approaches can be established to circularize these residues. 13×10^9 tonnes of organic by-products are estimated to be produced annually, which means that these residues represent a promising and abundant resource for the valorization in high-value-added by-products while mitigating the environmental problems (Chavan et al., 2022). Numerous studies highlight the potential of *Bacillus* sp. fermentation using circular substrates as cost-effective alternatives. *Bacillus* sp. is a well-known industrial microorganism used in recycling diverse agricultural and agro-industrial wastes, namely molasses, cassava waste, orange peel, corn steep liquor, sugarcane bagasse, tomato waste proteins, waste sunflower oil etc., into value-added products, (Bhange et al., 2016; Farias et al., 2021; Moayedi et al., 2016). However, compared to conventional media, these waste-derived substrates often suffer from imbalanced nutrient composition, logistics, feedstock inconsistency, and feedstock availability which must be carefully managed to achieve optimal productivity (Sarubbo et al., 2022).

Therefore, selecting a suitable agro-industrial waste or residue for biosurfactant or enzyme production requires considering factors such as raw material availability, transportation costs, minimal or no pretreatment requirements, and the avoidance of refined feedstock supplementation (Domínguez Rivera et al., 2019).

Beyond substrate selection, fermentation strategies are chosen based on substrate's nature, with solid-state fermentation (SSF) and submerged fermentation (SmF) being widely employed. While SSF is particularly efficient for fungal and yeast fermentation, it has also shown promise in certain *Bacillus* strains. For instance, *Bacillus pumilus* UFPEDA 448 yielded a higher concentration of lipopeptides under SSF when cultivated on an okara-based medium supplemented with sugarcane (Slivinski et al., 2012). Similarly, the thermophilic bacterium *Bacillus* sp. BBXS-2 successfully fermented nonsterile open wheat straw as a substrate, co-producing protease and amylase, offering a cost-effective approach for detergent applications (Qureshi et al., 2016). Despite SSF's advantages—such as high volumetric productivity, higher product concentrations, reduced effluent generation, and simpler fermentation equipment—its industrial scalability is hindered by challenges in downstream processing, limited oxygen transfer, difficulties in scaling up, heterogeneous substrate composition, and moisture control issues, necessitating a case-specific approach. Conversely, SmF, though requiring capital-intensive fermentation

infrastructure, enables homogeneous nutrient distribution and precise control over key cultivation parameters such as temperature, pH, and dissolved oxygen (Renge et al., 2012). For example, *Bacillus subtilis* LB1a and LB5a produced biosurfactant, protease, and amylase more effectively using cassava wastewater than with a synthetic medium. Interestingly, during bioreactor operation, the frequent occurrence of foam was identified as a contributing factor in protease recovery as higher value of enzyme found in foam, suggesting a simple and viable downstream process. However, as reported in the study, the enzyme yields in 3L bioreactors were lower than those observed in flasks for both protease and amylase (Barros et al., 2013). Thus, developing a successful large-scale fermentation process requires not only the advantages of controllable fermenter systems but also the optimization of key parameters, which are essential for in overcoming challenges posed by wastewater containing unwanted substances. In an experiment utilizing wheat bran and groundnut oil cake as feedstock in 600 mL and 5L bioreactors, *Bacillus amyloliquefaciens* exhibited a linear increase in amylase production with higher aeration, agitation, and biomass levels, indicating a growth-dependent pattern of α-amylase production. These findings, supported by Syu and Chen as well as El-Tayeb, highlight the significant influence of physical and biological factors in enhancing amylase yields (Gangadharan et al., 2011).

To date, numerous studies have investigated the use of agro-industrial residues and wastewater for the production of enzymes and biosurfactants, primarily evaluating their feasibility at the laboratory and pilot scales. These efforts have provided valuable insights into substrate characteristics, microbial compatibility, and product potential for integration into detergent formulations. However, scaling up from pilot to industrial production remains challenging and requires further technological innovation, comprehensive economic assessments, and alignment with regulatory standards to ensure process stability, consistent product quality, and commercial viability. As summarized in Table 3.3, the detergent industry emerges as a particularly promising application area for these sustainable bio-based ingredients obtained through residual fermentation.

Table 3.3 Fermentation using different types of waste by *Bacillus* sp.

Types of waste	Products	Strains	Types of fermentation	Remarks	References
Wheat bran and rice husk as carbon source	α-amylase	*Bacillus subtilis*	Solid-state fermentation	*B. subtilis*, isolated from hot springs. 7.3-fold higher enzyme production in wheat bran compared to rice husk	(Baysal et al., 2003)
Wheat bran	α-amylase	*Bacillus cereus* MTCC 1305	Solid-state fermentation	Highest enzyme production was observed with wheat bran (94±2 U/g) after 72 h	(Anto et al., 2006)
Potato starch waste as the sole carbon source	α-, β-, γ-amylase	*Bacillus amyloliquefaciens*	Shaking flasks	Using the medium containing 2% potato starch waste in shaking flasks (150 rpm) at 50°C produced the maximum α and β-amylase after 30 h, γ-amylase after 36 h	(Abd-Elhalem et al., 2015)
Rice bran as a carbon source	Cellulase	*Bacillus carboniphilus* CAS 3	Shaking flasks	At initial pH 9.0, and temperature 50°C, obtained 4040.4 U/mL of cellulose activity	(Annamalai et al., 2014)
Lignocellulosic wastes	Cellulase	*Bacillus halodurans* CAS 1	Shaking flasks	With an optimum pH, temperature of 9.0 and 60°C, an extracellular halotolerant, thermoalkaline cellulase produced	(Annamalai et al., 2013)
Wheat bran and lentils husk as a carbon source	Alkaline protease	*Bacillus* sp.	Solid-state fermentation	Greatest yields of 429.04 and 168.64 U/g were achieved in 0.1 M carbonate/bicarbonate buffer at pH 10	(Uyar & Baysal, 2004)
Cotton seed cake as a nitrogen source	Alkaline protease	*B. cereus* NS-2	Shaking flasks	Wheat bran supported maximal fibrinolytic protease production (148 U/mL), cotton cake enhanced the	(Bajaj et al., 2013)

				fibrinolytic protease production 315 U/mL, and *Bacillus* protease has the ability to remove blood stains.	
Waste cooking oil	Lipase	*Bacillus subtilis*	Shaking flasks	The optimal lipolytic activity was 4.96 U/mL in 84-hour fermentation	(Suci et al., 2018)
Wheat bran, banana waste, melon waste, watermelon waste, lentil husk, and rice husk as carbon sources	Lipase	*B. coagulans*	Solid-state fermentation	Melon waste supplemented with 1% olive oil was found to be the best substrate for lipase production (78.069 U/g)	(Alkan et al., 2007)
Chicken feather peptone (CFP) as a nitrogen source	Lipase and amylase	*Bacillus licheniformis* 016	Shaking flasks	The optimum concentration of CFP for lipase and amylase production was determined as 5 and 6 g/L, respectively	(Baltaci et al., 2020)
Chicken feathers as a complex substrate of carbon and nitrogen source	Alkaline proteases and thermostable amylase	*Bacillus licheniformis* NH1	Shaking flasks	Potential application as detergent additive	(Hmidet et al., 2009)
Industrial waste (feather	Keratinolytic protease,	*Bacillus subtilis* PF1	Shaking flasks	An overall 2.3% increase in proteases, 0.85% increase in amylase production and 1.2%	(Bhange et al., 2016)

meal, potato peel and rape seed cake)	amylase and biosurfactant			increase in biosurfactant production was achieved with optimised media.	
Corn steep liquor	Biosurfactant	*Bacillus subtilis*	Shaking flasks	10% (v/v) of Corn steep liquor, with a biosurfactant production of about 1.3 g/L	(Gudiña et al., 2015)
Soybean oil waste	Biosurfactant (lipoprotein)	*Bacillus pseudomycoides* BS6	Liquid culture	1.2 g crude biosurfactant was extracted from 1000 ml culture broth	(Li et al., 2016)
Cassava wastewater as an unconventional carbon source	Biosurfactant	*Bacillus subtilis* LB5a	40-L-Bioreactor	An average of 25.7 g of surfactant was recovered per batch (0.68 g of surfactant/L cassava wastewater	(Barros et al., 2008)
Wheat straw	Protease and amylase	*Bacillus sp. BBXS-2*	Solid-state fermentation	12,200 U/g and 6,900 U/g dry matter for protease and amylase, respectively after a 5-day-fermentation at 45°C, initial pH of 8.5, nonsterile open fermentation	(Qureshi et al., 2016)
Soybean flour and rice straw	Biosurfactant	*Bacillus amyloliquefaciens* XZ-173	Solid-state fermentation	A surfactin yield of 15.03 mg/gram dry substrate was attained in 1000-fold scale-up fermentation in a 50 L fermenter	(Fürst et al., 2013)

3.5.2 Energy-saving detergents with cold-adapted microbes

Cold-active enzymes have drawn a lot of attention from the detergent industry because their unique properties allow greater energy conservation (Nandanwar et al., 2020). In the past, wash performance relied heavily on the level of mechanical agitation in combination with water temperature. Hot water, potentially up to 95˚C, used to be preferred for washing

clothes. This caused an array of problems, such as high energy consumption and wear and tear on fabrics (Smulders & Sung, 2000; Verma et al., 2021).The introduction of enzyme-based detergent formulations redefined the optimal washing temperature, shifting it to a milder range of 30–60°C—lower than traditional, heat-dependent standards. This shift stems from the fact that enzyme activity decreases markedly at higher temperatures. Nevertheless, at these reduced temperatures, effective cleaning is still achieved through the catalytic efficiency of enzymes, even without substantial thermal input (Smulders & Sung, 2000). The common conditions for detergent applications are alkaline pH and low temperature (Al-Ghanayem & Joseph, 2020), and the percentage of global cold-water washing machines loads rose from 38 to 53% from 2010 to 2014 (Sarmiento et al., 2015). This has been achievable because of the development of cold enzymes detergent applications that are active at alkaline pH ranges with broad thermostability (5 - 60˚C). An array of cold-active enzymes including protease, lipase, cellulase, amylase, mannanase, and pectate lyase has already been incorporated into various commercial detergent formulations by leading companies such as Novozymes, DuPont, Genencor, and Jupiter. This widespread adoption underscores the undeniable industrial appeal and functional value of cold-active enzymes in enhancing detergent performance under energy-saving, low-temperature washing conditions (Al-Ghanayem & Joseph, 2020).

Cold enzymes are mainly produced by psychrophilic microorganisms (*Archaea, Bacteria, and Eukarya*) (Feller, 2013). In addition to the many different psychrophilic, there are numerous studies examining *Bacillus* species. *Bacillus subtilis* ITRCGG-3, which can produce cold-active proteases, was isolated from the Gangotri Glacier in the western Himalayas, where the temperature ranges from 2-5 ˚C in summer to below freezing in winter. The partial protease expressed by ITRCGG-3 exhibited unusual stability in the presence of SDS as a typical surfactant in detergent formulations and its activity was even increased by Tween 80 and a commercial detergents like Wheel. Furthermore, this enzyme demonstrated stability between 10 and 30˚C, pH from 9 to 11, with optimal activity at 20˚C and pH10 (Baghel et al., 2005). A pure cold-active protease isolated from *Bacillus subtilis* WLCP1 was found to be active at pH 10 and stable at pH 7–11, its highest activity was recorded at pHs 10 and 15˚C. The enzyme was also excellent at removing blood stains from fabrics (Furhan et al., 2019). A cold-active

amylase from *Bacillus cereus* GA6 was active in a wide range of temperatures from 4 to 37°C as well as the pH of 7-11, and showed maximum activity at 22°C, pH 9. The enzyme displayed considerable potential against urea, SDS and EDTA and compatibility with commercial laundry detergents (Kuddus, 2013). *Bacillus subtilis* N8 was observed for its ability to produce a cold active, alkaline, detergent stable α-amylase. The enzyme's optimal temperature and pH were 25°C and 8.0, respectively. This enzyme also resisted some chemical denaturants in the detergent industry, namely SDS, EDTA, Triton X-100 and urea (Arabacı & Arıkan, 2018). Psychrophilic *Bacillus sphaericus* MTCC 7526 produced a cold-active lipase which was preferable for use in detergents. Its optimal activity was at 15°C and pH 8.0 and its activity remained significant in the presence of acetone, DMSO and EDTA (Joseph & Ramteke, 2013). *Bacillus* sp. strain SY-7 was isolated from the sewage of oil producing cold-active lipase. This lipase showed good activity at pH 4.0-10.0 and 5-50°C, with optimal activity at pH 8.0 and temperature at 20°C. Moreover, the enzyme was measured for other properties pertinent to the laundry industry, such as maintaining its activity despite the presence of denaturants and commercial detergents (Yasemin et al., 2017).

Compared to cold enzymes, which represent a pioneering research field with some already commercialized, the term "cold-active biosurfactants" has only recently emerged in scientific literature (Table 3.4). This interest has been driven by the Low-Temperature Washing Campaign launched in 2013, yet research in this field remains limited (Perfumo et al., 2018). While this initiative offers significant environmental benefits, particularly in the context of the ongoing energy crisis, maintaining washing efficiency at low temperatures remains a challenge. Conventional surfactants are generally less effective in cleaning at lower temperatures. Below the Krafft temperature, some surfactants crystallize, resulting in the loss of essential surface activities such as dispersion, emulsification, and critical micelle formation (Gu & Sjöblom, 1992).

Cold-active biosurfactants, however, function effectively at low temperatures and can also be produced without requiring heating (Perfumo et al., 2018). This valuable property aligns well with the push for low temperature washing practices to conserve energy, offering a promising alternative to conventional surfactants. These biosurfactants originate from

extreme cold environments, where microorganisms have adapted to thrive in freezing conditions. These cold-adapted microbes are capable of producing biosurfactants with low Krafft temperatures —the minimum temperature at which surfactants can form micelles- making them suitable for diverse applications, particularly in cold and harsh environments.

Despite the growing interest, studies on biosurfactant-producing psychrophiles remain limited. Research has focused on Antarctic environments (Trudgeon et al., 2020), cold soils, sand, lake in polar regions and high altitudes (Coronel-León et al., 2015; Gesheva et al., 2010; Vasileva-Tonkova & Gesheva, 2007; Vollú et al., 2014), cold marine environments in Atlantic Canada (Cai et al., 2014), old seeps in the deep sea of South China (S. Wu et al., 2019), and cold seep sediments (Zhou et al., 2021), etc. These findings indicate that cold-adapted microorganisms belong to several genera, including *Bacillus, Pseudomonas, Burkholderia, Sphingomonas, Vibrio, Rhodococcus, Alcanivorax, Exiguobacterium, Halomonas, Acinetobacter, Streptomyces, Janthinobacterium, Psychrobacter*, and *Serratia* (Janek et al., 2013; Singh & Singh, 2022; Trudgeon et al., 2020; Zhou et al., 2021).

A major drawback that must be considered is that psychrophilic microorganisms are generally exhibit slow growth, making them less ideal for large-scale industrial production. This challenge is further exacerbated by their lower biosurfactant yields, doubling the difficulty of commercializing these compounds. However, promising developments have emerged from studies on certain Antarctic isolates, which have demonstrated the ability to produce biosurfactants even at 4°C using crude oil as their sole carbon source (Trudgeon et al., 2020).

Table 3.4. Cold-active enzymes and biosurfactants from *Bacillus* strains for detergent applications.

Bacillus species	Culture Medium / Conditions	Growth Temp and Time	Enzyme / Biosurfactant	Enzyme activity Characteristics	Scale	References
Bacillus sp. S1DI 10 (Himalayan Spring isolate)	Glucose–casein–peptone + salts; pH 7	20°C; ~48 h	Cold-active metallo-protease	Optimum: 10°C, pH 8; stable with 2% SDS and Tween-80;	Lab scale	(Singh et al., 2019)
Bacillus subtilis N8 (Turkey, alkaline soil)	Starch-based alkaline medium; 40 g/L glucose	15–25°C; ~48 h	Cold-active α-amylase	Optimum: 25°C, pH 8; stable pH 8–12 and 10–40 °C; resists SDS, EDTA, Triton X-100, urea	Lab scale	(Arabacı & Arıkan, 2018)
Bacillus cereus GA6 (Himalayan glacier)	Glycerol + ammonium acetate; pH ~10	20°C; 96 h	Cold-active α-amylase	Optimum: 22°C, pH 9; active 4–37°C, pH 7–11; stable with SDS, EDTA, urea; active in detergents	Lab scale	(Kuddus, 2013)
Bacillus sp. SY-7 (oil-mill sewage)	Tributyrin and olive oil broth	20°C; 72 h	Cold-active lipase	Active 5–50°C, pH 4–10; optimum at 20 °C, pH 8; stable in 5% SDS, detergents, metal ions	Lab scale	(Yasemin et al., 2017)
Bacillus subtilis SPB1 (Tunisian soil isolate)	Glucose, urea, NH_4Cl, 2% kerosene; DO control	30°C; 48–72 h	Biosurfact-ant (surfactin)	Stable pH 2–9; 70°C/1h retention; improves detergent stain removal by 33–45%	Pilot (2.6 L biore-actor)	(Bouassida et al., 2018; Ghribi & Ellouze-Chaabouni, 2011)

With the increasing interest in microbial biosurfactants and their potential applications across multiple sectors, including bioremediation, gas hydrate technologies, and green detergents, further research and development efforts are expected to address these challenges (Perfumo et al., 2018).

3.5.3 Advanced specialty detergents through protein engineering

Thanks to advances in molecular biotechnology, many challenges in the detergent industry can now be addressed through protein engineering, in addition to genetic engineering (Table 3.5) (Al-

Ghanayem & Joseph, 2020). The most effective strategy for producing biologically relevant molecules involves manipulating the genes encoding these molecules, coupled with approaches such as directed evolution, semi-rational design, or rational design. Among these, directed evolution is regarded as a promising platform for rapidly generating enzymes with novel properties. By leveraging random DNA manipulation, this method produces a vast pool of mutated proteins (Bansal & Kundu, 2022). The significance of this approach was recognized by the scientific community with the awarding of the 2018 Nobel Prize in Chemistry (Engqvist & Rabe, 2019).

Protein engineering has been successfully applied to *Bacillus* spp., addressing the requirement of specific criteria-thermostability, alkaline pH tolerance, and resistance to chemical oxidizing agents- for enzymes intended for use in laundry detergent formulations. Wild-type enzymes often fail to retain their functional properties under the harsh processing conditions typically encountered in such applications.

To enhance thermostability and broaden enzyme activity across a range of temperatures, a single round of random mutagenesis followed by recombination of improved variants was conducted on a mesophilic subtilisin-like protease from *Bacillus sphaericus*. This modification resulted in a 6.6-fold increase in the catalytic rate constant (*kcat*) at 10°C and a 9.6-fold improvement in catalytic efficiency (*kcat*/*KM*) compared to the wild-type enzyme (Wintrode et al., 2000). A psychrophilic enzyme named TA39 subtilisin (S39) was converted into the mesophilic subtilisin, savinase from Antarctic *Bacillus lentus* (clausii). The hybrid enzyme displayed its highest activity at 55°C and catalysed a wider substrate profile and showed a higher specificity toward synthetic substrates (Tindbaek et al., 2004). *B. gibsonii* alkaline protease (BgAP) was modified by a directed evolution campaign toward lower temperatures. After using three iterative rounds of Sequence Saturation Mutagenesis to broaden activity, one hybrid variant, MF1, was created. This variant showed greater activity at 15°C and 100 times superior thermal resistance at 60˚C (Martinez et al., 2013).

With respect to pH-dependent activity, *B. gibsonii* alkaline proteases (BgAP), which had an optimal pH of 11, underwent a post-translational autocatalytic deamidation process substituting positively charged asparagine and glutamine residues by negatively charged aspartic acid and

glutamic acid, respectively. This led to a twofold increase in pH-dependent activity at pH 8.6 (Jakob et al., 2013).

Bacillus subtilis DB104 was also successfully exploited as a host for a variant protease that could mediate the production of oxidative agents, such as peroxycarboxylic, as a side catalytic reaction (Despotovic et al., 2013). The recombination of three variants at position Gly165 exhibited an effective redirection from proteolysis using a standard suc-AAPF-pNA substrate to perhydrolysis of methyl-propionate, methyl-butyrate, and methyl-pentanoate as substrates when expressed in *Bacillus subtilis* DB104 (Despotovic et al., 2013). Methionine 197 located close to the active site of *B. licheniformis* amylase was replaced with a non-sulphur-containing amino acid, which resulted in the improvement of oxidation stability and better performance in the presence of bleach. The type of mutant amylase has been employed by Glencore International and Novozyme according to two commercial products Purafect OxAm® and Duramyl®, respectively (Gupta et al., 2003; Nielsen & Borchert, 2000).

Table 3.5. The application of protein engineering into detergent industry.

Strategies	Targeted improvement	Results	References
Directed Evolution	Thermostability and substrate specificity	• Random mutagenesis on *Bacillus sphaericus* protease: increased kcat (6.6-fold at 10°C) and kcat/KM (9.6-fold) • MF1 variant of *B. gibsonii* alkaline protease (BgAP): higher activity at 15°C, 100-fold improved thermal resistance at 60°C	(Wintrode et al., 2000) (Martinez et al., 2013)
Post-translational Modification	pH-dependent activity enhancement	• Deamidation of BgAP: two-fold increase in enzymatic activity at pH 8.6	(Jakob et al., 2013)
Recombination / Site-directed Mutagenesis	Oxidation stability	• Recombination at Gly165 in *B. subtilis* DB104: peroxycarboxylic acid production • Methionine 197 substitution in *B. licheniformis* amylase: improved bleach resistance	(Despotovic et al., 2013) (Gupta et al., 2003) (Nielsen & Borchert, 2000)
Semi-rational / Rational Design	Mentioned as complementary to directed evolution	• Complementary strategies to refine enzyme properties	(Bansal & Kundu, 2022)

3.5.4 Smart detergents for precision stain removal with CRISPR

The pursuit of high-efficiency detergents remains an enduring research objective, with significant advancements in the innovation and industrial integration of bio-based ingredients. The powerful gene-editing technology known as CRISPR has enabled the precise engineering of eco-friendly molecules such as enzymes and biosurfactants, paving the way for more sustainable, effective, and environmentally friendly detergent formulations (Chabhadiya et al., 2024; Song et al., 2022). Notably, CRISPR-based systems derived from the Cas9 protein—such as the gene-editing CRISPR-Cas9 system and the gene-regulation platforms CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi)—offer precise, versatile, and efficient approaches for enhancing microbial strains used in detergent production (Karlson et al., 2021; Song et al., 2022).

Unlike traditional genetic modification, which often depends on random mutations, CRISPR-Cas9 gene editing enables precise and efficient alterations at specific genomic sites. This technology harnesses the Cas9 enzyme system from the natural immune response of bacteria and manipulates it to function as molecular scissors that cut DNA at a specific location, guided by a single guide RNA (gRNA). Once the DNA is cut, the cell's natural repair mechanisms are triggered, facilitating simultaneously the insertion or deletion of genetic material or the replacement of faulty genes with new ones, thus enabling targeted and precise modifications (Charpentier, 2015; Cui et al., 2018).

Moreover, a modified version of this system, CRISPR-dCas9, has been repurposed for transcriptional regulation rather than gene editing. This system consists of three core components: a catalytically inactive Cas9 (dCas9) protein, a programmable single guide RNA (sgRNA) that targets promoter regions, and a transcriptional effector—either an activator (CRISPRa) or a repressor (CRISPRi). When the dCas9-sgRNA-effector complex binds to the promoter region of a target gene, it can inhibit transcription by blocking RNA polymerase binding or elongation (in the case of CRISPRi) or enhance transcription (in the case of CRISPRa) (Karlson et al., 2021; Song et al., 2022). These systems thus allow for precise modulation of gene expression levels, offering powerful tools for the rational engineering of microbial strains tailored for smart detergent applications.

The application range of CRISPR-based system is virtually limitless, encompassing fields such as medicine, agriculture, and industrial biotechnology, including the creation of genetically engineered organisms like bacteria, plants, and animals. There are several strategies using this tool anticipated to be applied in the detergent industry. CRISPR array sequencing enables bacterial strain genotyping and detects past virome infections, aiding CRISPR-Cas vaccine development to prevent fermentation failures (Yao et al., 2018), which frequently cause economic losses in industrial-scale enzyme or biosurfactant production. A particularly noteworthy advantage of CRISPR-based genetic tools, especially relevant to the detergent industry, is their ability to simultaneously target and regulate multiple genes using different single guide (sg) RNAs. This enables the concurrent production of multiple targeted products, as demonstrated by CRISPRi-optimized metabolic flows in *C. glutamicum*, which facilitated the first efficient L-lysine and squalene co-synthesis by regulating pyruvate metabolism. This strategy also serves as a reference for synergistic amino acid and terpene production (Wei & Li, 2023).

Additionally, by enabling expression level control of any genes of interest without altering the genomic sequence (Yao et al., 2018), CRISPR is able to control the proportion of individual enzymes in the mixture for optimization. This, in turn, enhances compatibility in detergent formulations and provides an effective solution for reducing enzyme production costs. Such versatility aligns seamlessly with the portfolios of start-up manufacturers, offering opportunities for cost savings and increased operational efficiency, like India, China (Li et al., 2012; Singh et al., 2016; Wei & Li, 2023). Fehler et al. (2022) et al. demonstrated that CRISPR-dCas9 boosts α-amylase production in *B. subtilis* by 2-3-fold through the knockdown of flagellar-associated genes, with potential to further enhance enzyme production, offering promising applications in biotechnology.

Yet, harnessing CRISPR-based technologies with *Bacillus* species for green detergent production remains a relatively novel application of this technology, as it has so far been used for genome editing/regulation in only a limited number of bacterial species, particularly those with challenging transformation or recombination processes. Among the *Bacillus* species, *B. subtilis*, *B. licheniformis*, *B. megaterium* and *B. cereus* represent a few examples that have been targeted, but for purposes other than detergent applications (Hartz et al., 2021; Song et al., 2022; Zhou et al., 2019).

One rare finding by Price et al. (2019) demonstrated the use of CRISPR-Cas9 to enhance *Bacillus subtilis* for industrial enzyme production, focusing on improving subtilisin E, a key detergent protease. In this case, the CRISPR system was first validated by knocking out the *amyE* gene, which encodes α-amylase, demonstrating successful gene editing. Next, they applied in situ modification to the *aprE* gene, which produces subtilisin E, aiming to enhance its thermostability and pH tolerance. Since the wild-type enzyme is vulnerable to detergent formulations and heat, they introduced a salt-bridge triad (Arg19-Glu271-Arg275) found in *Bacillus clausii* M-protease, known for its heat resistance. Using CRISPR-Cas9, they replaced specific residues (Gln125-Gln377-Gln381) in subtilisin E to form this stabilizing salt bridge, then tested the modified enzyme's thermostability and activity. Thus, future perspectives highlight the potential of CRISPR-based genetic tools as a simple, rapid, and effective approach for engineering to achieve sequence-specific genome editing in *Bacillus* species, tailored specifically for the detergent industry (Figure 3.3).

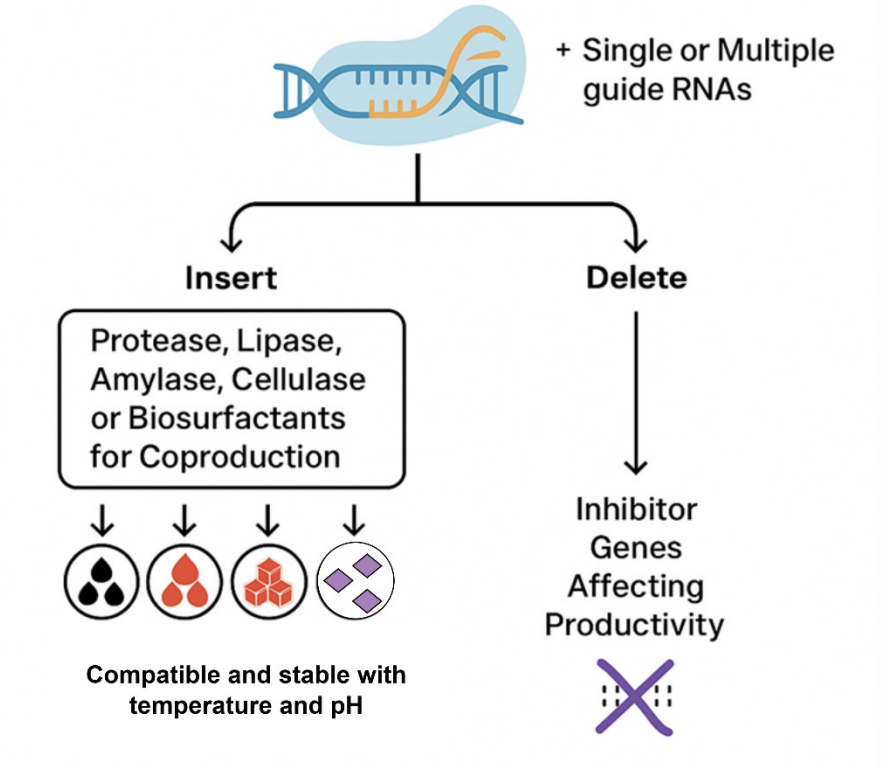


Figure 3.3 Potential applications of CRISPR-based genetic technologies in the detergent industry.

3.6 Conclusion

The increasing demand for environmentally friendly detergents has underscored the need for sustainable and efficient alternatives to conventional chemical formulations. *Bacillus* species have solidified their position as industrial microbial workhorses, demonstrating exceptional potential in enzyme and biosurfactant production for bio-based detergents. Their ability to generate a diverse array of extracellular enzymes including proteases, α-amylase, lipases, and cellulases along with biosurfactants such as lipopeptides, underscores their versatility and resilience under extreme conditions. These attributes not only enhance washing performance but also provide viable substitutes for synthetic surfactants with high toxicity. Although cost and efficiency remain significant challenges, continued research into innovative *Bacillus* strains and advanced biotechnological strategies will be essential for optimizing production and enhancing efficiency. Key approaches include optimizing fermentation processes with low-cost substrates and developing desirable "green chemicals" with novel characteristics and higher yields through gene-editing techniques, protein engineering, and the discovery of new candidates with unique features, such as cold-active biosurfactants and enzymes. While the widespread adoption of next-generation green detergents remains far off, advancements in microbial biotechnology, particularly within Bacillus species, will pave the way for more sustainable, high-performance detergent formulations, ultimately contributing to global environmental sustainability.

4 EVALUATION OF INDUSTRIAL WASTEWATERS AS LOW-COST RESOURCES FOR SUSTAINABLE ENZYME PRODUCTION BY *BACILLUS* SPECIES

Évaluation des eaux usées industrielles comme ressources à faible coût pour une production durable d'enzymes par les espèces de *Bacillus*

Vu-Mai-Linh Nguyen, Adama Ndao, Jean-François Blais and Kokou Adjallé

Centre Eau Terre Environnement (ETE), Institut National de la Recherche Scientifique (INRS), 490 Rue de la Couronne, Quebec City, QC G1K 9A9, Canada; vu_mai_linh.nguyen@inrs.ca (V.-M.-L.N.);

adama.ndao@inrs.ca (A.N.); jean-francois.blais@inrs.ca (J.-F.B.)

Correspondence: kokou.adjalle@inrs.ca; Tel.: +1-(418)-654-2610; Fax: +1-(418)-654-2600

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Lien entre l'article ou les articles précédents et le suivant :

Le premier article visait à explorer l'état actuel des connaissances sur les espèces prometteuses de *Bacillus* et leur potentiel à faire progresser et accélérer la production de détergents biosourcés. Les principaux points abordés étaient les suivants:

- La capacité des *Bacillus* à produire des biosurfactants et une large gamme d'enzymes indispensables à la formulation des détergents ;

- Leur aptitude à valoriser des substrats peu coûteux issus de déchets, contribuant ainsi à la réduction des coûts de production ;
- Leur facilité de manipulation génétique et d'ingénierie des protéines, permettant d'obtenir des enzymes plus efficaces et adaptées aux applications industrielles.

Ces constats ont conduit naturellement à la réalisation du second article, qui évalue le potentiel de souches commerciales de *Bacillus* pour la production d'enzymes en utilisant différentes eaux usées industrielles comme milieux de fermentation. Cette étude vise ainsi à valider expérimentalement l'hypothèse selon laquelle des résidus industriels fermentescibles peuvent constituer des ressources viables, à faible coût, pour une production durable d'enzymes destinées aux détergents.

4.1 Abstract

The increasing demand for industrial enzymes calls for cost-effective and sustainable production strategies. This study investigates the potential of industrial wastewater as an alternative fermentation medium for enzyme synthesis, aligning with the principles of the circular bioeconomy. Four wastewater types from Québec, Canada—beverage wastewater (BW), pulp and paper mill activated sludge (PPMS), food industry wastewater (FIW), and starch industry wastewater (SIW)—were evaluated for their potential to support protease, amylase, and lipase production using *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, and *Bacillus megaterium*. Initial screening identified SIW as optimal for amylase production with *B. amyloliquefaciens*, and PPMS for protease production with *B. megaterium*. Optimization using the Box–Behnken design was then performed, followed by scale-up experiments in 5 L bioreactors. *B. amyloliquefaciens* achieved 5.73 ± 0.01 U/mL of amylase at 48 h under 40 g/L total solids, 30 °C, and a 2% inoculum size (v/v), while *B. megaterium* produced the highest protease of 55.41 ± 3.54 U/mL at 24 h. Lipase production remained negligible across all media and strains. These findings demonstrate the feasibility of the potential of wastewater-based enzyme production, reducing reliance on expensive synthetic substrates, mitigating environmental burdens, and contributing to the transition to a circular bioeconomy.

Keywords: beverage wastewater; pulp and paper mill activated sludge; food industry wastewater; starch industry wastewater; *Bacillus licheniformis; Bacillus amyloliquefaciens; Bacillus megaterium;* protease; amylase; lipase; valorization; bioeconomy

La demande croissante en enzymes industrielles nécessite des stratégies de production à la fois rentables et durables. Cette étude examine le potentiel des eaux usées industrielles comme milieux de fermentation alternatifs pour la synthèse enzymatique, en accord avec les principes de la bioéconomie circulaire. Quatre types d'eaux usées provenant du Québec, Canada—les eaux usées de l'industrie des boissons (BW), les boues activées de l'industrie de la pâte et du papier (PPMS), les eaux usées de l'industrie alimentaire (FIW) et les eaux usées de l'industrie de l'amidon (SIW)—ont été évaluées pour leur capacité à soutenir la production de protéase, d'amylase et de lipase à l'aide de *Bacillus licheniformis*, *Bacillus amyloliquefaciens* et *Bacillus megaterium*. Le criblage initial a

identifié le SIW comme le substrat optimal pour la production d'amylase avec *B. amyloliquefaciens*, et le PPMS pour la production de protéase avec *B. megaterium*. Une optimisation selon le plan de Box–Behnken a ensuite été réalisée, suivie d'expériences de changement d'échelle dans des bioréacteurs de 5 L. *B. amyloliquefaciens* a atteint une activité amylasique de 5,73 ± 0,01 U/mL à 48 h sous 40 g/L de solides totaux, à 30 °C, avec une taille d'inoculum de 2 % (v/v), tandis que *B. megaterium* a produit une activité protéasique maximale de 55,41 ± 3,54 U/mL à 24 h. La production de lipase est restée négligeable dans tous les milieux et avec toutes les souches testées. Ces résultats démontrent la faisabilité de la production enzymatique à partir d'eaux usées, réduisant la dépendance aux substrats synthétiques coûteux, atténuant les impacts environnementaux, et contribuant à la transition vers une bioéconomie circulaire.

Mots-clés : eaux usées de l'industrie des boissons ; boues activées de l'industrie de la pâte et papier ; eaux usées de l'industrie alimentaire ; eaux usées de l'industrie de l'amidon ; *Bacillus licheniformis* ; *Bacillus amyloliquefaciens* ; *Bacillus megaterium* ; protéase ; amylase ; lipase ; valorisation ; bioéconomie

4.2 Introduction

One of the pressing environmental concerns today is the increasing volume of waste in both solid and liquid phases. This issue stems from the traditional linear economic model and the rapidly expanding global population (Maina et al., 2017; Vea et al., 2018). Waste originates from various sectors and can be broadly classified into industrial production and household consumption (Klitkou et al., 2019; Mahjoub & Domscheit, 2020). The continuous rise in organic waste production highlights the urgent need for a sustainable waste management system to reduce environmental pollution and greenhouse gas emissions (Ahmed et al., 2021; Godfrey et al., 2020; Lee et al., 2024).

Recently, a roadmap for achieving a circular and sustainable bioeconomy has been introduced to address these grand challenges by leveraging innovative technologies to convert bioresources into renewable energy and high-value-added products (Inamuddin, 2021; Klitkou et al., 2019). The success of the transition to a bioeconomy depends on advancements in technology, cost-effectiveness, and the availability of sustainable biomass (Scarlat et al., 2015). In this context, industrial wastewater and sludge—such as starch wastewater, beverage wastewater, and pulp and paper mill activated sludge, etc., which are rich in diverse nutrients and components—have emerged as valuable renewable substrates for ensuring the availability of sustainable biomass (Kathi et al., 2023).

In contrast to conventional treatment plants, which primarily treat wastewater to reduce organic and suspended solid loads for discharge compliance, various wastewater sources can instead be valorized into value-added products such as fertilizers, biofuels, biopesticides and enzymes, etc. (Kathi et al., 2023; Ravindran et al., 2018). However, in practice, effectively addressing the heterogeneity of biowaste and the presence of unwanted chemicals to maximize productivity requires further comprehensive studies adopting a sector-specific approach.

The valorization of organic wastes for enzyme production exemplifies a closed-loop process within the bioeconomy, where microbes transform organic residues into high-value enzymes. These enzymes are then utilized across various industrial sectors—such as biofuel production, food processing, pharmaceuticals, and bioremediation (Singh et al., 2016)—contributing to

resource efficiency, waste minimization, and circularity by reintegrating biological materials into the production cycle.

Different sets of enzymes can be obtained successfully through microbial submerged fermentation, which is well suited to the liquid nature of wastewater. Among the most important industrial enzyme producers, members of the genus *Bacillus* are microorganisms of choice for their simple, scalable cultivation, supported by well-studied biochemistry, physiology, and genome (Schallmey et al., 2004). Their ability to efficiently export metabolites across the cytoplasmic membrane, including proteases, α-amylase, lipase, cellulase, etc. (Chandra et al., 2020; Özbek Yazıcı & Özmen, 2020; Ravindran et al., 2018; S. Singh et al., 2021), simplifies downstream processing compared to bacteria that produce proteins intracellularly (Contesini et al., 2018). Notably, as their fermentative metabolism enables the rapid utilization of a broad range of substrates (Dong & Zhang, 2014), *Bacillus* spp. emerge as key players in zero-waste initiatives and green manufacturing.

Given the increasing interest in harnessing diverse industrial wastewater streams for biotechnological applications, selecting the most suitable substrate for a specific purpose requires a carefully designed and systematic strategy. This study introduces a pragmatic approach to evaluating the enzymatic potential of industrial wastewaters by employing well-characterized commercial bacterial strains during the initial screening phase. Utilizing these strains accelerates the screening process, ensures reproducible enzyme production, and provides a standardized platform for comparing diverse waste streams. This approach is particularly valuable in cases where the native microbial community is limited or where the physicochemical properties of the wastewater hinder effective microbial isolation. Although a potential drawback of using defined commercial strains is the risk of underestimating the full bioconversion capacity of certain wastes, this trade-off is a deliberate and calculated aspect of the research design. Crucially, it is addressed in subsequent stages, where the most promising waste streams can be revisited using native isolates or strains more suitable for targeted optimization. Importantly, the scalability of this strategy has been demonstrated in 5 L bioreactors—even when using selected commercial strains—as effective waste–strain combinations identified during screening can be readily adapted for larger-scale bioprocess applications. This initial phase of the stepwise framework

enables systematic substrate evaluation, setting the stage for future strain optimization and scalable enzyme production.

4.3 Materials and Methods

4.3.1 Industrial wastewater sources

Drawing from literature highlighting the capacity of *Bacillus* sp. to produce a wide range of enzymes using agro-industrial, brewery, and starch-based waste substrates (Choubane et al., 2015; Mazhar et al., 2016; Sánchez Blanco et al., 2016), four distinct industrial wastewater samples were collected from local facilities in Québec, Canada, to evaluate their potential as fermentation media: beverage wastewater (BW) from Brasserie Rural (Cookshire-Eaton), pulp and paper mill activated sludge (PPMS) from Kruger Inc. (Trois-Rivières), food industry wastewater (FIW) from Diana Food Canada Inc. (fruit processing from cranberries, blueberries, and strawberries) (Champlain), and starch industry wastewater (SIW) from ADM-Ogilvie (Candiac). These samples were collected directly from the effluent output points prior to any tertiary treatment. In all cases, a basic pretreatment stage was performed, consisting of coarse screening to remove large particulates and debris. Immediately after collection, the samples were stored in sterile containers, kept at 4 °C, and transported to the laboratory within 24 h. Upon arrival, their pH was measured (ranging from 2.45 to 6.65), and samples were either processed immediately or stored at 4 °C for no more than 72 h prior to use in fermentation experiments.

To characterize their suitability for microbial growth and enzyme production, a suite of analytical techniques was employed. Total organic carbon (TOC) and total nitrogen (TN) were quantified using a Shimadzu VCPH analyzer, calibrated with standard solutions to ensure precision within ±2% error. This provided insight into the carbon and nitrogen pools available for bacterial metabolism. Mineral composition—including phosphorus, sodium, iron, potassium, calcium, and sulfur—was determined via inductively coupled plasma optical emission spectrometry (ICP-OES) using an ICP-5110 Dual View spectrometer, with calibration against multi-element standards to achieve detection limits below 0.01 mg/L. Sugar profiles (glucose, fructose, lactose, sucrose, galactose, xylose, and trehalose) were analyzed using high-performance liquid chromatography

(HPLC) equipped with a refractive index detector, employing an Aminex HPX-87H column and a mobile phase of 5 mM H_2SO_4 at 0.6 mL/min. These measurements elucidated the nutrient diversity and concentration, critical for fermentation conditions to specific enzyme production goals.

4.3.2 Microorganisms

Three industrially relevant *Bacillus* strains were selected for their enzymatic versatility: *Bacillus licheniformis* ATCC 14580, known for producing amylase, protease, and lipase; *Bacillus amyloliquefaciens* ATCC 23842, recognized for high amylase and protease yields; and *Bacillus megaterium* (laboratory strain), capable of protease and lipase synthesis. These strains were chosen for their ability to secrete enzymes extracellularly, facilitating recovery and reducing downstream processing costs (Schallmey et al., 2004).

4.3.3 Inoculum Preparation

The pre-seed followed a two-stage protocol to ensure optimal bacterial adaptation and vigor. Initially, a loopful of bacterial culture was transferred from an agar plate to 10 mL of nutrient broth (10 g/L peptone, 3 g/L beef extract, 5 g/L NaCl, pH 7) in a 50 mL Erlenmeyer shake flask. The inoculated flask was incubated at 30 °C with shaking at 180 rpm for 16–18 h, reaching the late exponential phase (approximately 10^8 CFU/mL for *B. licheniformis* and *B. megaterium*, 10^7 CFU/mL for *B. amyloliquefaciens*). Five (5) mL of this culture was subsequently transferred to 250 mL of the respective sterilized wastewater in a 1 L flask and incubated under identical conditions to acclimate the bacteria to the wastewater's nutrient profile, enhancing their metabolic readiness for fermentation.

In this study, all wastewaters were sterilized in an autoclave for 15 min at 121 °C before any experiments.

4.3.4 Screening of wastewater sources for enzyme production

Screening experiments assessed the wastewaters' capacity to support enzyme production by culturing each *Bacillus* strain in 200 mL of BW, PPMS, FIW, or SIW, alongside a nutrient broth

control. Cultures were maintained at 30 °C, pH 7, and 180 rpm for 48 h in an orbital shaker. Post-incubation, samples were analyzed for bacterial growth (CFU/mL) and enzyme activities (protease, amylase, lipase), providing a basis for selecting the most promising strain–wastewater combinations.

4.3.5 Growth conditions

Bacterial growth was quantified by serial dilution in 0.8% (*w*/*v*) saline, followed by spread plating 100 µL aliquots onto nutrient agar plates. After incubation at 30 °C for 24 h, colonies were enumerated to calculate CFU/mL, with all measurements conducted in duplicate to ensure reproducibility (standard deviation <5%).

4.3.6 Total solids (TSs) and total volatile solids (TVSs)

A well-homogenized aliquot of 25 to 50 mL of the sample was transferred into a pre-weighed porcelain crucible. The sample was dried at 105 °C for 24 h in a drying oven to remove moisture. The crucible was then cooled in a desiccator and weighed to determine total solids (TSs).

To determine total volatile solids (TVSs), the dried sample was further combusted at 550 °C for 2 h in a muffle furnace. The crucible was again cooled in a desiccator and weighed. TVSs were calculated as the weight loss between 105 °C and 550 °C.

TSs (g/L) = (weight after 105 °C drying − tare weight)/volume of sample (L)

TVSs (g/L) = (weight after 105 °C drying − weight after 550 °C ashing/volume of sample (L)

4.3.7 Suspended solids (SSs) and suspended volatile solids (SVSs)

To quantify the suspended solids (SSs), a known volume (typically 25 to 100 mL) of the sample was filtered through a pre-weighed glass fiber filter (Whatman GF/C or equivalent). The filter containing the retained solids was dried at 105 °C for 24 h, cooled in a desiccator, and weighed.

For suspended volatile solids (SVSs), the dried filter was ashed at 550 °C for 2 h in a muffle furnace, cooled in a desiccator, and reweighed.

SSs (g/L) = (weight after 105 °C drying − tare weight of filter)/volume of sample (L)

SVSs (g/L) = (weight after 105 °C drying − weight after 550 °C ashing)/volume of sample (L)

All measurements were performed in triplicate, and results are expressed as mean ± standard deviation.

4.3.8 Protease assay

Protease activity was measured using a casein hydrolysis method. A reaction was set up by mixing 1 mL of diluted enzyme with 1 mL of 1% casein in 50 mM Tris-HCl buffer (pH 7) and incubating it at 50 °C for 10 min. To stop the reaction, 2 mL of 15% trichloroacetic acid was added, and the mixture was centrifuged for 10 min at 10,000 rpm at 4 °C. After centrifugation, 0.5 mL of the supernatant was taken and mixed with 2.5 mL of 2% sodium carbonate and 0.25 mL of 1 N Folin's reagent. The mixture was incubated at room temperature for 30 min. Absorbance was measured at 660 nm using a spectrophotometer. One unit of protease activity is the amount of enzyme that releases 1 μg of tyrosine under these conditions (Ramkumar et al., 2018).

4.3.9 Lipase assay

Lipase activity was determined using *p*-nitrophenyl palmitate (pNPP) as the substrate. The assay buffer was prepared by mixing 25 mL of 100 mM Tris-HCl (pH X), 5 mL of 100 mM $CaCl_2 \cdot 2H_2O$, 0.15 mL of Triton X-100, and 19.85 mL of distilled water. To prepare the substrate solution, 1 mL of 20 mM pNPP was dissolved in 19 mL of the assay buffer. For the assay, 2.76 mL of the substrate solution was mixed with 0.24 mL of enzyme solution. The reaction was carried out at 30 °C for 30 min, and the release of *p*-nitrophenol was monitored by measuring the absorbance at 410 nm. One unit (U) of lipase activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute under the assay conditions (Shart & Elkhalil, 2020).

4.3.10 Amylase assay

Amylase activity was determined via the DNS method. The reaction mixture, containing 0.1 mL of appropriately diluted enzyme and 0.9 mL of 1.0% (*w*/*v*) corn starch in 50 mM Tris-HCl buffer

(pH 7.0), was incubated at 50 °C for 10 min. The amount of reducing sugars liberated was determined using the dinitrosalicylic (DNS) acid method. After the reaction, 1.5 mL of DNS reagent was added, and the mixture was boiled for 10 min before being diluted with 5 mL of distilled water. The absorbance was then measured at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per minute. A standard curve was plotted using D-glucose. The α-amylase activity is represented by the mean value of two determinations, each performed in duplicate (Kherouf et al., 2021).

4.3.11 Optimization of fermentation parameters using Box–Behnken design

The study of optimal conditions for enzyme production in 1 L shaking flasks was conducted using a set of 17 runs designed according to the Box–Behnken design. Three factors—total solids (TSs), inoculum size, and temperature—were investigated, with the design points positioned at the middle of the edges of the experimental domain, typically coded as −1, 0, and +1. This experimental approach efficiently models the response surface, requiring fewer experimental runs compared to traditional factorial techniques. Enzyme activities were measured as responses to the design, and a model for optimal conditions was developed. The optimal conditions determined from previous work at the flask level serve as the baseline for further optimization in a 5 L bioreactor.

The relationship between the studied variables and the resulting activities of enzymes will be considered via the following second-order polynomial equation:

$$Y = \beta 0 + \sum_{i-1}^{k} \beta i Xi + \sum_{i-1}^{k} \beta ii Xi Xi + \sum_{i-1}^{k-1} \sum_{j-2}^{k} Bij Xi Xj$$

where Y is the predicted enzyme yield (U/mL), β0 is the intercept (a constant), and βi, βii, and βij are regression coefficients for linear, squared, and interaction effects, respectively. Xi and Xj are the parameters under consideration. Data regression analysis was performed using Design Expert® (Version 7.0.0) software.

4.3.12 Bench scale—5 Liter bioreactors fermentation

Scaling up protease production in a 5 L Sartorius Biostat B plus bioreactor represents a critical transition step in the research process, aimed at maintaining enzyme yield and activity for large-scale production.

The fermentation studies were conducted using a 5 L glass bioreactor with a working volume of 3 L, controlled via a programmable logic control (PLC) system to monitor key parameters such as dissolved oxygen (DO), pH, temperature, agitation, aeration rate, and antifoam. Nutrient concentrations of wastewater were optimized based on prior shake flask studies, and the wastewater media were autoclaved at 121 °C and 15 lbs/in^2 pressure for 30 min to ensure sterility. Aseptic conditions were maintained during the transfer of the inoculum, which was incubated at 30 °C for 15 h. The selected microorganisms and wastewater media, based on earlier experiments, were cultured under specific conditions for each microorganism in the bioreactor. Dissolved oxygen levels were maintained at 30–50% by adjusting aeration and agitation. Samples were collected every 12 h to monitor progress, with the goal of achieving cell concentrations of 10^8 CFU/mL for *B. megaterium*, 10^7 CFU/mL for *B. amyloliquefaciens*, and optimizing enzyme production.

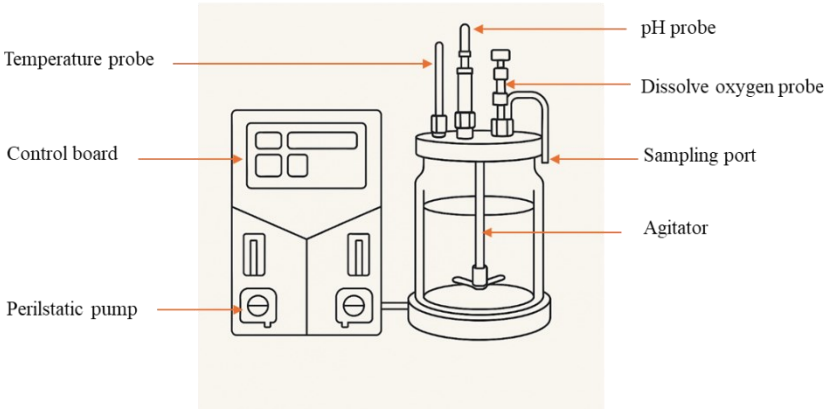

Figure 4.1 Schematic diagram of the 5 L Sartorius bioreactor.

4.4 Results and Discussion

4.4.1 Characterization of wastewaters

The nutrient profiles of the four wastewaters were pivotal in determining their suitability for enzyme production. SIW's high sugar content (e.g., 31.9 g/L glucose, 11.4 g/L fructose, 9.1 g/L lactose) and balanced TOC (207 g C/L) and TON (17.5 g N/L) positioned it as an ideal substrate for amylase production, as *Bacillus* species efficiently metabolize sugars into amylolytic enzymes (Abd-Elhalem et al., 2015). Its acidic pH (3.99) required neutralization to 7, aligning with the optimal range for *Bacillus* growth (pH 6.5–7.5). BW offered a nutrient-rich profile (TOC 345 g C/L, TON 11 g N/L), but its lower sugar levels suggested versatility rather than specificity for amylase synthesis. PPMS, with modest TOC (14 g C/L) but elevated calcium (22 g/L) and iron (0.4 g/L), supported protease production, as these minerals enhance enzyme stability and microbial metabolism (Eijsink et al., 2011; O'Hara & Hageman, 1990). FIW's high TOC (387 g C/L) was offset by negligible nitrogen (0.339 g N/L) and phosphorus (0.039 g/L), resulting in a C/N ratio unfavorable for bacterial growth, rendering it impractical without supplementation.

Table 4.1 Wastewater characteristics.

Media	SIW	BW	PPMS	FIW
pH	3.99 ± 0.12	5.33 ± 0.19	6.65 ± 0.35	2.45 ± 0.46
TSs (g/L)	13.2 ± 0.35	69 ± 2.65	10.3 ± 0.36	0
TSVs (g/L)	4.4 ± 0.53	47.1 ± 4.22	6.09 ± 0.37	0
SSs (g/L)	4.44 ± 0.57	13.5 ± 2.18	9.4 ± 0.36	0
SSVs (g/L)	2.4 ± 0.46	3.2 ± 0.37	2.35 ± 0.18	0
TOC (g C/ kg TS)	207 ± 30.32	345 ± 18.03	14 ± 1.00	387 ± 31.58
TON (g N/ kg TS)	17.5 ± 0.46	11 ± 0.87	1.4 ± 0.15	0.339 ± 0.11
P org (g/ kg TS)	3 ± 0.23	4.7 ± 0.62	2.3 ± 0.36	0.039
Na (g/ kg TS)	11.3 ± 1.18	0.12 ± 0.86	10.2 ± 0.56	0.051
Fe (g/ kg TS)	0.1 ± 0.02	0.02 ± 0.02	0.4 ± 0.17	0.001

K (g/ kg TS)	7 ± 0.00	7.3 ± 0.44	1.4 ± 0.30	0.56 ± 0.12
Ca (g/ kg TS)	3.6 ± 0.36	0.2 ± 0.09	22 ± 2.65	0.086 ± 0.01
S (g/ kg TS)	4.2 ± 0.10	1.3 ± 0.26	3.9 ± 0.95	0.0145 ± 0.01
Glucose (g/ kg TS)	31.9 ± 0.85	0.96 ± 0.19	1.9 ± 0.75	270 ± 67.27
Fructose (g/ kg TS)	11.4 ± 3.90	0.38 ± 0.12	1.5 ± 0.30	34 ± 1.00
Lactose (g/ kg TS)	9.1 ± 0.78	0.65 ± 0.11	0.83 ± 0.05	3.5 ± 0.78
Sucrose (g/ kg TS)	6.4 ± 0.66	0.15 ± 0.08	0.97 ± 0.20	<0.4
Galactose (g/ kg TS)	8.7 ± 0.82	0.3 ± 0.04	1.4 ± 0.13	<0.400
Xylose (g/ kg TS)	7.6 ± 0.10	0.7 ± 0.14	1.1 ± 0.29	3.5 ± 0.70
Trehalose (g/ kg TS)	5.7 ± 0.82	1.3 ± 0.31	0.53 ± 0.08	<0.4

4.4.2 Screening of wastewater sources for potential enzyme production

Based on the characteristics of these wastewaters, only minimal pretreatment was required, primarily consisting of pH adjustment to 7 with sodium hydroxide following sterilization at 121 °C for 15 min. Additionally, 1% Tween 20 was supplemented in the media to enhance lipase production. This approach enables the evaluation of enzyme production potential while closely maintaining the original nature of the wastewater, offering insights into its feasibility as a fermentation medium with minimal modification.

4.4.3 Bacterial growth on wastewater

On nutrient broth, the number of *B. licheniformis* and *B. megaterium* cells reached approximately 10^8 CFU/mL after 24 h and maintained to 48 h, and that of *B. amyloliquefaciens* was slightly lower, but still around 10^6–10^7 CFU/mL (Figure 4.2).

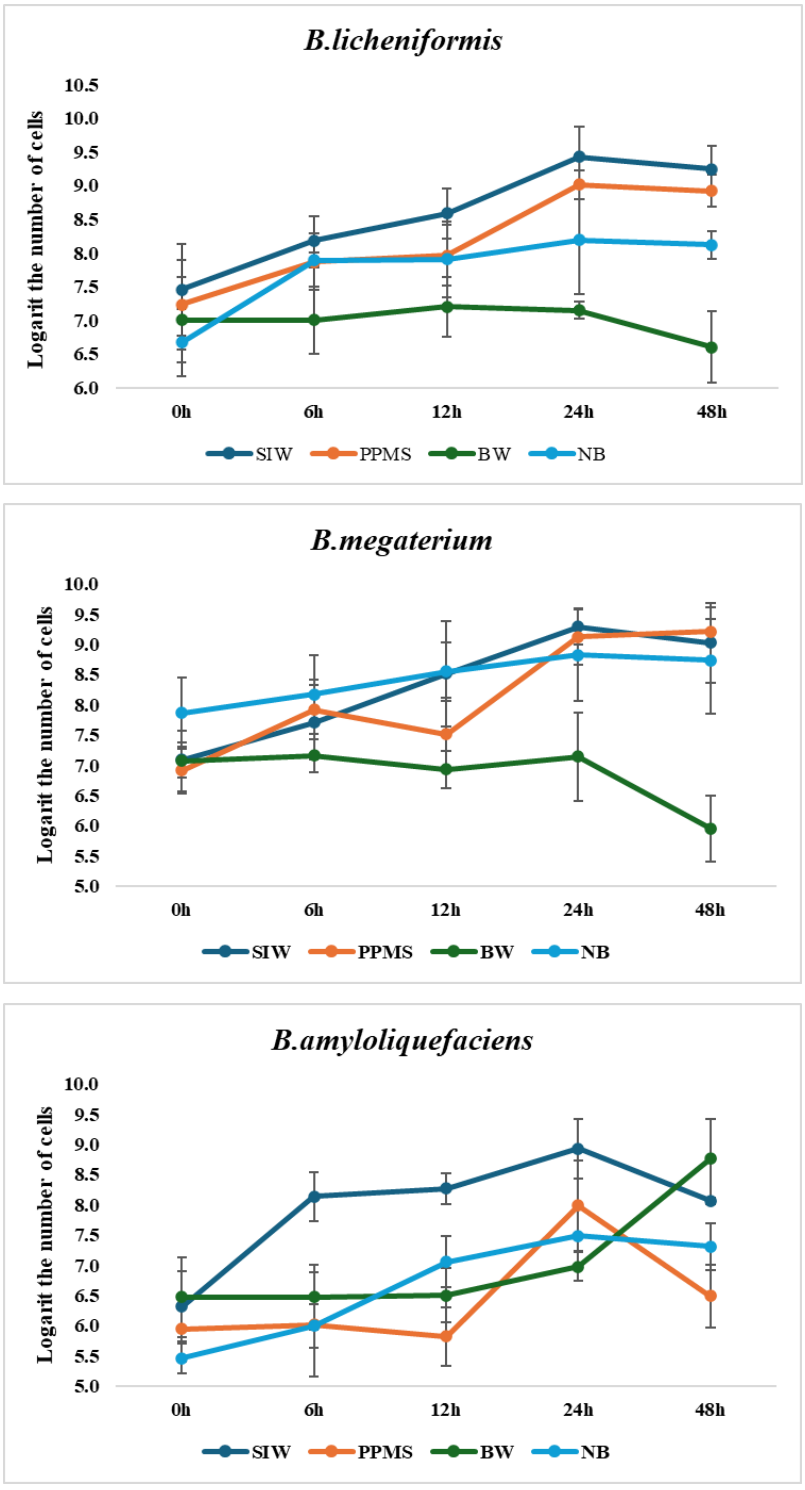

Figure 4.2 Comparison of bacterial growth on various wastewaters and synthetic medium (NB).

FIW failed to support the growth of all strains, likely due to an imbalance in the carbon/nitrogen ratio leading to nitrogen starvation, which may inhibit bacterial growth while favoring yeast. Wastewaters with extremely unfavorable conditions require more complex treatment strategies to support effective microbial growth and enzyme production, such as mixing different wastewaters to adjust pH, compensate for nutrient deficiencies, and achieve a more balanced composition (Lad et al., 2022). Therefore, the growth data for this wastewater were not included in the figure.

Although all three strains could grow on BW, their cell densities were lower. Notably, *B. amyloliquefaciens* exhibited an increased growth rate between 24 h and 48 h, suggesting its ability to utilize nutrients from this wastewater over a prolonged period. In contrast, the other strains showed minimal growth and a significant decline at 48 h.

The bacterial growth on SIW and PPMS was significantly comparable to that in synthetic medium, which means that these wastewater sources contain sufficient digestible nutrients for the division of cells, as nutrient availability and growth rate have a positive proportional relationship (Atlas, 2010). On both media, the peak cell densities of all three tested bacteria were observed at 24 h, with the exponential phase occurring between 12 and 24 h.

In conclusion, compared to the pure synthetic media, the growth of the studied strains on SIW and PPMS was basically similar, confirming that these types of wastewaters can provide enough digestible nutrients to support bacterial proliferation.

4.4.4 Enzyme production potential

After 48 h of incubation, *Bacillus* strains exhibited varying enzyme yields when cultivated on different types of wastewaters (Table 4.2). *B. amyloliquefaciens* was capable of producing both amylase and protease across all tested wastewaters, with amylase production particularly pronounced on SIW, reaching 4.26 U/mL alongside 11.64 U/mL of protease. This suggests its ability to synthesize amylase even under low-sugar conditions. In contrast, amylase activity from *B. licheniformis* and *B. megaterium* was detected mainly on SIW, indicating a more limited capacity for amylase production. While *B. amyloliquefaciens* did not exhibit any lipase activity in the tested

media, *B. licheniformis* and *B. megaterium* showed minimal lipase production (0.01 U/mL) on PPMS. On the same medium, both strains also produced substantial amounts of protease, reaching 49.59 U/mL and 53.12 U/mL, respectively. On BW, only protease activity was detected in all three strains, while amylase production was observed exclusively in *B. amyloliquefaciens*. However, the enzyme levels were lower compared to those obtained on the other wastewater substrates, indicating a limited compatibility between this wastewater and the selected strains.

Table 4.2. Enzyme activities on studied wastewaters.

***Bacillus* Strains**	**Amylase (U/mL)**	**Protease (U/mL)**	**Lipase (U/mL)**
B. amyloliquefaciens SIW	4.26 ± 0.45	11.64 ± 1.76	0
B. licheniformis SIW	1.21 ± 0.01	25.69 ± 1.46	0
B. megaterium SIW	1.95 ± 0.0	34.31 ± 4.27	0.01 ± 0.0007
B. amyloliquefaciens PPMS	0.34 ± 0.12	40.52	0
B. licheniformis PPMS	0	49.59 ± 2.53	0.01 ± 0.0004
B. megaterium PPMS	0.12 ± 0.03	53.12 ± 11.46	0.01 ± 0.0
B. amyloliquefaciens BW	1.73 ± 0.17	25.14	0
B. licheniformis BW	0	8.85	0
B. megaterium BW	0	3.97 ± 2.5	0

In this study, *B. licheniformis* was selected for its potential in wastewater treatment and value-added applications, as its ability to produce novel enzymes and valuable bioactive compounds has attracted significant attention since the first report in 1945. To date, its unique genetic background and safety profile have made it highly relevant for biological applications in the food industry, pharmaceuticals, and bioremediation, resulting in the patenting of various *B. licheniformis* strains, along with associated methods and applications (Muras et al., 2021; Singh et al., 2016). Evaluating its performance alongside other strains in wastewater treatment demonstrated the potential of SIW as an amylase- and protease-inducing medium, while PPMS

specifically promotes protease production. Further studies with modifications may enhance the efficiency of *B. licheniformis* even further.

In conclusion, *B. megaterium* and *B. licheniformis* appear to be more efficient protease producers across various wastewater types. However, all strains had limited lipase production, suggesting that these studied wastewater sources are not suitable for selected *Bacillus* strains synthesizing lipase. Although BW shows high nutrient potential for enzyme production, SIW and PPMS appear to be more conducive to the desirable enzyme production, with higher enzyme yields observed in these media by the selected bacteria.

Based on the combined results of bacterial growth and enzyme activities, *Bacillus amyloliquefaciens* was selected to cultivate on SIW for amylase production, while *Bacillus megaterium* was chosen to ferment on PPMS for protease and lipase production. These strains were selected for their optimal enzyme production capabilities under the respective wastewater conditions.

4.4.5 Optimization of enzyme production

In the previous study, *Bacillus megaterium* demonstrated the ability to produce protease and minimal lipase when cultivated on PPMS and PPMS supplemented with Tween 20, respectively. To comprehensively assess this potential, a design-of-experiments approach was employed to identify key factors influencing protease and lipase production. A similar objective was pursued for *Bacillus amyloliquefaciens* on SIW. Among various response surface methodologies, the Box–Behnken design was chosen, considering three factors: temperature, total solids, and inoculum size. The Box–Behnken design was particularly advantageous due to its ability to reduce the number of experimental runs for a known bioprocess while maintaining three balanced levels, thereby avoiding extreme conditions unsuitable for bacterial growth and enzyme production. This statistical approach ensured the generation of a reliable dataset for model development, with a total of 17 experimental runs conducted across three enzyme production conditions.

Enzyme production is highly sensitive to cultural parameters, with various factors influencing yields to different extents depending on the bacterial strain and the specific enzyme produced. In this study, the primary goal is to develop a cost-effective enzyme production process by valorizing

wastewater. Therefore, key variables and their relevant levels were selected based on their impact on both yield efficiency and economic feasibility, as well as their simplicity and practicality.

Temperature is one of the most critical physiological factors affecting microbial growth and enzyme production. It directly influences metabolic activity, enzymatic reaction rates, and microbial adaptability. Excessive temperatures can denature proteins and reduce bacterial viability, whereas temperatures that are too low slow down metabolism and enzyme synthesis. Furthermore, temperature regulation requires energy—higher temperatures demand more power, while lower temperatures may compromise microbial efficiency. Given these considerations, a moderate range of 30–37 °C suitable was chosen, as it supports optimal microbial activity while minimizing excessive energy costs. This range is commonly used in bioprocesses with *Bacillus* species due to its balance between efficiency and economic sustainability.

Nutritional factors, particularly media composition, play a key role in microbial fermentation. Instead of relying on pure chemical substrates, this study utilized low-cost industrial wastewaters as nutrient sources. However, careful selection was necessary to maximize nutrient availability while minimizing the impact of undesirable components. To support this, TSs were employed as an adjustment parameter, helping to ensure an efficient fermentation process without the need for additional chemical supplementation. As the wastewaters investigated in this study originated from established industrial operations, their quality could vary between batches, but typically within a manageable range. TSs thus provided a relatively stable and practical parameter for preliminary assessment. TSs reflect the sum of suspended and dissolved substances, encompassing both essential nutrients for microbial growth (such as organic carbon, nitrogen, and phosphorus) and potentially inhibitory compounds (such as phenolics and long-chain fatty acids). Therefore, the relationship between TSs, nutrient availability, and the presence of inhibitory substances was considered in selecting and optimizing wastewaters for enzyme production. In this study, optimization of TSs did not aim to eliminate undesirable substances, but rather to adjust the solids concentration to balance nutrient availability and toxicity risk. A TSs level that is too low may fail to support sufficient microbial growth due to nutrient limitation, whereas an excessively high TSs could introduce inhibitory effects that suppress enzyme production. Thus, adjusting TSs to an optimal range was critical for maximizing microbial performance and enzyme

productivity while avoiding the need for further pretreatment, thereby enabling a more cost-effective and efficient valorization strategy.

Finally, inoculum size is another crucial factor, as it determines microbial adaptation to the fermentation environment and ultimately influences enzyme yields. This is particularly important in unconventional conditions such as wastewater-based fermentation, where microbial resilience plays a key role in process efficiency.

4.4.6 Optimization of protease production

Protease activity peaked at 42.56 U/mL (33.5 °C, 25 g/L TS, 5% inoculum) (Table 4.3). The comparison between predicted and experimental values showed no significant difference, indicating a good model fit and statistical significance ($p \leq 0.05$) (Table 4.4).

Table 4.3 Optimization of protease production by *Bacillus megaterium* on PPMS using Box–Behnken design.

Std	Run	Temperature (°C)	TSs (g/L)	Inoculum Size (% *v*/*v*)	Protease (U/mL)
11	1	33.5	15	2	36.19
9	2	30	20	2	38.22
6	3	33.5	25	2	38.60
8	4	33.5	20	3.5	39.34
1	5	33.5	20	3.5	39.30
7	6	33.5	20	3.5	38.78
4	7	33.5	20	3.5	38.60
16	8	33.5	20	3.5	40.06
12	9	30	25	3.5	39.19
3	10	30	15	3.5	39.42

5	11	30	20	5	42.56
17	12	33.5	15	5	41.46
15	13	33.5	25	5	42.56
2	14	37	15	3.5	40.02
14	15	37	25	3.5	39.32
10	16	37	20	2	39.23
13	17	37	20	5	40.15

Table 4.4. Statistical analysis of Box–Benkhen design for protease production in *Bacillus megaterium*.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	27.06	2	13.53	16.67	0.0002	significant
B—TSs	0.8363	1	0.8363	1.03	0.3273	
C—inoculum size	26.23	1	26.23	32.31	<0.0001	
Residual	11.36	14	0.8116			
Lack of Fit	10.07	10	1.01	3.11	0.1424	Not significant
Pure error	1.29	4	0.3233			
Cor total	38.42	16				

The first-order polynomial Equation (1) was fitted to the experimental protease activity, resulting in the following regression equation, which describes the relationship between the tested factors and enzyme production.

$$\text{Protease} = 39.59 + 0.32 * \text{B} + 1.81 * \text{C} \quad 1)$$

where A represents temperature, B represents total solids, and C represents inoculum size.

The results of the analysis indicate that the regression model is statistically significant, with a model F-value of 16.67 and only a 0.02% chance that this result could occur due to random noise. The lack-of-fit F-value of 3.11 suggests that the model fits the data well. Although the predicted R^2 (0.5336) and adjusted R^2 (0.6620) values are in reasonable agreement—indicating a consistent model fit—the moderate R^2 values suggest that the model explains a fair portion of the variability in the response. This may limit its predictive accuracy to some extent and implies that additional influential factors may not have been captured in the current experimental design. Nonetheless, the Adeq precision value of 11.277 supports the model's adequacy and reliability for guiding optimization within the design space.

The final linear equation highlights the relative positive impact of factors B and C on protease activity, with C having a stronger effect and factor A excluded due to its insignificance. The model is effective for predicting protease activity based on two out of three studied factors, and its linear nature suggests that no higher-order terms are needed for this analysis.

Since temperature had no significant effect on protease production, the lowest tested temperature was chosen to minimize energy consumption. Total solids and inoculum size positively affected the model; therefore, the maximum total solid concentration was maintained. However, the highest inoculum level was impractical for industrial applications, and the inoculum size was adjusted to 2% (v/v) to ensure feasibility in large-scale operations.

The suggested conditions (30 °C, 25 g/L total solids, and 2% inoculum size (v/v)) were tested in triplicate to validate the model. The experimental results closely aligned with the predicted values, confirming the model's reliability despite the moderate predicted R^2 value of 38.1 U/mL. Under these optimized conditions, *Bacillus megaterium* produced 39.6 ± 3.53 U/mL of protease, demonstrating the model's effectiveness in predicting enzyme production.

4.4.7 Optimization of lipase production

As *B. megaterium* exhibited low lipase activity on PPMS alone, and non-ionic surfactants such as Tween are commonly used as low-cost inducers to stimulate bacterial lipase production (Boekema

et al., 2007), an additional step was included. Tween 20, containing medium-chain fatty acids, was selected for inclusion in the single-run optimization experiment, as the long-chain structure of Tween 80 had previously demonstrated a lower induction effect under the specific conditions of PPMS. In the presence of Tween 20 and Tween 80, the observed lipase activities were 0.07 ± 0.0012 U/mL and 0.02 ± 0.07 U/mL, respectively.

The Box–Behnken design (BBD) results revealed that maximum lipase production of 0.097 U/mL was achieved at 30 °C with 25 g/L total solids and 3,5% inoculum size in sludge (v/v). The correlation is expressed by the following equation:

$$\text{Lipase} = 0.0278 - 0.0135 * \text{A} + 0.0253 * \text{B} - 0.0163 * \text{AB} \quad (2)$$

where A represents temperature, B represents total solids, and C represents inoculum size.

The results indicate that, although A and B were reported to play a role in the model, the coefficients for both factors are relatively small, indicating that their individual effects on lipase production are modest. Additionally, factor C had no significant influence on the model, confirming that its exclusion does not affect predictive accuracy.

The model F-value of 47.16 indicates statistical significance, with only a 0.01% chance that such a high F-value occurred due to noise. The lack-of-fit F-value of 1.96 implies that the lack of fit is not significant relative to pure error, with a 26.93% chance that this large F-value could arise due to noise. A non-significant lack of fit is desirable, as it indicates the model fits the data well.

The predicted R^2 of 0.7554 is in reasonable agreement with the adjusted R^2 of 0.8964, with the difference being less than 0.2, which suggests a good model fit. Furthermore, the Adeq precision ratio of 23.323 indicates a high signal-to-noise ratio, confirming that the model can be reliably used to navigate the design space.

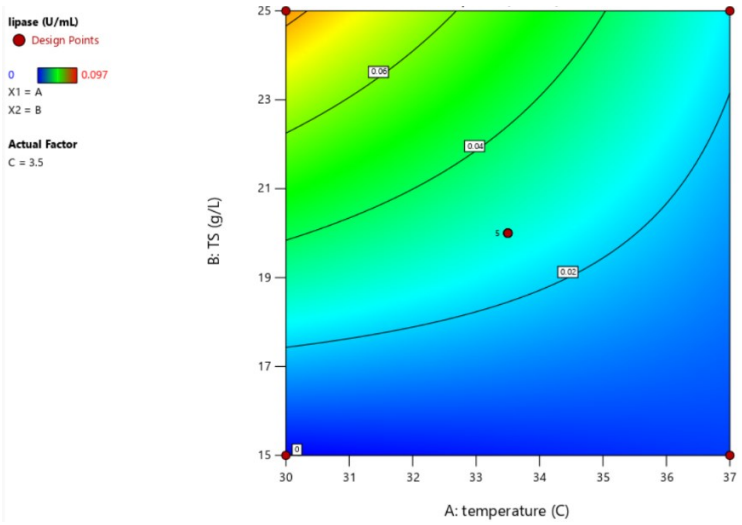

Figure 4.3 Response surface of the optimal cultivation conditions for lipase production.

To confirm the statistical tests, while the significant factors—temperature (30 °C) and total solid concentration (25 g/L)—were maintained as per the model, the inoculum size was reduced to 2% from the initial 3.5%, as this factor had no significant impact on lipase production. This adjustment was made to improve the feasibility of large-scale optimization. Under these specific conditions, a lipase activity of 0.091 U/mL was recorded, showing an approximate 10% difference from the predicted value of 0.083 U/mL, confirming the model's validity. The results from this study with PPMS, supplemented with 1% Tween 80, are quite similar to the findings for *Bacillus megaterium* AKG-1, which produced 0.116 U/mL of lipase in nutrient broth with added soybean oil (data converted for comparison based on the definition of one enzyme unit) (Sekhon et al., 2006). Although lipase activity increased significantly from 0.01 U/mL before optimization, the final yield of approximately 0.1 U/mL remains too low for practical industrial applications, as recognized in other studies. For example, *Bacillus megaterium* F25 was reported to secrete lipase at a concentration of 0.583 U/mL in the presence of Tween 80 in shaking flask cultures (Karaman et al., 2024). As the experiment did not meet the necessary criteria for the project, further trials will not proceed.

4.4.8 Optimization of amylase production

The response variances of α-amylase (U/mL) are described in Table 4.5. The quadratic model was obtained from experimental amylases to explain the statistical interactions between each parameter and their effect on the process of α-amylase production. The correlation between the enzyme production and the optimization factors A, B, and C through the Box–Behnken design has presented in expressions of coded factors according to the following equation:

$$\begin{aligned}\text{Amylase} = {} & 4.02 - 0.425 * \text{A} + 0.575 * \text{B} - 0.2325 * \text{C} + 0.09 * \text{AB} - 0.155 * \\ & \text{AC} - 0.31 * \text{BC} - 0.566 * \text{A2} - 0.141 * \text{B2} - 0.426 * \text{C2}\end{aligned} \quad (3)$$

where A, B, and C are the coded values of temperature, total solids, and inoculum size, respectively.

Table 4.5. Optimization of amylase production by *Bacillus amyloliquefaciens* on SIW using Box–Behnken design.

Std	Run	Temperature (°C)	TSs (g/L)	Inoculum Size (% *v*/*v*)	α-Amylase (U/mL)
9	1	33.5	15	2	2.75
6	2	37	20	2	3.08
10	3	33.5	25	2	4.53
16	4	33.5	20	3.5	3.85
13	5	33.5	20	3.5	4.23
3	6	30	25	3.5	4.26
17	7	33.5	20	3.5	4.19
8	8	37	20	5	2.21
11	9	33.5	15	5	3
15	10	33.5	20	3.5	3.8
14	11	33.5	20	3.5	4.04
1	12	30	15	3.5	3.3
2	13	37	15	3.5	2.19
5	14	30	20	2	3.54
7	15	30	20	5	3.29
12	16	33.5	25	5	3.54
4	17	37	25	3.5	3.51

The given quadratic response surface model describes the influence of temperature (A), total solids (B), and inoculum size (C) on α-amylase activity (Y), incorporating linear, interaction, and quadratic terms (Table 4.6). The negative coefficient for temperature (−0.425) indicates that increasing temperature reduces α-amylase activity, with a strong quadratic effect (−0.566)

suggesting an optimal temperature exists beyond which activity declines. In contrast, total solids has a positive linear effect (+0.575), enhancing activity, though the quadratic term (−0.141) implies diminishing returns at higher concentrations. The negative linear effect of inoculum size (−0.2325) and its quadratic term (−0.426) indicate a non-linear impact, where excessive inoculum may reduce enzyme activity. Interaction terms, such as the positive AB (+0.09) and negative AC (−0.155) and BC (−0.31), reveal that combined factors can enhance or inhibit α-amylase production, highlighting the need to optimize multiple parameters rather than individual factors in isolation. Overall, the model suggests that α-amylase activity is highly sensitive to these factors, and response surface methodology can be applied to determine optimal conditions for maximizing enzyme production.

Table 4.6 Statistical analysis of Box–Behnken design for amylase production in *B.amyloliquefaciens*.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	7.42	9	0.8249	31.77	<0.0001	significant
A—temperature	1.44	1	1.44	55.66	0.0001	
B—TSs	2.65	1	2.65	101.88	<0.0001	
C—inoculum size	0.4325	1	0.4325	16.66	0.0047	
AB	0.0324	1	0.0324	1.25	0.3008	
AC	0.0961	1	0.0961	3.70	0.0958	
BC	0.3844	1	0.3844	14.81	0.0063	
A^2	1.35	1	1.35	51.96	0.0002	
B^2	0.0837	1	0.0837	3.22	0.1156	
C^2	0.7641	1	0.7641	29.43	0.0010	
Residual	0.1817	7	0.0260			
Lack of fit	0.0311	3	0.0104	0.2748	0.8415	Not significant
Pure error	0.1507	4	0.0377			
Cor total	7.61	16				

The statistical analysis confirms that the model is highly significant, as indicated by the model F-value of 31.77, with only a 0.01% chance that such a high value could result from noise. The *p*-values reveal that temperature (A), total solids (B), inoculum size (C), the interaction term BC, and the quadratic terms A^2 and C^2 significantly influence α-amylase activity, while other terms may have a negligible effect. If multiple insignificant terms are present, model reduction could enhance efficiency without disrupting the hierarchical structure. The lack-of-fit F-value of 0.27 suggests that the model fits the data well, as the lack of fit is statistically insignificant. Furthermore, the predicted R^2 (0.9037) and adjusted R^2 (0.9454) are very close to 1, indicating that the model explains over 90% of the variation in α-amylase activity (Figure 4.4). The small difference between these values (less than 0.2) confirms the model's predictive reliability and suggests that it generalizes well without overfitting. The high adjusted R^2 also indicates that the model effectively captures the relevant predictors while minimizing the impact of unnecessary terms. Additionally, the Adeq precision ratio of 19.057, which far exceeds the minimum desirable threshold of 4, reflects a strong signal-to-noise ratio, reinforcing the model's suitability for exploring the design space. These statistical indicators suggest that the model provides a reliable basis for optimizing and predicting α-amylase activity under different experimental conditions.

The experimental validation of the model's optimal conditions confirmed that the maximum α-amylase production of 4.05 ± 0.05 U/mL can be achieved by incubating *Bacillus amyloliquefaciens* at 30 °C, using a medium containing 25 g/L total solids from starch wastewater and a 2% inoculum size (Figure 4.5). This result is highly consistent with the model's predicted data (4.19 U/mL), further supporting the accuracy and reliability of the designed model.

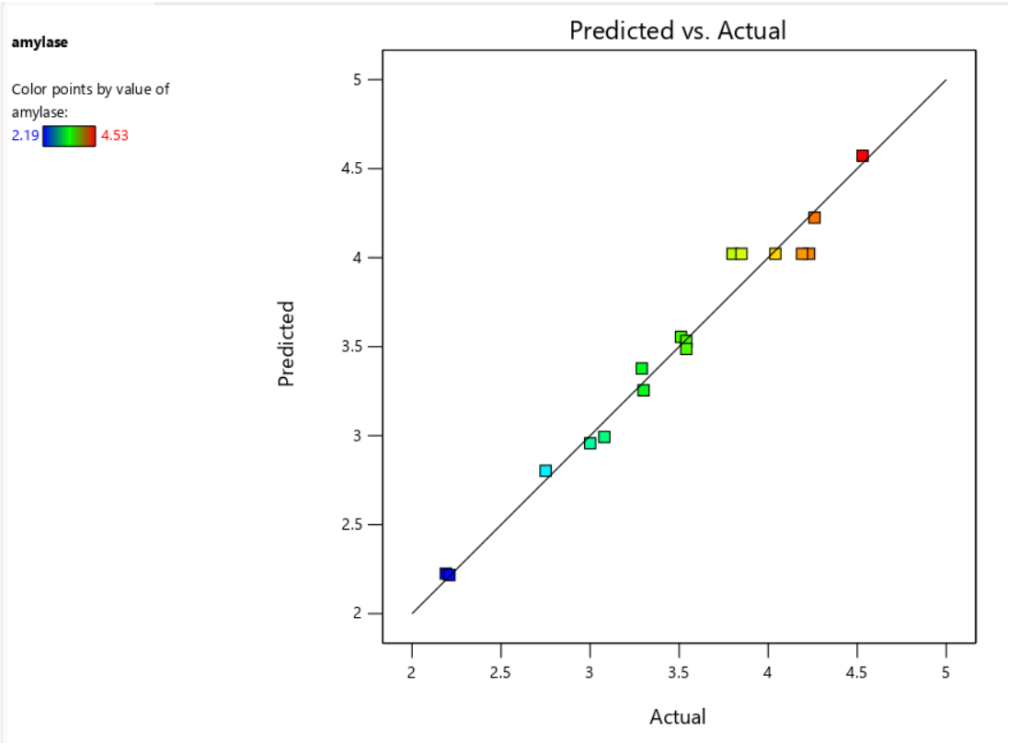

Figure 4.4 Model predictions as a function of the experimental amylase activity (U/mL).

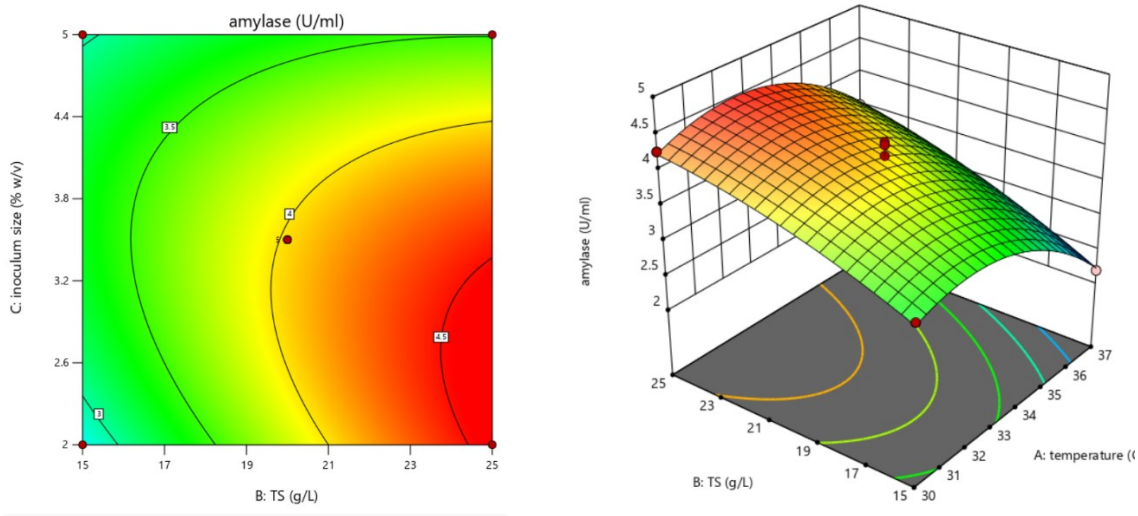

Figure 4.5 Response surface of optimal conditions for amylase production.

4.4.9 Enzyme production in 5 L bioreactor

4.4.9.1 Production of protease using *Bacillus megaterium*

Protease production was assessed at 24 h intervals, showing significantly higher yields in 5 L bioreactors compared to shake flasks, highlighting the advantages of controlled fermentation conditions. In the first batch, *Bacillus megaterium* produced 52.91 U/mL after 24 h, decreasing to 43.73 U/mL at 48 h. In a second batch, protease activity peaked at 57.91 U/mL at 24 h—coinciding with maximum cell density—before declining to 40.18 U/mL at 48 h. The observed decline in activity after 24 h may be attributed to protease instability, product degradation, or nutrient depletion in the medium. These results are consistent with previous findings, although the maximum yields reported here are slightly lower than those in some literature. Differences can largely be explained by variations in medium composition, inoculum characteristics, and strain origin. For instance, Rajkumar et al. (2011) reported a significantly higher yield of 78.5 U/mL from a *B. megaterium* strain isolated from red seaweed, using a complex medium enriched with glucose, yeast extract, gelatin, lactose, and seawater, and adjusted to an initial pH of 9. The incubation was carried out at 40 °C for 42 h. These optimized conditions, particularly the alkaline pH and complex nitrogen sources, may have favored protease expression.

In contrast, a marine-derived *B. megaterium* strain from the Gulf of Thailand showed a much lower activity (6.57 ± 0.25 U/mL) after 15 h at pH 5.0 and 30 °C (Uttatree et al., 2017). This emphasizes the strong influence of environmental and nutritional parameters on protease productivity. An important observation from this study is the shorter fermentation time and higher yield in bioreactor cultures compared to shake flasks (see Figure 4.6). This improvement is likely due to better aeration, mixing efficiency, and pH/temperature control in bioreactors, which collectively enhance microbial growth and enzyme production, as also noted by Priya et al. (2014).

Similarly, *B. megaterium* AU02, an organic solvent-tolerant strain isolated from dairy effluents, produced 43.6 U/mL in shake flasks after 49 h using a medium containing skim milk and calcium chloride. Under optimized conditions in a 7 L fermenter, this value increased to 53 U/mL at approximately 32 h, aligning closely with the current findings. Overall, the current study demonstrates the potential of *B. megaterium* for efficient protease production under relatively

simple culture conditions, with bioreactor cultivation significantly enhancing productivity. Further optimization of media components, particularly nitrogen and carbon sources, along with fed-batch or continuous fermentation strategies, could improve yields even further and facilitate industrial-scale applications.

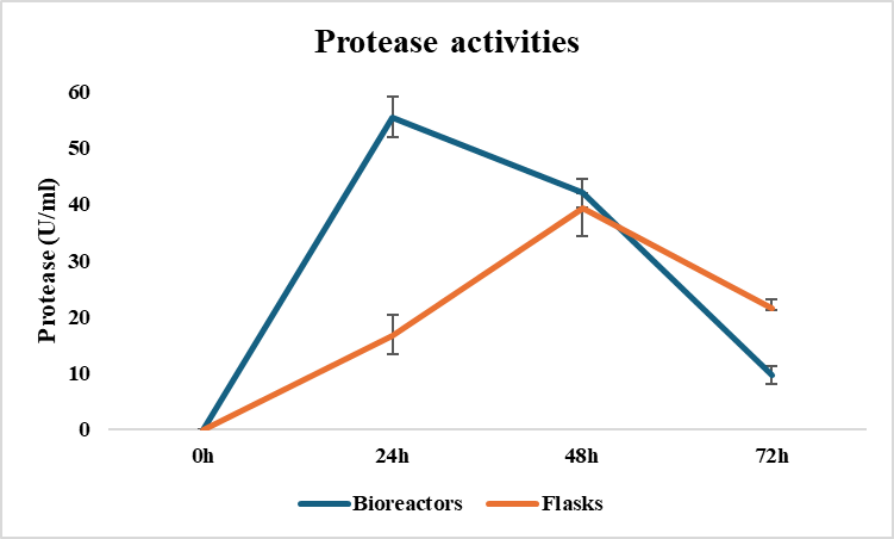

Figure 4.6 Kinetics of protease production in flasks and bioreactors.

4.4.9.2 Bioreactor production of amylase using *Bacillus amyloliquefaciens*

The optimization model identified 25 g/L of total solids as the optimal concentration for protease production. However, it also suggested that increasing total solids could enhance amylase production, with a positive trend observed up to higher concentrations, despite diminishing returns. To validate this, an additional experiment was conducted using 40 g/L of total solids. Results from 1 L shake flasks confirmed that this concentration provided an optimal balance between maximizing amylase yield and maintaining manageable medium viscosity. Exceeding this concentration resulted in increased viscosity, which negatively impacted oxygen diffusion and mixing, critical parameters for microbial growth and enzyme production, especially in scaled-up fermentation systems. Based on this optimization, two 5 L bioreactor experiments were conducted using 40 g/L total solids, 30 °C incubation temperature, and a 2% inoculum size to evaluate amylase production under controlled and scalable conditions. In the first bioreactor trial, samples were collected every 12 h over a 72 h period. *Bacillus amyloliquefaciens* began secreting

amylase during the exponential phase, with activity levels rising from 0.05 U/mL at 12 h to 4.15 U/mL at 36 h and peaking at 5.74 U/mL at 48 h. By 72 h, activity had declined to 4.34 U/mL, suggesting that maximum enzyme production occurred during the transition from the late exponential to early stationary phase, with a decline likely due to proteolytic degradation or depletion of key nutrients. The peak amylase activity in the second batch reached 5.72 U/mL, nearly identical to the first trial, indicating that extending the fermentation beyond 48 h offered no additional benefit and could even reduce enzyme yield due to stability issues. These findings confirm that bioreactor cultivation, with precise control over temperature and dissolved oxygen, significantly enhances amylase production compared to flask-scale fermentations, even under the same incubation duration (Figure 4.7). This underscores the scalability and industrial potential of the process.

B. amyloliquefaciens is widely recognized for its industrial utility, with applications spanning food processing, pharmaceuticals, agriculture, biofuels, and environmental biotechnology (WoldemariamYohannes et al., 2020). Its ability to convert diverse agro-industrial residues into high-value products like amylases aligns with current trends in circular bioeconomy and waste valorization. Numerous substrates such as kitchen waste (Bhatt et al., 2020), bread waste (Abd-Elhalim et al., 2023), wheat bran, rice husk, maize starch, and starchy tuber residues have been explored as low-cost feedstocks for enzyme production. However, reported amylase yields vary considerably across studies, influenced by multiple factors including strain genetics, substrate composition, pretreatment methods, fermentation strategies (submerged vs. solid-state), and differences in enzyme assay protocols.

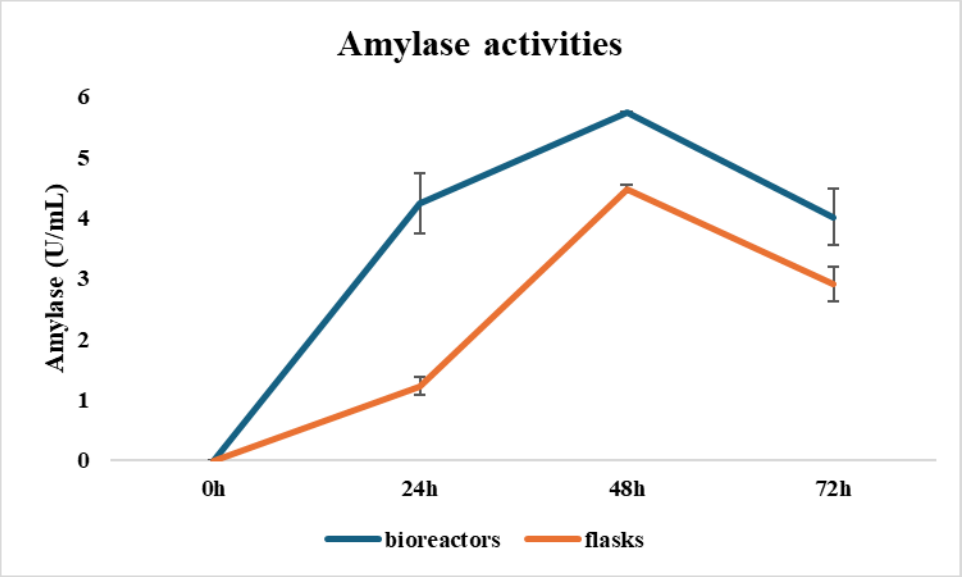

Figure 4.7 Kinetics of amylase production in flasks and bioreactors.

4.1.Conclusion

This study investigated four local wastewater types in Quebec as alternative media for enzyme production, focusing on protease, amylase, and lipase using *Bacillus* species as part of a pragmatic screening strategy for potential waste substrates. Among them, SIW effectively supported amylase synthesis by *B. amyloliquefaciens*, achieving 5.73 ± 0.01 U/mL at 48 h under 40 g/L total solids, 30 °C, and a 2% inoculum size in a 5 L bioreactor. Meanwhile, *B. megaterium* produced both protease and lipase in PPMS, with significant protease activity warranting further scale-up experiments. The highest protease yield, 55.41 ± 3.54 U/mL, was recorded at 24 h under 25 g/L total solids, 30 °C, and a 2% inoculum in duplicated bioreactors. The simple preparation of wastewater—considering total solids, temperature, and inoculum size—and the adaptability of these enzymes for high yields in 5 L bioreactors, underscores the industrial relevance of this approach. The reproducibility observed at this scale implies a strong potential for larger-scale development. However, physical constraints encountered during scale-up, including limitations in oxygen transfer, mixing efficiency, temperature control, and foaming are anticipated to manifest more severely in complex media such as wastewater compared to synthetic substrates, owing to their heterogeneous and variable nature. Despite these challenges, repurposing wastewater not only mitigates environmental impact but also transforms waste into valuable

bioproducts, aligning with the circular bioeconomy and enhancing sustainability by improving resource efficiency and lowering production costs.

This study is conducted from a foundational stage; therefore, selecting a subset of wastewater sources from several options serves as an initial step in identifying those with potential for enzyme production by designated *Bacillus* species. However, this approach inherently carries the risk of overlooking novel isolates that could enhance enzyme productivity due to their adaptation to specific environmental conditions. Consequently, the findings of this study should be regarded as preliminary and may serve as a reference for future experiments incorporating newly identified isolates.

5 VALORIZATION OF PAPER PULP MILL SLUDGE FOR PROTEASE PRODUCTION BY INDIGENOUS *BACILLUS TROPICUS* P4

Valorisation des boues de l'industrie papetière pour la production de protéase par la souche indigène *Bacillus tropicus* P4

Vu-Mai-Linh Nguyen[1], Adama Ndao[1], Jean-François Blais[1], Kokou Adjallé[1*]

[1] Centre Eau Terre Environnement (ETE), Institut National de la Recherche Scientifique (INRS), 490 Rue de la Couronne, Quebec City, QC G1K 9A9, Canada

Corresponding author: Tel: (418) 654 2610, Fax: (418) 654 2600, email: Kokou.Adjalle@inrs.ca

(This paper has been submitted.)

Lien entre le deuxième et le troisième article:

Le deuxième article avait pour objectif de sélectionner les effluents industriels les plus prometteurs parmi quatre types étudiés, en évaluant les combinaisons possibles entre différentes souches commerciales de *Bacillus* et les eaux usées. Les résultats majeurs incluent :

- *Bacillus megaterium* a produit des protéases et des lipases sur les boues de papeterie (PPMS), tandis que *B. amyloliquefaciens* a généré des amylases sur les eaux usées de l'industrie de l'amidon (SIW) ;
- Grâce à un plan de Box-Behnken, les conditions optimales pour la production de protéase par *B. megaterium* ont été déterminées : 30 °C, 25 g/L de solides totaux et 2 % d'inoculum, avec l'ajout de 1 % de Tween 20 pour induire la production de lipase ;
- Pour *B. amyloliquefaciens*, les conditions optimisées pour la production d'amylase sur SIW étaient de 25 °C, 40 g/L de solides totaux et 2 % d'inoculum ;

- La scalabilité de la production d'amylase et de protéase a été validée à l'échelle de bioréacteurs de 5 L.

Bien que ces souches commerciales aient démontré leur capacité à exploiter certains effluents pour produire des enzymes, le potentiel des souches indigènes reste largement inexploité. En particulier, le SIW, avec un pH acide, s'avère peu favorable à la croissance des *Bacillus*, tandis que le PPMS, au pH plus neutre, apparaît comme un milieu propice à l'isolement de souches locales capables de produire des enzymes d'intérêt. Le troisième article s'inscrit dans cette logique en visant à isoler des bactéries indigènes productrices de protéase à partir du PPMS et à évaluer leur capacité enzymatique dans ce substrat complexe.

5.1 Abstract

This study explores the potential of using paper pulp mill sludge (PPMS) as an economical substrate for producing high-value protease enzymes with an indigenous bacterial strain, *Bacillus tropicus* P4. Isolated directly from PPMS, B. *tropicus* P4 showed high protease-producing ability, approximately 134 U/mL after 48 hours—more than three times the yield of the benchmark strain (B. *megaterium*). Among various additives tested to boost enzyme production, Tween 80 emerged as the most effective, increasing enzyme activity by more than threefold compared to the control. Scale-up experiments in bioreactors of 5L and 150L confirmed that B. *tropicus* P4 maintains high protease yields under typical cultivation conditions with minimal modifications, specifically the addition of Tween 80 (1%) and increased total solids concentration (25 g/L). In the 5L bioreactor, enzyme production peaked at approximately 755 U/mL within 24 hours, while the 150L bioreactor consistently achieved high enzyme activity (~848 U/mL). These results support the feasibility of a simple and scalable approach for converting industrial sludge into high-value protease enzymes, contributing to resource recovery and circular bioeconomy strategies.

Keywords: Pulp and paper mill activated sludge, enzymes, valorization, protease, Bacillus tropicus P4, 5L bioreactor, 150L bioreactor, scale-up, bioeconomy.

Cette étude explore le potentiel d'utilisation des boues issues d'une usine de pâte à papier (PPMS) comme substrat économique pour la production d'enzymes protéases à forte valeur ajoutée à l'aide d'une souche bactérienne indigène, *Bacillus tropicus* P4. Isolée directement des PPMS, *B. tropicus* P4 a démontré une capacité élevée de production de protéase, atteignant environ 134 U/mL après 48 heures—soit plus de trois fois le rendement obtenu avec la souche de référence (*B. megaterium*). Parmi les différents additifs testés pour stimuler la production enzymatique, le Tween 80 s'est révélé le plus efficace, augmentant l'activité enzymatique de plus de trois fois par rapport au témoin. Des essais de changement d'échelle dans des bioréacteurs de 5 L et 150 L ont confirmé que *B. tropicus* P4 maintient des rendements élevés en protéase dans des conditions de culture classiques, moyennant seulement des ajustements mineurs, notamment l'ajout de Tween 80 (1 %) et une concentration accrue en solides totaux (25 g/L). Dans le bioréacteur de 5 L, la production enzymatique a culminé à environ 755 U/mL en 24 heures, tandis que le bioréacteur de

150 L a atteint de manière constante une activité élevée (~848 U/mL). Ces résultats démontrent la faisabilité d'une approche simple et évolutive pour transformer les boues industrielles en enzymes protéases à haute valeur ajoutée, contribuant ainsi à la valorisation des ressources et aux stratégies de bioéconomie circulaire.

Mots-clés : boues activées de pâte à papier, enzymes, valorisation, protéase, *Bacillus tropicus* P4, bioréacteur 5 L, bioréacteur 150 L, changement d'échelle, bioéconomie.

5.2 Introduction

The pulp and paper industry is one of the largest consumers of freshwater and generates a substantial amount of effluent worldwide. Water consumption ranges from 20,000 to 60,000 gallons per ton of product, leading to significant wastewater production (Pokhrel & Viraraghavan, 2004). Although wastewater treatment is well-established, it generates a large amount of sludge. Approximately 40–50 kg of dry primary and secondary (activated) sludge is produced per ton of paper. The projected rise in global paper and paperboard production from 400 million tons in 2012 to 550 million tons by 2050 could further increase wastewater sludge generation by 48–86% (Kaur et al., 2020).

Valorizing primary sludge, which is fiber-rich and accounts for 70% of total sludge in a paper mill, presents a promising waste-to-resource opportunity (Quintana et al., 2024). However, secondary sludge also known as pulp and paper mill activated sludge (PPMS) holds most of the organic load from the treatment process because of microbial degradation and assimilation (Geng et al., 2007; Kaur et al., 2020). These microbes help reduce dissolved organic matter, chemical oxygen demand (COD), and biochemical oxygen demand (BOD), but their activity also incorporates or adsorbs recalcitrant compounds onto the microbial biomass, making the sludge difficult to process and dewater (Kamali & Khodaparast, 2015). Managing PPMS remains a major challenge, as improper disposal can lead to soil, air, and water pollution from toxic compounds such as chlorinated organics, resin acids, heavy metals, and other contaminants (Faubert et al., 2016; Ghribi et al., 2016).

Beyond conventional disposal methods, microbial valorization of waste-activated sludge aligns with a "zero-sludge" strategy for wastewater treatment. Due to its diverse composition, sludge environments harbor a wide variety of resistant and adapted bacteria, making it a promising source of industrially relevant enzymes. Several studies have confirmed that paper mill sludge supports microbial diversity (Karn et al., 2013; Maki et al., 2011).

Despite their high production costs, industrial enzymes remain in strong demand particularly proteases, which are experiencing significant global growth. In 2024, the protease market was valued at USD 3.4 billion and is projected to reach USD 5.01 billion by 2030, reflecting a compound

annual growth rate (CAGR) of 5.7% (https://virtuelmarketresearch.com). Bacteria and bacterial proteases are becoming increasingly important across various industries, including food and beverages, pharmaceuticals, detergents, and animal feed (Kumar et al., 2012).

This study aims to isolate novel bacteria from PPMS that can act as microbial catalysts to convert sludge into high-value-added products such as proteases. These cost-effective enzymes could serve as green chemicals in various end-use industries, contributing to a circular bioeconomy.

5.3 Materials and methodology

5.3.1 Sample collection and storage

Pulp and paper mill activated sludge (PPMS) from Kruger Inc. (Trois-Rivières, Québec, Canada) was collected locally in Québec, Canada, and stored at 4°C.

5.3.2 Medium preparation

In this study, PPMS had the following composition (g/L): total solids (TS), 10.3 ± 0.36; total suspended volatile solids (TSV), 6.09 ± 0.37; suspended solids (SS), 9.4 ± 0.36; suspended volatile solids (SSV), 2.35 ± 0.18; total organic carbon (TOC), 14 ± 1.00; total organic nitrogen (TON), 1.4 ± 0.15; organic phosphorus (P_org), 2.3 ± 0.36; sodium (Na), 10.2 ± 0.56; iron (Fe), 0.4 ± 0.17; potassium (K), 1.4 ± 0.30; calcium (Ca), 22 ± 2.65; sulfur (S), 3.9 ± 0.95; glucose, 1.9 ± 0.75; fructose, 1.5 ± 0.30; lactose, 0.83 ± 0.05; sucrose, 0.97 ± 0.20; galactose, 1.4 ± 0.13; xylose, 1.1 ± 0.29; and trehalose, 0.53 ± 0.08 and pH 6.65 ± 0.35.

As the sludge was investigated in previous experiments in our laboratory (Nguyen et al., 2025), it was found that achieving higher protease activity requires a more concentrated supply of nutrients compared to the untreated sludge. By increasing the total solids content through centrifugation from 10.3 to 25 g/L, essential nutrients are effectively enriched, thereby creating a more favorable environment for fermentation. This pretreatment step offers a balance between nutrient availability and process feasibility, minimizing complications such as poor mixing or limited mass transfer associated with excessively high solid concentrations.

5.3.3 Isolation of microorganisms

A total of 0.5 mL of PPMS was aseptically transferred into 4.5 mL of sterile distilled water to obtain a 10^{-1} dilution. Serial dilutions were then performed up to 10^{-5} by transferring 0.5 mL from each dilution into 4.5 mL of sterile distilled water in subsequent tubes. From the 10^{-3}, 10^{-4}, and 10^{-5} dilutions, 100 µL of each sample was inoculated onto nutrient agar plates (10 g/L peptone, 3 g/L beef extract, 5 g/L NaCl; pH 7.0) supplemented with 1% (w/v) casein by using the spread plate technique. The plates were then incubated at 30 °C for 48 hours to allow for microbial growth and colony formation.

5.3.4 Screening of protease-producing strains

The screening of protease-producing isolates was conducted in two successive stages. Initially, a qualitative assessment was performed using spot inoculation on selective agar plates, followed by quantitative evaluation through cultivation on sludge medium.

To evaluate enzyme production potential, *Bacillus megaterium* (BM) was used as a reference control, as it initially demonstrated the ability to produce alkaline proteases in PPMS during the early stage of the project (Nguyen et al., 2025). This comparison highlights the efficiency of wastewater utilization through two distinct approaches—using a commercial strain versus indigenous isolates. For the qualitative screening of protease-producing isolates, spot inoculation was performed on selective agar plates containing (per liter): K_2HPO_4, 2 g; glucose, 1 g; peptone, 5 g; and casein, 10 g. The plates were incubated at 30 °C for 48 hours. Following incubation, the plates were flooded with 25% (w/v) trichloroacetic acid (TCA) and maintained at 45 °C for 15 minutes to precipitate unhydrolyzed proteins, thereby enhancing the visibility of proteolytic zones. The formation of clear zones surrounding the colonies was considered indicative of proteolytic activity.

For quantitative screening, isolates demonstrating clear proteolytic zones were cultured in PPMS liquid media under the following conditions: incubation at 30 °C, pH 7.0, for 48 hours in an orbital shaker set at 180 rpm. Following fermentation, protease production was assessed to identify the most promising isolates for further characterization.

5.3.5 DNA Extraction and 16S rRNA Gene Amplification

Genomic DNA was extracted using the Promega Genomic DNA Purification Kit (USA). The 16S rDNA region was amplified by PCR with universal primers 27F (5′-TAACACATGCAAGTCGAACG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR conditions included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplified product was sequenced and compared to reference sequences using BLASTN (https://www.ncbi.nlm.nih.gov/BLAST) to determine taxonomic identity based on sequence similarity.

5.3.6 Protease inducers

The effect of different inducers, casein, wheat bran, Tween 20, and Tween 80, on protease production was evaluated by incorporating them into the culture medium, followed by incubation in shake flasks at 30°C, pH 7.0, and 180 rpm for 48 hours, after which protease activity was determined.

5.3.7 Inoculum preparation

To prepare an active inoculum for large-scale experiments, a loopful of bacterial culture from a nutrient agar plate was transferred into 10 mL of nutrient broth in a 50 mL Erlenmeyer flask. The culture was incubated at 30°C with shaking at 180 rpm for 12 hours, allowing the cells to reach the exponential phase with a cell density of approximately 10^8 CFU/mL. Subsequently, 5 mL of this culture was inoculated into 250 mL of PPMS medium in a 1 L flask and incubated under the same conditions. This step facilitated the development of a metabolically active inoculum, well-adapted to the nutrient composition of the wastewater used in 5 L bioreactor experiments. For the 150 L bioreactor setup, which required 1.8 L of inoculum, an additional scale-up step was implemented. Two 5 L Erlenmeyer flasks, each containing 1.2 L of PPMS, were inoculated and incubated under identical conditions to produce the required volume of active culture.

5.3.8 Bioreactor studies

Fermentation was conducted in a Sartorius Biostat B 5 L glass bioreactor with a 3 L working volume containing PPMS (25 g/L total solids) supplemented with 1% (v/v) Tween 80. The system, equipped with a PLC for automated control of dissolved oxygen (DO), pH, temperature, agitation, aeration, and antifoam, was operated at 30 °C for 48 hours.

Scale-up was performed in a 150 L stirred-tank reactor with a 90 L working volume under the same operating conditions. In both bioreactor systems, DO levels were maintained between 30–50%, and samples were collected every 12 hours to monitor protease production.

The 5 L fermentation trials were carried out in two independent replicates, while the 150 L scale-up experiment was conducted as a single run.

5.3.9 Protease assay

To determine protease activity, the reaction mixture was prepared by combining 1 mL of appropriately diluted enzyme solution with 1 mL of 1% (w/v) casein dissolved in 50 mM Tris-HCl buffer (pH 7.0). The mixture was incubated at 50°C for 10 minutes to allow proteolysis. Following incubation, enzymatic activity was halted by adding 2 mL of 15% (w/v) trichloroacetic acid (TCA), which precipitated the undigested proteins. The resulting mixture was then centrifuged at 10,000 rpm for 10 minutes at 4°C to separate the supernatant containing the soluble peptides. From the clear supernatant, 0.5 mL was mixed with 2.5 mL of 2% (w/v) sodium carbonate solution, followed by the addition of 0.25 mL of 1 N Folin–Ciocalteu reagent. This mixture was incubated at room temperature for 30 minutes. Absorbance was then measured at 660 nm using a spectrophotometer. One unit of protease activity is defined as the amount of enzyme required to liberate 1 μg of tyrosine under the specified assay conditions (Nguyen et al., 2025).

5.4 Results and Discussion

5.4.1 Isolation protease-producing bacteria

Isolation was conducted as described in the Methodology section. After 2 days of incubation with the diluted sludge, visible bacterial colonies developed on Petri plates. On NA medium supplemented with 1% casein, four morphologically distinct strains were observed and designated as P1, P2, P3, and P4 (Figure 5.1). These strains were then purified on fresh NA plates and stored in a 10% glycerol solution at -20°C.

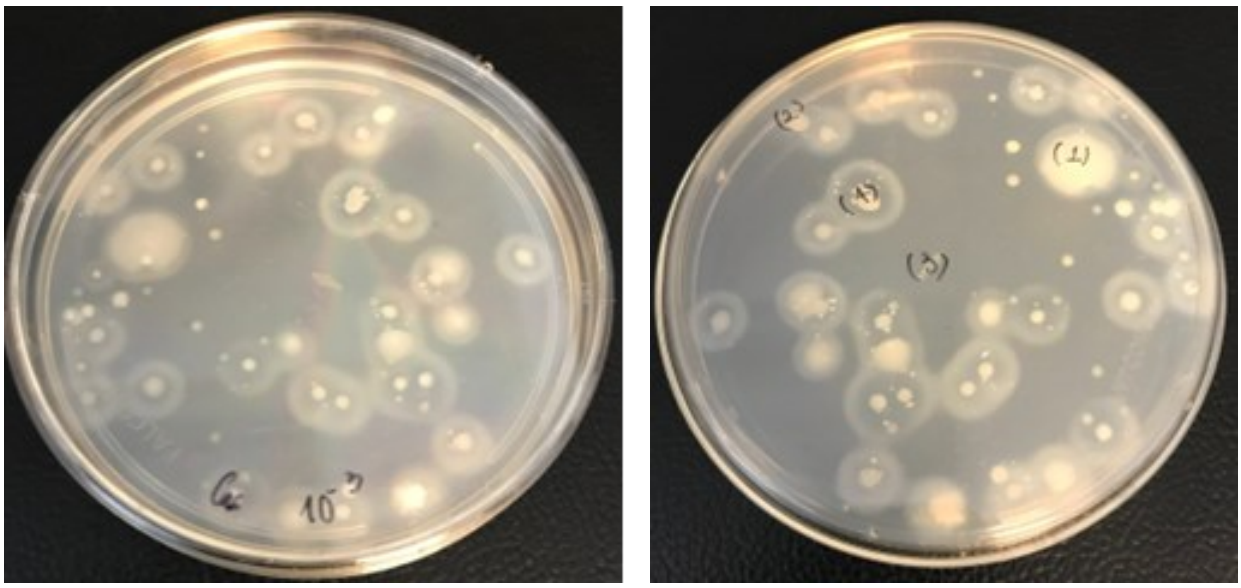

Figure 5.1 Isolation of bacterial strains on casein agar plates for preliminary screening of protease production. Distinct colonies were selected based on growth characteristics for further enzymatic analysis.

5.4.2 Screening and comparative assessment

Following isolation, a rapid and sensitive agar plate assay was employed to assess extracellular protease activity among the four bacterial isolates and the reference strain, *Bacillus megaterium*. After two days of incubation at 30°C, isolate P4 exhibited the largest halo, indicating the highest protease activity, followed by P1. In contrast, *Bacillus megaterium* merely caused a slight discoloration of the medium, suggesting lower protease activity, while P2 and P3 showed no detectable activity, as no halos were observed (Figure 5.2).

Since P1 and P4 exhibited substantial protease activity, these two strains were selected for further investigation of protease production in flasks. For this purpose, 200 mL of sludge containing 25

g/L total solids was used in 1 L flasks, and protease production was monitored spectrophotometrically to ensure precise measurement. The cultivation was carried out under standardized conditions, with periodic sampling to assess enzyme activity throughout the incubation period.

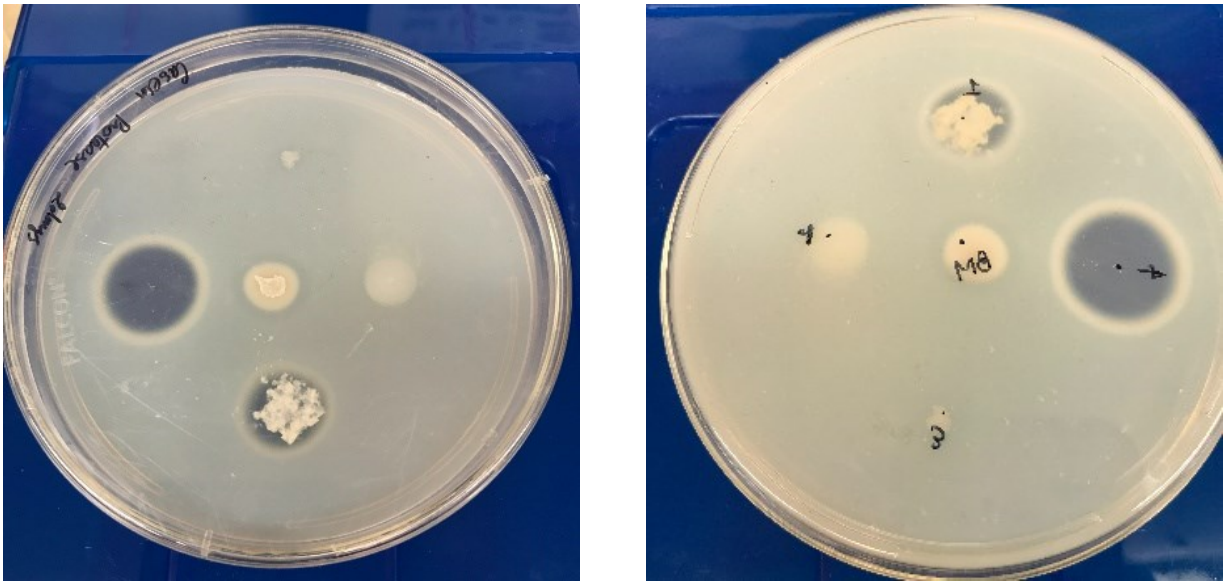

Figure 5.2 Top and bottom views of agar plates showing bacterial colonies and the formation of clear halo zones around them, indicating enzymatic activity.

Table 5.1 Protease activities of isolates (U/ml)

	0h	24h	48h
P1	-	10.32 ± 0.23	61.28 ± 5.66
P4	-	131.2 ± 0.11	133.84 ± 5.66
BM	-	16.71 ± 3.27	39.6 ± 3.53

(-) not detectable

Notably, *Bacillus megaterium* is widely recognized for its protease-producing ability across a broad range of synthetic and agro-industrial substrates, including mustard oilseed cake, rice bran, wheat bran, corn husk, gram husk, and soybean oil cake.(Saxena & Singh, 2014). For instance, Mishra et al. (2017) reported that *Bacillus megaterium* MTCC-9205 cultivated in soybean powder medium yielded a maximum of 46.75 ± 1.22 U/mL acidic protease and 38.47 ± 1.32 U/mL alkaline protease after 96 hours of incubation. These findings are consistent with the results of the present study, in

which *B. megaterium* produced 39.6 ± 3.53 U/mL protease after 48 hours, indicating that PPMS exhibits comparable effectiveness in supporting enzyme production. The inclusion of *B. megaterium* as a control thus provides a meaningful benchmark to assess the performance of a commercial strain under identical conditions. Interestingly, after 48 hours of cultivation in a sludge-based medium, the indigenous isolate P4 achieved a protease yield of approximately 134 U/mL over three times higher than *B. megaterium*—demonstrating exceptional enzymatic potential and robust environmental adaptation.

Although PPMS presents a cost-effective alternative for alkaline protease production, it also introduces challenges such as unfavorable growth conditions and stress-induced enzyme expression. Nevertheless, PPMS harbors a diverse microbial community, serving as a valuable reservoir of protease-producing candidates (Karn et al., 2013; Maki et al., 2011). In this context, only four isolates were obtained from the PPMS on the selective media, and only two demonstrated protease production, this limited diversity suggests that the PPMS environment is relatively harsh for or the survival of protease-producing bacteria. By contrast, this also implies a high potential for discovering valuable strains with strong adaptive capabilities and robust enzymatic potential. The sludge-derived isolate P4 demonstrated exceptional performance in a wastewater-based medium, further underscoring its capacity to fully harness the nutrient potential of this substrate. These findings underscore the strategic value of sourcing native strains from underexplored, selective environments, where harsh conditions drive the evolution of microorganisms with exceptional metabolic versatility and stress resilience. Consequently, wild isolates P4 was selected for further investigation to gain deeper understanding into its enzymatic capabilities.

In nutrient agar, the isolates P4 forms ivory-yellow, circular, smooth and creamy colonies. The isolate P4 was tested for its cultural conditions and demonstrated the ability to grow across a broad temperature range (15°C to 45°C), indicating its mesophilic nature. While growth was weak at the temperature of 15°C, P4 exhibited robust growth with larger colony diameters at higher temperatures, even at 45°C. However, no growth was observed at 50°C. Additionally, it thrived across pH levels ranging from 6 to 9, highlighting its adaptability to diverse environmental conditions.

5.4.3 Identification of the isolate microorganism (P4)

The 16S rDNA nucleotide sequence (1543 bp) of the isolate was analyzed using BLAST against the NCBI GenBank database, revealing 98.62% similarity with several members of the *Bacillus cereus* group, including *Bacillus tropicus* MCCC 1A01406 (NR_157736.1), *Bacillus paramycoides* MCCC 1A04098 (NR_157734.1), and *Bacillus nitratireducens* MCCC 1A00732 (NR_157732.1). Due to the high sequence similarity among these species, additional functional and ecological traits were considered. While *B. paramycoides* BP-N07 was described as forming slimy white colonies (Wang et al., 2023), and no reports are available on protease production or colony morphology for *B. nitratireducens*, *B. tropicus* has been isolated from diverse habitats, including marine sediments, vegetable waste, poultry feather waste, rhizospheric soils, and sewage effluents (Das & Ghosh, 2023; Liya et al., 2023; Shen et al., 2022).

Several *B. tropicus* strains have demonstrated industrially significant traits. Strain Y14, isolated from kitchen wastewater, produces a serine alkaline protease (PrA) with high thermal and chemical stability, comparable to commercial enzymes such as Alcalase (Das & Ghosh, 2023). Strain LS27 exhibited robust keratinolytic activity (~401.7 U/mL) and was effective in eco-friendly dehairing of hides and silver recovery from waste photographic film (Liya et al., 2023). Additionally, Gxun-17 could completely degrade feather waste within 48 hours, while other strains have shown cellulase or pectinase activity when cultivated on agro-industrial residues (Shen et al., 2022; Thakur et al., 2021). Beyond enzyme production, *B. tropicus* has also been reported to degrade chlorpyrifos, pentachlorophenol, and low-density polyethylene, as well as reduce toxic heavy metals like Cr(VI) and Pb(II) (Aregbesola et al., 2021; KumarMalik et al., 2024; Samanta et al., 2020).

Taken together, the molecular identity, ecological versatility, and wide functional profile support the assignment of the isolate as *Bacillus tropicus* P4. These findings are consistent with prior reports highlighting *Bacillus* species, including *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus sp., Bacillus tequilensis, Bacillus thuringiensis, and Bacillus cereus* as dominant enzyme producers in harsh or waste-rich environments (Ghribi et al., 2016).

5.4.4 Protease inducers

To assess the effect of each inducer, relative protease activities were expressed as percentages, calculated by comparing the activity from inducer-supplemented media to the baseline activity obtained from a medium containing only sludge (set as 100%). This approach allowed for the quantification of each inducer's contribution to protease enhancement relative to the unsupplemented control.

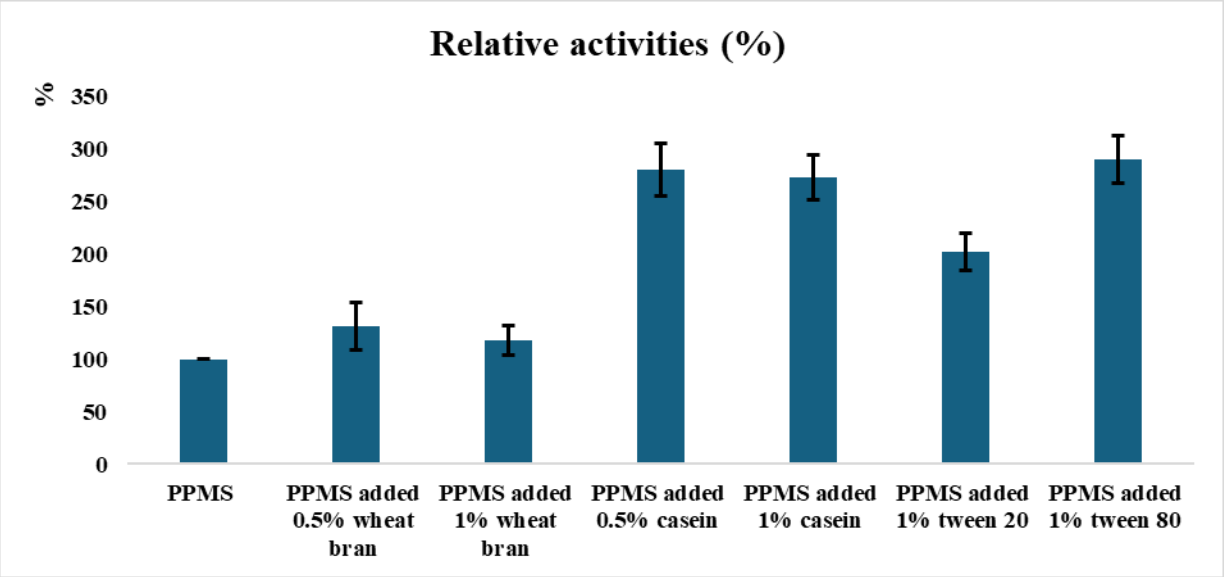

Figure 5.3 Relative activities between different inducers for protease production

The outcome of this experiment provides valuable insights into the influence of various inducers on protease production by *Bacillus tropicus* P4. All tested additives enhanced enzyme activity compared to the sludge-only control, indicating their stimulatory effect. Among them, wheat bran showed a modest impact, with relative activities increasing by 29.7% at 0.5% supplementation and 15.4% at 1%, possibly due to substrate inhibition, limited oxygen diffusion, or metabolic repression at higher concentrations. In contrast, Tween 80 (1%) exhibited the highest relative activity (290.58 ± 22.73 %), followed closely by 0.5% casein (280.93 ± 25.07%) and 1% casein (273.36 ± 20.93%). These findings suggest that non-ionic surfactants and proteinaceous inducers are particularly effective in stimulating protease secretion, likely by enhancing cell membrane permeability (Tween 80) and providing readily metabolizable nitrogen sources or signaling molecules (casein) (Figure 5.3).

Several inducers are known to stimulate protease production, including a variety of carbon and nitrogen sources, biosurfactants, and environmental stress conditions (Gupta et al., 2002; Rao et al., 1998). In this study, the primary objective was to enhance protease production in a cost-effective and sustainable manner by utilizing waste biomass as the main substrate. To support this goal, a selection of inexpensive and readily available inducers was chosen, targeting both economic feasibility and improved enzyme yield. This included wheat bran, a lignocellulosic agro-industrial by-product rich in carbohydrates and residual proteins, serving as a low-cost carbon source that also acts as a natural inducer in many microbial systems (Espoui et al., 2022; Limkar et al., 2019; Sandhya et al., 2005). Casein, a milk-derived protein, was selected as a nitrogen-rich inducer due to its proven ability to stimulate protease gene expression by mimicking the natural substrates of proteolytic enzymes (Dutt et al., 2008). Additionally, Tween 20 and Tween 80, non-ionic biosurfactants, were incorporated for their ability to enhance enzyme secretion by increasing membrane permeability and improving oxygen transfer, particularly beneficial in submerged fermentation (Asha & Palaniswamy, 2018).

Tween 80 has been widely recognized for its positive influence on microbial protease synthesis. For instance, in *Streptomyces badius*, Tween 80 led to a slight increase in protease activity during the peak enzyme production phase (Liu et al., 2011). Additionally, a comparative study on rumen microbial enzymes demonstrated that Tween 80 significantly enhanced protease activity compared to Tween 60, likely due to its superior solubilizing ability associated with its marginally higher hydrophilic-lipophilic balance-HLB (HLB of 15 vs. 14.9) (Kamande et al., 2000). Similarly, a study by Grbavčić et al. (2011) reported that Tween 80 boosted protease production in *Pseudomonas aeruginosa* by up to 157%. These findings are consistent with reports on *Bacillus* species, including *Bacillus sp. L21* (Tari et al., 2006) and *Bacillus cereus* FT1 (Asha & Palaniswamy, 2018), further reinforcing its role as a potent enhancer of protease biosynthesis. In the present study, the addition of 1% Tween 80 resulted in a marked increase in protease activity by *B. tropicus* P4—from 148.52 ± 20.76 U/mL in the control to 474.22 ± 18.23 U/mL, representing more than a threefold enhancement.

Tween 80 is a widely used non-ionic surfactant known for its ability to induce enzyme production, particularly in microbial systems (Sánchez Muñoz et al., 2022). Its amphipathic nature allows it to

interact with lipid bilayers, increasing cell membrane permeability, which facilitates nutrient uptake and secretion of extracellular enzymes such as proteases (Chidi Evans & Abdulkadir, 2012). While surfactants can sometimes be aggressive to microbial cells, even low concentrations of Tween 80 were effective, supporting its potent induction capacity (Sánchez Muñoz et al., 2022). Moreover, its amphipathicity may contribute to exposing hydrophobic enzyme regions or active sites, thereby improving enzyme–substrate interactions through hydrophobic binding (Chidi Evans & Abdulkadir, 2012).

Beyond enzyme induction, Tween 80 plays a critical role in industrial fermentation. It reduces surface tension in submerged cultures, which improves broth homogeneity and enhances nutrient and oxygen transfer—essential factors for maximizing microbial productivity, especially at scale (Tari et al., 2006). It also possesses low polarity, low toxicity, and high compatibility with microbial systems, along with a high solubilization capacity. Combined with its cost-effectiveness and significantly lower prices at industrial scale, these attributes make Tween 80 a highly viable option for large-scale applications (Cheng et al., 2017).

Finally, the observed compatibility between Tween 80 and proteases is particularly beneficial in detergent formulations, where enzyme–surfactant interactions are crucial to performance (Chidi Evans & Abdulkadir, 2012; Gupta et al., 2002). Taken together, these characteristics make Tween 80 a versatile, efficient, and economical additive for both research and industrial enzyme production systems.

5.4.5 Bioreactor studies

Scale-up is a critical next step in validating whether the conditions optimized at laboratory scale can be effectively applied to industrial bioprocesses. Despite its importance, achieving a successful scale-up presents undeniable challenges and often requires additional experimentation to gain deeper process understanding. Therefore, protease production by *Bacillus tropicus* P4 was first conducted in repeated batches in a 5 L bioreactor, and subsequently scaled up to a 150 L system.

5.4.5.1 5 L bioreactor production

In two independent batch fermentations, *B. tropicus* P4 exhibited a consistent pattern of protease production, with peak activity observed at 24 hours. The first batch reached a peak of 685.96 ± 28.79 U/mL, while the second batch exhibited a slightly higher peak at 755.02 ± 53.55 U/mL. Following this peak, enzyme activity gradually declined in both batches, with values dropping to 595.38 ± 1.51 U/mL and 555.29 ± 11.44 U/mL at 48 hours for the first and second batches, respectively. This decline may be attributed to nutrient depletion, enzyme degradation, or the accumulation of inhibitory metabolites. Overall, the 24-hour mark appears to be the optimal harvesting time for maximal protease yield under the tested conditions. A reduction in the time required to achieve peak enzyme production—from 48 hours in flask cultures to 24 hours in bioreactor systems—was observed in this study and has been consistently reported in several previous studies. This phenomenon is commonly attributed to enhanced aeration, efficient mixing, and precise control of pH and temperature in bioreactor systems, which collectively promote faster microbial growth and metabolic activity (Singh et al., 2011). Despite the shorter production time, the bioreactor process demonstrated improved efficiency, yielding approximately 1.5-fold higher productivity. No protease activity was detected at 6 hours; however, by 12 hours, the strain had already produced a significant amount of enzyme, suggesting that protease synthesis began during the late lag phase. Following the peak, enzyme activity declined gradually until 48 hours of incubation.

Although various cultural parameters—such as incubation temperature, broth pH, incubation time, and the type and concentration of carbon and nitrogen sources—can be optimized to maximize protease production, the primary goal in the context of valorizing agro-industrial waste or residues is to minimize or avoid pretreatment steps and reduce reliance on refined raw materials, thereby enhancing the cost-effectiveness of enzyme production processes. With this principle in mind, the present study was conducted under basic cultivation conditions commonly used for *Bacillus* species, including an incubation temperature of 30 °C, an inoculum size of 2%, and a medium pH adjusted to 7.0. The only modifications were made for the PPMS condition, in which the total solids concentration was increased to 25 g/L and the medium was supplemented with 1% Tween 80 to enhance enzyme production. In the study, the pH of fermentation batches

was allowed to float, as protease productivity was found to be comparable between pH-controlled (pH 7) and uncontrol conditions. (data not shown). Interesting, the natural rise in pH-reaching approximately to 8.2-8.3 by the end of fermentation-not also served as an indicator of protease production efficiency but also helped detect potential risks such as contamination, while reducing the need for chemical pH adjustments. Furthermore, the ability of the system to maintain high protease activity under these elevated pH conditions confirms the enzyme's tolerance to alkaline environments, as importantly preferred characteristics for detergent applications.

Table 5.2. Time course of protease production in bioreactor studies

Hours	1st batch	2nd batch
12	557.24 ± 36.83	484.18 ± 53.68
24	685.96 ± 28.79	755.02 ± 53.55
30	672.44 ± 4.4	691.73 ± 47.27
36	610.67 ± 28.91	649.07 ± 37.46
48	595.38 ± 1.51	555.29 ± 11.44

5.4.5.2 150 L bioreactor production

In the 150L bioreactor experiment, both cell density and enzyme titer increased significantly during the first 24 hours, indicating that protease production was associated with active growth. Notably, while bacterial growth plateaued after 24 h, protease levels remained relatively stable throughout the remainder of the fermentation, declining only slightly from 847.64 U/L to 710.58 U/L by 48 h (Figure 5.4). This stability reflects a well-optimized large-scale process, where environmental conditions—such as pH, oxygen transfer, and agitation—likely contributed to both cellular viability and extracellular enzyme stability. The data suggest that the transition from exponential to stationary phase was smooth, allowing for prolonged protease accumulation without significant degradation. Additionally, although the protease productivity achieved in the present study does not reach exceptionally high levels reported in some studies (3000-8000 U/mL), it exceeds the yields in several publications producing by *Bacillus subtilis* PCSIR-5, *Bacillus licheniformis, Bacillus sp. Y, Bacillus subtilis IH-72.* Moreover, it is comparable to that of *Bacillus coagulans* PSB- 07 (760.4 U/mL), or *Bacillus* sp.GA CAS10 (842.102 U/mL) (Kamal et al., 2017). Remarkably, the protease activity produced in our study using 25 g/L PPMS added with 1%

Tween 80 under the basic conditions (30 °C, 2% inoculum, and initial pH 7.0) is similar to 903 ± 4 U/mL obtained by *Bacillus licheniformis* when using wet-oxidation-pretreated waste activated sludge at pH 7.5 and 30% inoculum (Moreno et al., 2024).

These findings indicate that the 150 L system not only support stable biomass levels but also provided favorable conditions for protease retention, underlining its potential for robust industrial-scale enzyme production. Moreover, the consistent enzyme performance at scale validates the fermentation strategy, which relies on industrial wastewater as a low-cost substrate, requiring minimal pre-treatment and employing operationally simple, low-energy, and low-chemical-input fermentation conditions.

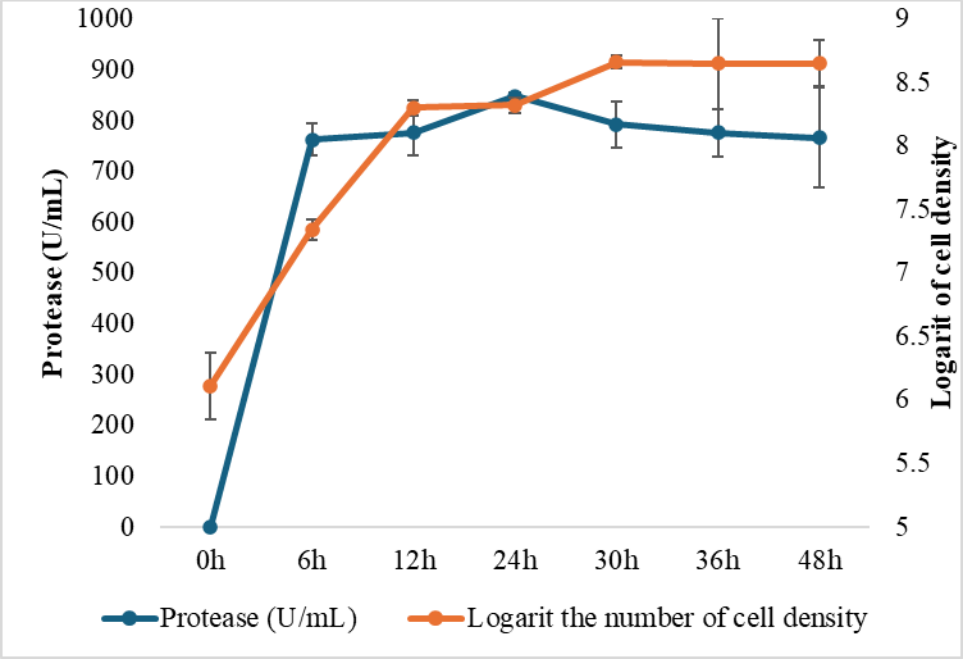

Figure 5.4 The growth and production kinetics in the 150L bioreactor

5.5 Conclusion

This study highlights the potential of *Bacillus tropicus* P4, isolated from PPMS, as an efficient microbial catalyst for protease production. By optimizing simple cultivation conditions and supplementing with Tween 80, protease yields were significantly enhanced—even at pilot scale. These findings support a scalable, cost-effective strategy for converting paper pulp sludge into valuable enzymes, offering a practical step toward circular bioeconomy goals. Using waste as a

resource not only reduces environmental burden but also opens new pathways for sustainable enzyme production across industries.

6 LIPASE PRODUCTION BY ISOLATE *ACINETOBACTER TANDOII* FOR VALORIZATION OF PAPER PULP MILL SLUDGE

Production de lipase par l'isolat *Acinetobacter tandoii* pour la valorisation des boues de l'industrie papetière

Vu-Mai-Linh Nguyen[1], Adama Ndao[1], Jean-François Blais[1], Kokou Adjallé[1,]

[1] Centre Eau Terre Environnement (ETE), Institut National de la Recherche Scientifique (INRS), 490 Rue de la Couronne, Quebec City, QC G1K 9A9, Canada

Corresponding author: Tel: (418) 654 2610, Fax: (418) 654 2600, email: Kokou.Adjalle@inrs.ca

(This paper is being prepared for submission.)

Lien entre le troisième et le quatrième article:

Le troisième article portait sur l'isolement de bactéries indigènes productrices de protéase à partir du PPMS, ainsi que sur l'évaluation de leur performance à l'échelle pilote dans un bioréacteur de 150 L. Cette étude a permis d'identifier *Bacillus tropicus* P4 comme une souche particulièrement efficace, capable de produire des niveaux élevés de protéase dans des conditions de fermentation à grande échelle.

Dans la continuité de cette démarche, le quatrième article visait à compléter cette exploration en se concentrant cette fois sur les bactéries productrices de lipase isolées du même substrat (PPMS). L'objectif était d'identifier les souches les plus prometteuses pour la production de lipase et de valider leur potentiel à l'échelle du bioréacteur de 150 L. Cette approche permet d'élargir le spectre enzymatique exploitable à partir de micro-organismes indigènes adaptés aux milieux résiduels complexes, tout en renforçant les perspectives d'intégration dans des formulations de détergents biologiques.

6.1 Abstract

This study evaluates the lipolytic potential of *Acinetobacter tandoii* L3, a strain isolated from pulp and paper mill sludge, using minimally treated secondary activated sludge as a sustainable and low-cost fermentation substrate. Lipase production was monitored under standard conditions in shake flasks and scaled up to 5 L and 150 L bioreactors. In the 150 L bioreactor, lipase activity was first detected at 3 h, showed a substantial increase by 6 h (0.52 U/mL), and peaked at 9 h (0.62 U/mL). Unlike the 5 L bioreactor, where activity declined sharply post-peak, the 150 L system-maintained levels above 0.53 U/mL up to 48 h. This sustained activity suggests that improved oxygen transfer and agitation enhanced enzyme stability. These findings highlight the early and robust lipase production capacity of *A. tandoii* L3 and its potential in the circular valorization of paper mill sludge into industrially relevant bioproducts.

Keywords: secondary pulp and paper activated sludge, lipase, valorization, scale-up

Cette étude évalue le potentiel lipolytique d'*Acinetobacter tandoii* L3, une souche isolée des boues d'une usine de pâte et papier, en utilisant des boues activées secondaires faiblement traitées comme substrat de fermentation durable et à faible coût. La production de lipase a été suivie dans des flacons agités dans des conditions standard, puis à l'échelle des bioréacteurs de 5 L et 150 L. Dans le bioréacteur de 150 L, l'activité lipasique a été détectée dès 3 h, a fortement augmenté à 6 h (0,52 U/mL), et a atteint un pic à 9 h (0,62 U/mL). Contrairement au bioréacteur de 5 L, où l'activité chutait brutalement après le pic, le système de 150 L a maintenu des niveaux supérieurs à 0,53 U/mL jusqu'à 48 h. Cette activité soutenue suggère que l'amélioration du transfert d'oxygène et de l'agitation a renforcé la stabilité enzymatique. Ces résultats mettent en évidence la capacité de production lipasique précoce et robuste de *A. tandoii* L3, ainsi que son potentiel pour la valorisation circulaire des boues papetières en bioproduits d'intérêt industriel.

Mots-clés : boues activées secondaires de pâte et papier, lipase, valorisation, changement d'échelle

6.2 Introduction

Pulp and paper mill activated sludge (PPMS) is no longer viewed as a persistent waste that pollutes soil and air or requires expensive treatment. Instead, it has emerged as a promising feedstock for sustainable energy and value-added products, driven by advancements in bioconversion technologies. As a renewable resource, secondary activated sludge offers several attractive features. Produced as a byproduct of industrial wastewater treatment, it is abundantly available—accounting for approximately 30% (Bajpai, 2015) of the 450 million tons of sludge generated annually by the global pulp and paper industry (Kumar & Verma, 2024) —and thus holds great potential to support a biomass-based economy.

Moreover, PPMS is rich in diverse organic components, including proteins (22–52%), lignin (20–58%), carbohydrates (0–23%), cellulose (2–28%), hemicellulose (12%), and lipids (2–10%), along with trace amounts of heavy metals. This complex composition makes it a suitable candidate for a wide range of biotechnological treatments aimed at producing various value-added products, such as biochemicals and biopolymers (e.g., polyhydroxyalkanoates, proteins and peptides, amino acids, enzymes, biosurfactants), bioenergy carriers (e.g., biogas, biohydrogen, bio-oil, biochar), bioethanol, volatile fatty acids (VFAs), biofertilizers, and biopesticides (Bajpai, 2015; Bengtsson et al., 2008; Kaluža et al., 2014; Kaur et al., 2020; Pervaiz & Sain, 2012; Xu & Lancaster, 2008; Zhang et al., 2018). However, its valorization is challenged by the presence of recalcitrant and potentially inhibitory substances, as well as the inherent heterogeneity of the sludge, all of which can reduce microbial efficiency and process stability.

In particular, a study by Karn et al. (2013) demonstrated the successful extraction of a significant amount of lipase using 0.1% Triton X-100, with the enzyme primarily associated with microbial cells embedded in activated sludge flocs rather than present in the extracellular medium. This confirms that lipase activity originates from living microbes within the sludge, rather than from externally added or residual free enzymes—eliminating the possibility of redundant enzyme supplementation during treatment. This also highlights the potential to discover novel lipase producers in this underexplored niche. Moreover, the elevated activity reinforces the microbes'

effectiveness in lipid degradation, underscoring their active metabolic role within the sludge ecosystem.

Lipases rank as the third most commercially significant group of enzymes due to their broad substrate specificity, high catalytic efficiency, and versatility across various industries, including food, biofuels, pharmaceuticals and detergents. The demand for lipases continues to grow, driven by the shift toward eco-friendly processing, biocatalysis in green chemistry, and sustainable industrial applications. Microbial lipases are especially valued for their high production yields, amenability to genetic engineering, and resilience under harsh industrial conditions, further bolstered by ongoing technological advancements (Kim et al., 2023).

Considering these perspectives, the present study aims to valorize PPMS by isolating and identifying efficient lipase-producing strains for the production of high-value enzymes. This approach leverages well-adapted microbial communities, reducing the risks typically associated with unconventional substrates like PPMS, while supporting the zero-waste paradigm and advancing sustainable development.

6.3 Materials and methods

6.3.1 Sample collection and pretreatment strategy

PPMS used in this study was obtained from Kruger Inc. in Trois-Rivières, Québec, and promptly refrigerated at 4 °C to prevent degradation. The sludge displayed a near-neutral pH of 6.65 ± 0.35 and contained a diverse profile of organic and inorganic components, including total solids (10.3 ± 0.36 g/L), total suspended volatile solids (6.09± 0.37 g/L), suspended solids (9.4 ± 0.36 g/L), and suspended volatile solids (2.35 ± 0.18 g/L). Nutrient analysis revealed total organic carbon at 14 ± 1.00 g/L, total organic nitrogen at 1.4 ± 0.15g/L, and organic phosphorus at 2.3 ± 0.36 g/L. Major mineral ions included sodium (10.2 ± 0.56 g/L), iron (0.4 ± 0.17 g/L), potassium (1.4 ± 0.30 g/L), calcium (22 ± 2.65 g/L), and sulfur (3.9 ± 0.95 g/L). The carbohydrate composition comprised glucose (1.9 ± 0.75 g/L), fructose (1.5 ± 0.30 g/L), lactose (0.83 ± 0.05 g/L), sucrose (0.97 ± 0.20 g/L), galactose (1.4 ± 0.13 g/L), xylose (1.1 g/L), and trehalose (0.53 ± 0.13 g/L).

To improve its suitability for fermentation-based enzyme production, the sludge was pretreated through a concentration step. Building on earlier findings indicating that nutrient dilution limited lipase activity, the sludge was centrifuged to elevate the total solids content to 25 g/L. This pretreatment strategy established a practical balance between nutrient concentration and process operability. By enriching key nutrients, it enhanced the suitability of the sludge matrix for microbial growth and metabolic activity. Concurrently, maintaining an optimal solids content ensured a manageable viscosity, thereby mitigating common challenges associated with high-solids substrates, such as restricted oxygen transfer and inadequate mixing.

A few reports have shown that the extent of lipase production by *Acinetobacter* species varies with the initial pH of the medium, depending on the strain, although neutral pH is more commonly optimal (Allimoun et al., 2015). Therefore, in the present study, a fixed pH of 7.0 was maintained throughout the experiments.

6.3.2 Isolation of bacteria producing lipases

To initiate microbial isolation, a 10^{-1} dilution of the PPMS sample was prepared by adding 0.5 mL of sludge to 4.5 mL of sterile distilled water. This was followed by a series of tenfold serial dilutions up to 10^{-5}, achieved by sequentially repeating the same dilution step. From the 10^{-3}, 10^{-4}, and 10^{-5} dilutions, 100 µL aliquots were spread onto nutrient agar plates (10 g/L peptone, 3 g/L beef extract, 5 g/L NaCl; pH 7.0), each supplemented with 1% (v/v) Tween 20. Plates were incubated at 30 °C for 48 hours to promote microbial growth and the development of visible colonies.

6.3.3 Spotted agar method

Selective agar plates were prepared using a basal medium containing (per liter): 10 g peptone, 5 g NaCl, and 0.1 g $CaCl_2 \cdot 2H_2O$. Tween 20 was sterilized separately by autoclaving at 121 °C for 15 minutes and subsequently added to the basal medium at a concentration of 1 mL per 100 mL of cooled, sterile medium.

Isolates were inoculated onto the surface of the prepared medium using the spot inoculation technique. Lipase activity was assessed based on visual indicators: either the development of an opaque zone resulting from the formation of insoluble calcium laurate (a salt of lauric acid released by enzymatic hydrolysis), or a clear halo surrounding the colony, indicating further degradation of the precipitated fatty acid salt (Ugras & Uzmez, 2016).

6.3.4 Screening of lipase-producing strains

Isolates exhibiting lipolytic activity on spotted agar were selected for further screening in liquid culture. Each isolate was inoculated into PPMS-based medium supplemented with 1% (v/v) Tween 20 and incubated at 30 °C, pH 7.0, for 48 hours under continuous agitation at 180 rpm using an orbital shaker. Following incubation, lipase activity in the culture supernatants was measured spectrophotometrically to identify the most efficient producers for further characterization as shown in 6.3.9.

6.3.5 DNA extraction and 16S rRNA gene amplification

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. The 16S rDNA region was amplified by polymerase chain reaction (PCR) using universal primers 27F (5′-TAACACATGCAAGTCGAACG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR amplification was carried out in a thermal cycler with the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min (Wang et al., 2012).

The amplified PCR products were sequenced, and the resulting 16S rDNA sequences were aligned with reference sequences available in the NCBI GenBank database using the BLASTN tool (https://www.ncbi.nlm.nih.gov/BLAST). The identity of the isolate was determined based on the highest sequence similarity with known bacterial strains.

6.3.6 Lipase inducers

To investigate the impact of different inducers on lipase production, three PPMS-based media were prepared: one supplemented with Tween 20 (as used in the initial screening), one with Tween 80, and one without any added inducer. Each medium was inoculated and incubated in shake flasks at 30 °C, with an initial pH of 7.0, under agitation at 180 rpm for 48 hours. After incubation, lipase activity was quantified spectrophotometrically to assess and compare the effectiveness of Tween 20 and Tween 80 against the non-supplemented control.

6.3.7 Inoculum development for bioreactor cultivation

A sequential inoculum preparation strategy was employed to support fermentation in both 5 L and 150 L bioreactors, each requiring a 2% (v/v) inoculum. The process was initiated by transferring a single bacterial colony from a nutrient agar plate into sterile nutrient broth within an Erlenmeyer flask. This starter culture was incubated at 30 °C with agitation at 180 rpm for 16 hours to achieve exponential-phase growth, reaching an approximate cell density of 10^8 CFU/mL.

An aliquot equivalent to 2% of the final culture volume was then used to inoculate 250 mL of PPMS-based medium in a 1 L Erlenmeyer flask, followed by incubation under identical conditions. This intermediate step served to physiologically adapt the microbial cells to the composition of the sludge-based medium before their transfer to the 5 L bioreactor.

For the 150 L bioreactor trial, further scale-up was necessary to generate the required inoculum volume. Two 5 L Erlenmeyer flasks, each containing 1.2 L of PPMS medium, were inoculated with the adapted culture and incubated under the same conditions. This final step ensured the availability of a sufficient volume of metabolically active, sludge-adapted inoculum for large-scale fermentation.

6.3.8 Fermentation process and scale-up

In both 5 L and 150 L bioreactor systems, the core operating principle was to maintain dissolved oxygen (DO) levels within the 30–50% range to support optimal microbial activity. The working volumes of PPMS medium were 3 L and 90 L respectively for the 5 L and 150 L reactors. Prior to

fermentation, the PPMS was sterilized by autoclaving at 121 °C and 15 psi for 30 minutes, then adjusted to pH 7.0. Once cooled, each bioreactor was aseptically inoculated with 2% (v/v) of a pre-adapted bacterial culture and maintained at 30 °C for 48 hours. Throughout the process, samples were taken every 12 hours to monitor fermentation kinetics, with particular attention to lipase production.

In the 150 L bioreactor, the agitation capacity reaches up to 800 rpm with an aeration rate of 200 L/h, while in the 5 L reactor, agitation is set at 400 rpm and aeration at 8 L/h.

6.3.9 Lipase assay

Lipase activity was assessed using *p*-nitrophenyl palmitate (*p*NPP) as the substrate. The assay buffer was prepared in a ratio of 25:5:0.15:19.85 for 100 mM Tris-HCl (pH X), 100 mM $CaCl_2 \cdot 2H_2O$, Triton X-100, and distilled water, respectively. The substrate solution was prepared by mixing *p*NPP (1 mL of 20 mM) with 19 mL of the assay buffer.

For the reaction, 2.76 mL of the substrate solution was combined with 0.24 mL of enzyme solution. The mixture was incubated at 30 °C for 30 minutes, and the release of *p*-nitrophenol was quantified by measuring absorbance at 410 nm.

One unit (U) of lipase activity was defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per minute under the assay conditions (Shart & Elkhalil, 2020).

6.4 Results and Discussion

6.4.1 Isolation of lipase-producing bacteria

Isolation was performed following the established methodology, and after 48 hours of incubation on the selective medium, four distinct morphological colony types were observed and designated as L1 through L4 (Figure 6.1). These isolates were subsequently purified on nutrient agar and preserved in 10% glycerol at –20 °C for further characterization.

6.4.2 Screening

To identify the most promising lipase-producing strain, the lipolytic potential of the four isolates was initially assessed using the spotted agar method. This preliminary screening was conducted on a selective medium containing Tween 20 and calcium ions (Ca^{2+}). Bacillus megaterium was employed as a control strain in the lipase activity assay. All four isolates in the present study exhibited visible precipitate formation on agar plates, indicating lipase production comparable to that of *B. megaterium* (Figure 6.2). Although the number of isolates obtained here is fewer than the nine reported by Ghribi et al. (2016) from press sludge—a mixture of primary and secondary sludge subjected to acid treatment (pH ~3, temperature ~30 °C)—in a pulp and paper mill in Trois-Rivières, it is worth noting that no lipase-producing strains were recovered from secondary sludge in their study. In the presence of lipase, ester bonds in Tween 20 are hydrolyzed, releasing free fatty acids and glycerol. The liberated fatty acids subsequently react with calcium ions in the medium to form insoluble calcium salts, resulting in the appearance of opaque or whitish precipitate zones around the colonies. These zones serve as simple and effective qualitative indicators of lipase activity, making this method suitable for initial screening (Ugras & Uzmez, 2016).

In contrast, our findings contribute to the emerging body of research on lipase-producing strains isolated from secondary sludge specifically. A similar effort by Tripathi et al. (2022) reported six lipase-producing bacteria, with activities ranging from 3.93 to 6.34 U/mL, isolated from secondary sludge at a pulp and paper mill in India. These findings support the idea that secondary sludge can serve as a valuable source of lipolytic microorganisms and may represent a promising reservoir for industrially relevant enzymes.

However, the diameter of the precipitate zones was not sufficiently distinct to allow for accurate comparative analysis. Despite this limitation, the method effectively verified the lipolytic capability of all isolates.

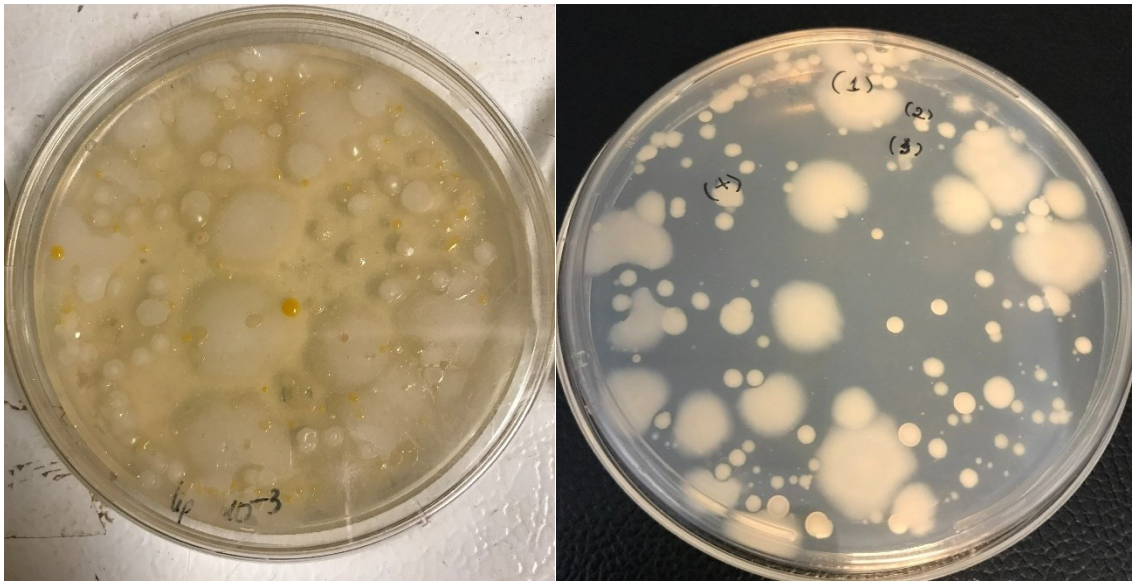

Figure 6.1 Isolates on Tween 20 agar plates after 48h incubation for preliminary screening of lipase production

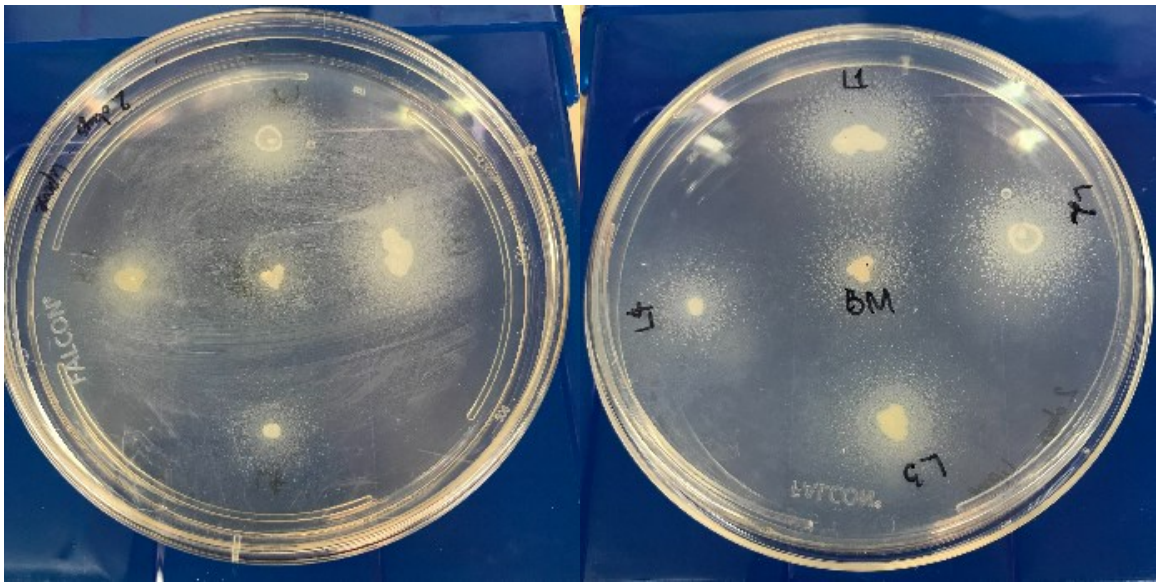

Figure 6.2 Top and bottom views of agar plates showing bacterial colonies and the precipitation formation, indicating lipase activity.

To further evaluate lipase production under liquid culture conditions, the four isolates were inoculated into sludge-based broth supplemented with 1% (v/v) Tween 20. Cultures were incubated in shake flasks at 30 °C and 180 rpm for 48 hours. Samples were collected at 24 and 48 hours, and lipase activity was quantitatively measured using a spectrophotometric assay, providing more precise data for comparison among the isolates.

Table 6.1. Lipase activities of isolates (U/mL)

	0h	24h	48h
L1	-	0.04 ± 0.001	0.043 ± 0.001
L2	-	0.053 ± 0.001	0.053 ± 0.0003
L3	-	0.044 ± 0.002	0.386 ± 0.01
L4	-	0.08 ± 0.007	0.083 ± 0.002
BM	-	0.02 ± 0.002	0.046 ± 0.007

(-) No detectable lipase activity

At 24 hours, all isolates exhibited higher lipase activity than *Bacillus megaterium*, with L4 showing the highest value (0.080 U/mL), approximately four times greater than that of *B. megaterium* (0.020 U/mL). L1, L2, and L3 also showed modest increases, though the differences were relatively minor. However, at 48 hours, a clear divergence emerged among the isolates. While *B. megaterium*, L1, and L2 showed little to no change in activity, and L4 exhibited only a slight increase, L3 demonstrated a substantial surge in lipase production, reaching 0.386 U/mL—nearly nine times its 24-hour value and over eight times higher than the next best performer, L4. This dramatic increase indicates that L3 possesses strong time-dependent lipase expression, positioning it as the most promising candidate for further development in lipase-related applications.

In the present study, *Bacillus megaterium* demonstrated lipase-producing potential, as indicated by the formation of clear zones on agar plates (Figure 6.2). However, its performance was markedly lower during fermentation using PPMS compared to isolates, suggesting that lipase production is strongly influenced by the composition of the growth medium. This finding contrasts with previous studies, where *B. megaterium* exhibited high lipase activity—particularly thermostable lipase—when cultivated with various substrates such as soybean oil, coconut oil, wheat bran, neem seed cake, cotton seed cake or palm oil press fibers and effluent (Chandra et al., 2020; Lima et al., 2004; Sekhon et al., 2006). These observations highlight the critical role of substrate selection and underscore the importance of isolating indigenous strains adapted to specific waste types for the successful valorization of agro-industrial residues.

The isolate L3 forms distinct, small colonies on nutrient agar, characterized by a round, smooth, pale-yellow appearance with a glossy and moist surface. Colonies typically measure approximately 0.1 mm in diameter and often appear as tiny dots yet remain easily distinguishable due to their compact and uniform morphology. L3 exhibits moderate temperature tolerance, with growth observed between 15 °C and 40 °C and an optimal range between 30 °C and 37 °C. The strain displays limited pH adaptability, growing only under slightly alkaline conditions (pH 7 to 9), with no growth detected outside this range. These findings suggest that L3 is best suited to environments with moderate temperatures and mildly alkaline pH, indicating its potential applicability in targeted biotechnological or environmental processes.

6.4.3 Identification of the isolates

The 16S rDNA gene sequence of the isolate was a continuous stretch of 1005 base pairs, which was analyzed using the BLAST tool on the National Center for Biotechnology Information (NCBI) database. The sequence showed a 100% match with Acinetobacter tandoii DSM 14970, which is registered in GenBank under accession number NR 117630.1. The findings in this study share consistency with previous research suggesting that activated sludge could be a common habitat for *Acinetobacter tandoii*. In fact, the presence of *Acinetobacter tandoii* in activated sludge from a plant in Australia (Carr et al., 2003) further supports the notion that activated sludge environments are favorable for the growth of this species. Additionally, *A. tandoii* has been isolated from diverse sources including soil, freshwater, and treated effluents (Abdel-El-Haleem, 2003).

Importantly, this study is the first to report lipase production by *Acinetobacter tandoii*, adding to its known functional repertoire in pollutant degradation, such as nitrogen removal and phenol degradation through the ortho and β-ketoadipate pathways (Ouyang et al., 2020; Van Dexter & Boopathy, 2019). Based on these findings, the isolate was designated *Acinetobacter tandoii* L3.

More broadly, species within the genus *Acinetobacter* are recognized for secreting robust lipases that demonstrate high activity even during short fermentation periods, particularly when grown on oily or waste-based substrates. These characteristics make them attractive candidates for

industrial enzyme production. However, their potential has often been underappreciated compared to genera like *Pseudomonas* and *Burkholderia*, possibly due to the complex taxonomy of *Acinetobacter* and its relatively recent emergence as a focus of biotechnological research (Snellman & Colwell, 2004).

Beyond lipase production, many *Acinetobacter* species possess notable metabolic versatility. While some, like *A. baumannii*, are known for their clinical relevance and multidrug resistance (Muleshkova et al., 2025), numerous non-pathogenic strains have shown valuable capabilities in synthesizing high-value biochemicals such as polyhydroxyalkanoates (PHAs), biosurfactants like emulsan, and long-chain lipids including wax esters (Abdel-El-Haleem, 2003; Alizadeh-Sani et al., 2018; Kannisto et al., 2017). These species thrive on a wide variety of carbon sources, including inexpensive and waste-derived substrates such as used cooking oil, crude glycerol, and even transformer oil. Strains like *A. junii* and *A. baylyi* ADP1 have demonstrated high PHA accumulation (up to 72% of cell dry weight) and wax ester production (up to 19%) under optimized conditions (Anburajan et al., 2019; Kannisto et al., 2017).

In the context of environmental biotechnology, *Acinetobacter* spp. contribute significantly to hydrocarbon degradation, heavy metal detoxification, phosphate removal from wastewater, and even plant growth promotion through phosphate solubilization and siderophore production (Boswell et al., 1999; Sharma, 2022; Wang et al., 2021; Zhang et al., 2019). Collectively, these traits underscore the biotechnological relevance of the genus and highlight the potential of *A. tandoii* L3 for future applications in both industrial enzyme production and environmental remediation.

6.4.4 Effect of temperature on lipase production

The effect of temperature on lipase production by *Acinetobacter tandoii* L3 was evaluated through a time-course experiment conducted at 30 °C and 37 °C. At 30 °C, lipase activity increased rapidly during the early incubation phase, peaking at 0.32 U/mL at 12 hours. This was followed by a gradual decline, with activity decreasing to 0.24 U/mL at 24 hours and further dropping to 0.15 U/mL by 48 hours. In contrast, enzyme production at 37 °C remained substantially lower throughout the experiment, with a maximum of only 0.05 U/mL observed at 12 and 24 hours, and

no further increase beyond that point. These results suggest that 37 °C is suboptimal for both microbial growth and lipase synthesis in this strain, likely due to thermal stress affecting gene regulation or enzyme stability.

Overall, these findings confirm that *A. tandoii* L3 exhibits optimal lipase production at 30 °C, consistent with reports on other *Acinetobacter* species such as *A. baylyi* G40, *Acinetobacter* sp. AU07, *Acinetobacter radioresistens*, *Acinetobacter* sp. K5b4. and BK43 (Allimoun et al., 2015; Anbu et al., 2011; Chen et al., 1998; Furini et al., 2018; Gururaj et al., 2016). In contrast, BK44 demonstrated notable temperature sensitivity, losing approximately 25% of its enzyme activity when the temperature increased from 25 °C to 30 °C. Furthermore, both BK43 and BK44 showed a dramatic decrease in lipase production at 37 °C, indicating thermal instability (Anbu et al., 2011). Conversely, some strains exhibit thermotolerance, as demonstrated by *Acinetobacter* sp. SU15, which produced maximal lipase activity at 40 °C (Ugras & Uzmez, 2016). Several *Acinetobacter* species are psychrophilic or psychrotrophic—for instance, *A. calcoaceticus* LP009, which shows optimal lipase activity at 15 °C (Pratuangdejkul & Dharmsthiti, 2000).

These findings emphasize the temperature-dependent nature of lipase production, especially when compared with previous reports that have demonstrated optimal enzyme activity at both low and high temperatures across various bacterial species. In the case of *Acinetobacter tandoii* L3, however, lipase production was highly sensitive to temperature fluctuations, underscoring the necessity of maintaining precise temperature control during fermentation. Such control is crucial for the successful optimization of bioreactor conditions aimed at maximizing enzyme yield for industrial-scale lipase production.

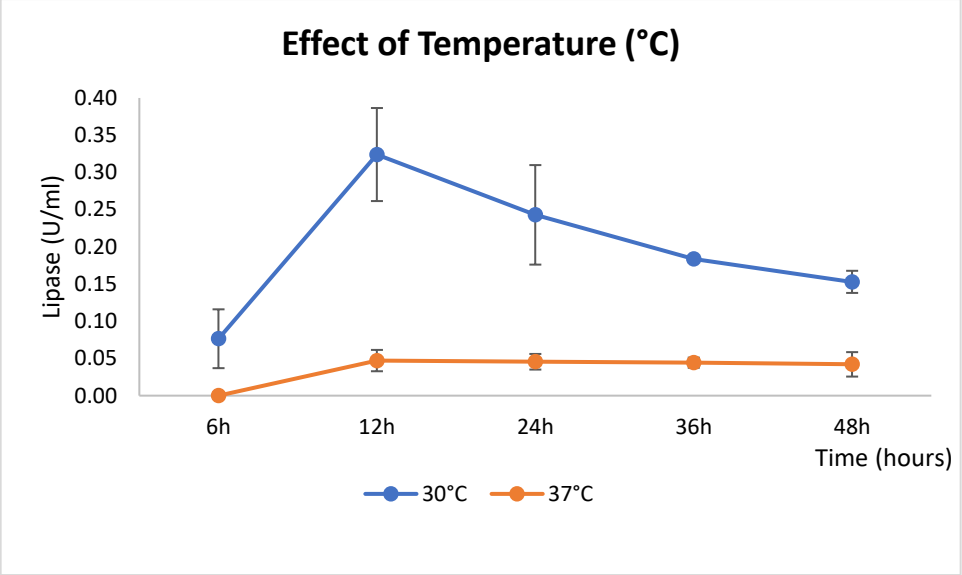

Figure 6.3 Effect of temperature on lipase production

6.4.5 Effect of inducers on lipase production

The time-course data indicate that the sludge medium alone supported the highest lipase activity, with a peak at 24 hours (0.55) followed by a slight decrease at 48 hours (0.52), suggesting that native components in the sludge effectively induce enzyme production without additional supplements. In contrast, the addition of 1% Tween 20 resulted in lower activity at 24 hours (0.33), with a modest increase by 48 hours (0.40), indicating a delayed and moderate induction effect. Similarly, Tween 80 initially showed higher activity than Tween 20 at 24 hours (0.43), but the activity declined to 0.35 at 48 hours, suggesting a temporary enhancement followed by potential inhibition.

Inducers such as natural oils and surfactants mimic the natural substrates of lipases, thereby activating the transcription of lipase-encoding genes and promoting enzyme synthesis (Gupta et al., 2004). Tween is a commonly used substrate known to enhance bacterial lipase production, particularly in members of the genus *Acinetobacter* (Boekema et al., 2007), while also functioning as an emulsifier that facilitates oxygen transfer in large-scale fermentation. For these reasons, it was included in the present experiment. Surfactants like Tween 80 (C18:1) can act as both inducers and emulsifiers, enhancing lipase secretion and oil dispersion in the medium. Similarly, Tween 20 (C12:0) also serves as an inducer, although it is generally considered less effective than Tween

80 in promoting lipase production (Allimoun et al., 2015; Wang et al., 2012). Although Tween 20 and Tween 80 are well-established surfactant inducers, the results suggest that lipase production by *Acinetobacter tandoii* L3 may be less reliant on these agents. Furthermore, their presence at 1% concentration may even suppress lipase expression or negatively impact cell activity over time under the tested conditions.

Other common inducers include natural and waste-derived oils, such as olive oil, castor oil, coconut oil, and waste cooking oils. For instance, in *Acinetobacter sp.* K5b4, the inclusion of olive oil as an inducer led to an increase up to 2.7 mU/mL in lipase activity compared to control conditions (not detectable) (Allimoun et al., 2015). In *Acinetobacter sp.* AU07, optimizing the fermentation medium with 2% (v/v) castor oil significantly enhanced lipase production, increasing the yield from 14.5 U/mL in shake flasks to 48 U/mL in a 3 L bioreactor—a more than 3-fold increase (Gururaj et al., 2016). In solid-state fermentation systems, the addition of 5% coconut oil and 5% olive oil elevated lipase production to 75.4 U/mg protein, representing a 5-fold increase over the baseline (Khoramnia et al., 2011). Although these inducers demonstrate remarkable induction potential at flask scale, their application at an industrial scale poses several challenges. Poor solubility in complex media like sludge broth can lead to uneven distribution, resulting in inconsistent induction and enzyme production. Oils, especially when used with biosurfactant-producing strains, can also cause excessive foaming during agitation, which may hinder aeration, increase contamination risk, and even cause overflow in bioreactors. Furthermore, oils can form surface layers or increase medium viscosity, impeding oxygen transfer in aerobic fermentations (Khoramnia et al., 2011). Their presence in the fermentation broth may also complicate downstream processing, particularly when lipase is extracellular, as the oils may co-purify with the enzyme, necessitating additional separation steps. Taken together, and in line with the primary aim of ensuring cost-effectiveness, oils were excluded from the present study.

Overall, these findings highlight that the sludge medium alone remains the most effective and practical approach for sustaining lipase production under the tested conditions.

Table 6.2. Effect of inducers on lipase production

	U/mL		
Media	**0h**	**24h**	**48h**
PPMS	-	0.55 ± 0.005	0.52 ± 0.006
PPMS added 1% tween 20	-	0.33 ± 0.005	0.39 ± 0.003
PPMS added 1% tween 80	-	0.43 ± 0.045	0.35 ± 0.026

(-) No detectable lipase activity

The lipase activity in this study was evaluated in the time interval between 24 to 48 h of culture.

6.4.6 Bioreactor studies

6.4.6.1 Fermentation in 5L bioreactors

Under basic cultivation conditions—30 °C, initial pH 7, 2% (v/v) inoculum size—and using pulp and paper mill sludge (PPMS) with a total solids concentration of 25 g/L as the sole substrate, lipase production by *Acinetobacter tandoii* L3 was assessed in repeated batch fermentations. Both batches exhibited a consistent kinetic pattern, with maximum lipase activity occurring at 9 hours (0.56 U/mL in the first batch and 0.55 U/mL in the second batch). This peak appeared significantly earlier than that observed in flask-scale experiments, where maximum activity typically occurred beyond 12 hours. This suggests that the tested bioreactor conditions may have promoted accelerated microbial metabolism or enhanced substrate accessibility. The transition from shake flasks to 5 L bioreactors resulted in both an increase in peak lipase activity and a reduction in the time required to reach that peak. These improvements indicate that enhanced oxygen transfer and agitation in the bioreactor played a critical role in boosting lipase production.

Following the peak, enzyme activity declined sharply, with a 50% reduction recorded at 12 hours and continued decrease thereafter. The narrow production time frame observed here not only underscores the importance of precise harvest timing but also presents potential for reducing production costs by shortening fermentation duration, minimizing energy input, and enhancing bioreactor throughput—particularly in the context of using PPMS without the need for supplemented inducers.

Table 6.3. Time course of lipase production in bioreactor studies

	U/mL	
Time course (hours)	**1st batch**	**2nd batch**
0h	-	-
6h	0.27 ± 0.002	0.47 ± 0.003
9h	0.56 ± 0.007	0.55 ± 0.005
12	0.27 ± 0.005	0.37 ± 0.016
24	0.14 ± 0.01	0.12 ± 0.009
30	0.11 ± 0.004	0.11 ± 0.005
36	0.1 ± 0.005	0.1 ± 0.004
48	0.05 ± 0.003	0.1 ± 0.003

(-) No detectable lipase activity

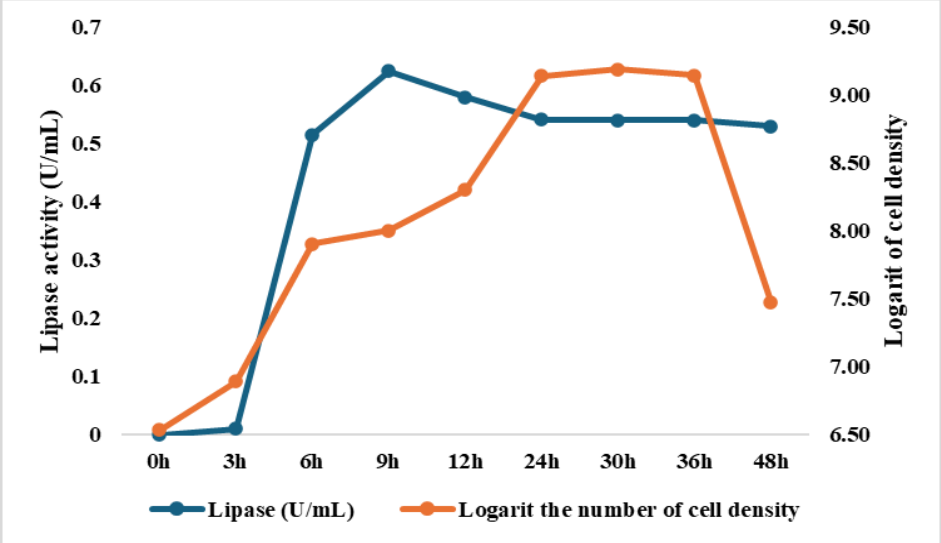

Figure 6.4 Time course of lipase production in bioreactor studies

6.1.6. Fermentation in 150L bioreactor

Lipase activity by *Acinetobacter tandoii* L3 was first detected as early as 3 hours in the 150 L bioreactor and reached 0.52 U/mL by 6 hours—a level that was undetectable at the same timepoint in the 5 L system. Activity peaked at 9 hours, reaching 0.62 U/mL, which aligned with the peak observed in the 5 L bioreactor, highlighting the reproducibility of L3's lipolytic profile across scales. Notably, unlike the sharp post-peak decline observed in the smaller system, lipase activity in the 150 L bioreactor remained relatively stable, maintaining values above 0.53 U/mL up to 48 hours. This extended activity window may be attributed to enhanced control over agitation, aeration, and mixing at the larger scale, likely resulting in improved oxygen transfer and nutrient distribution that supported both enzyme stability and sustained production. In scaling up lipase production from 5 L to 150 L, key operational parameters such as agitation and aeration were adjusted to maintain efficient oxygen transfer and substrate mixing. The agitation speed was increased from 400 rpm in the 5 L reactor to a capacity of 800 rpm in the 150 L system, while the aeration rate rose from 8 L/h (1.6 vvm) to 200 L/h (1.33 vvm). Although the volumetric aeration rate slightly decreased at larger scale, the improved mixing and oxygen distribution afforded by higher agitation likely contributed to enhanced substrate accessibility and better biomass–enzyme interaction (Ndao et al., 2017).

Meanwhile, cell density continued to rise until 30 hours, reaching a log CFU/mL value of 9.19 before a slight decrease was observed. Achieving peak lipase production within 9 hours using *Acinetobacter* species is uncommon, as most reported strains require significantly longer incubation periods, typically ranging from 48 to 72 hours during the late logarithmic phase (Patel et al., 2020). For example, *Acinetobacter* sp. YMP reached its maximum lipase yield after 120 hours under specific culture conditions (Attar et al., 2012). However, a few strains have demonstrated earlier lipase production, during the stationary phase. Notably, *Acinetobacter* sp. BK44 and *A. baylyi* G40 achieved peak activity at 12 hours in flask and bioreactor cultures, respectively, while strain AU07 reached its maximum lipase production after 16 hours in a bioreactor system (Anbu et al., 2011; Furini et al., 2018; Gururaj et al., 2016).

This notably short production timeframe observed in L3 places it among the most efficient *Acinetobacter* lipase producers currently reported, reinforcing its industrial relevance. Furthermore, the temporal decoupling between peak enzyme activity and maximum biomass accumulation suggests that enzyme synthesis is not strictly growth associated. Collectively, these results demonstrate the robustness and scalability of L3 for large-scale lipase production using PPMS as a cost-effective substrate.

The lipase level obtained in this study agree with previously published data. For example, *Acinetobacter sp.* UBT1 showed an increase in lipase production from 0.042 U/mL to 0.243 U/mL when deoiled seed cakes were used as inducers instead of olive oil in nutrient broth, based on unit conversion for comparative purposes (Patel et al., 2020). In another study, *Acinetobacter baylyi* G40 demonstrated high enzymatic activity with olive oil (0.358 U/mL·min^{-1}), followed closely by grape seed oil (0.352 U/mL·min^{-1}) and canola oil (0.348 U/mL·min^{-1}) in a medium containing 0.5% peptone, 0.1% yeast extract, and 0.4% NaCl (Anbu et al., 2011). Notably, there are only a few reports documenting substantial lipase production by *Acinetobacter* species. Among them, *Acinetobacter sp.* AU07 achieved a remarkable activity of 48 U/mL in a 3 L bioreactor operated at 30 °C and pH 7.0, using a 0.5% (v/v) inoculum and 2% (v/v) castor oil as the inducer. Under these optimized conditions, the strain reached peak enzyme production within just 12 hours, compared to 16 hours in shake flask cultivation, where a lower activity of 14.5 U/mL was recorded (Gururaj et al., 2016). In contrast, *Bacillus thermocatenulatus* produced only 0.31 U/mL of lipase under bioreactor conditions—a 70% reduction compared to shake flask cultures—even with the addition of 1% gum Arabic and 2.5% olive oil (Schmidt-Dannert et al., 1994). These findings underscore the variability and potential risk of reduced lipase productivity during scale-up, even when synthetic media and optimized inducers are used. In this context, the successful use of PPMS as a fermentation medium in the present study not only achieved comparable or superior enzyme yields but also demonstrated the feasibility of utilizing a low-cost, waste-derived substrate in bioreactor-scale production.

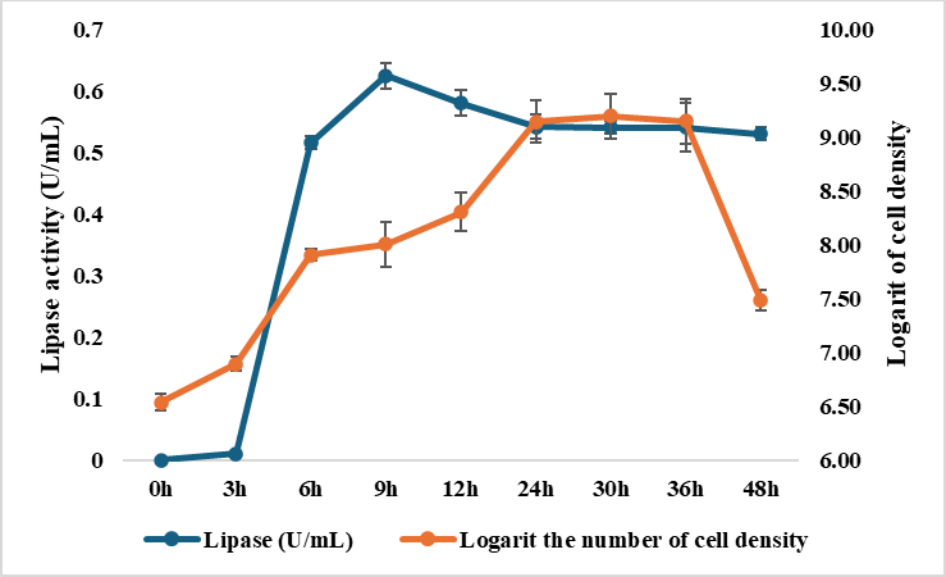

Figure 6.5 The growth and production kinetics in the 150L bioreactor

6.5 Conclusion

The findings of this study demonstrate that *Acinetobacter tandoii* L3 efficiently produces lipase using minimally treated pulp and paper mill sludge, showing early onset and considerable enzyme activity. Notably, lipase production reached a substantial level by 6 h and peaked at 9 h in the 150 L bioreactor, with stable activity sustained for up to 48 h. This performance surpasses that of the 5 L system and reflects the benefits of improved process control at larger scale. The ability to achieve rapid and consistent enzyme yields using unrefined industrial waste confirms *A. tandoii* L3 as a promising candidate for cost-effective and scalable enzyme production within circular bioeconomy frameworks.

7 DISCUSSION, CONCLUSION

7.1 General discussion

As interest grows in leveraging industrial wastewater streams for biotechnological innovation, identifying the most effective substrate for enzyme production necessitates a structured and strategic approach. This study outlines a practical, phased methodology that begins with the preliminary assessment of detergent-compatible enzymatic potential using well-established commercial bacterial strains. This initial screening enables rapid and reliable comparisons across diverse wastewaters. In later phases, selected streams showing the potential can be explored in greater detail with native isolates, tailored for specific optimization goals. Such a sequential framework not only enhances substrate selection efficiency but also provides a scalable foundation for sustainable enzyme production from industrial waste resources.

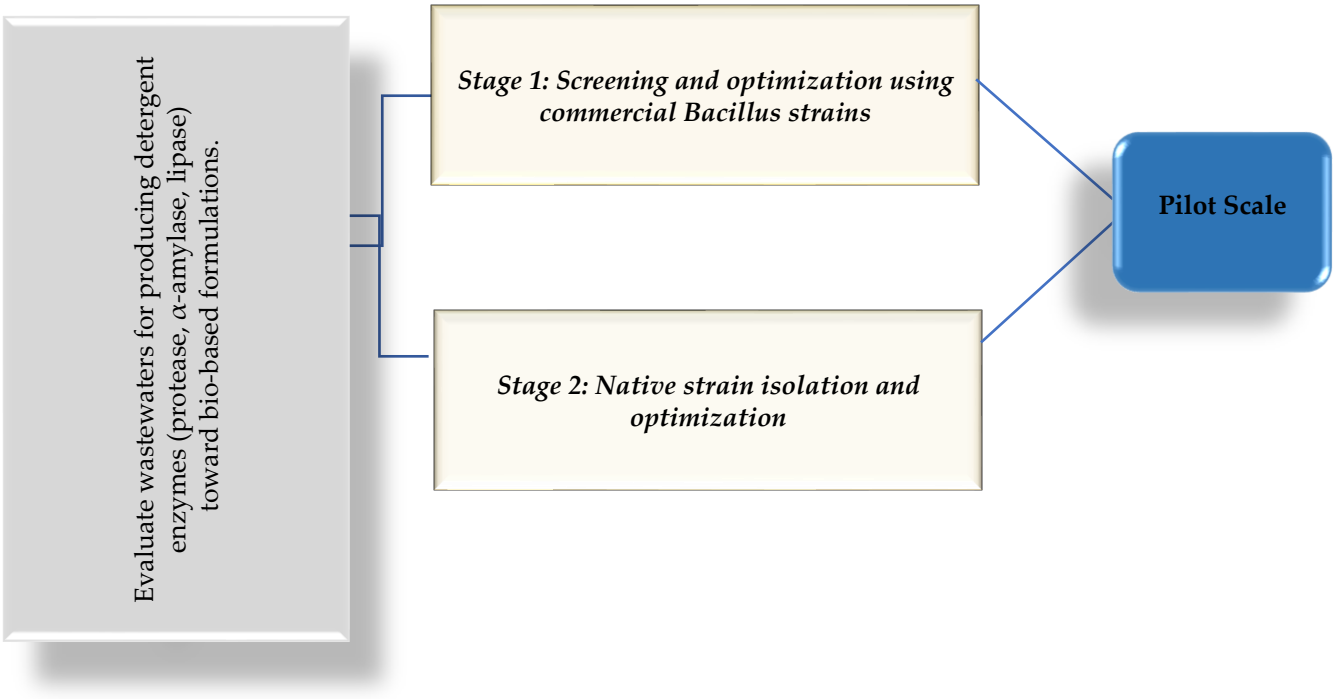

Figure 7.1 Thesis plan: Integrative approach for detergent enzyme production

7.2 Selected commercial strains

Among the microbial producers explored for this purpose, *Bacillus licheniformis*, *B. megaterium*, and *B. amyloliquefaciens* have emerged as leading candidates due to their ability to secrete robust, detergent-compatible enzymes—specifically alkaline proteases, α-amylases, and lipases—and their proven resilience in nutrient-limited, waste-derived media.

Bacillus licheniformis has a longstanding industrial legacy in the detergent sector, dating back to 1960 when Novo Industry A/S commercialized subtilisin Carlsberg from this species as BIOTEX—the first detergent enzyme product (Razzaq et al., 2019). Its alkaline proteases are known for their oxidative and surfactant stability, tolerating compounds such as Triton X-100 and Tween 20 with minimal loss of activity (Hmidet et al., 2009), while calcium ions have been shown to enhance thermostability above 65 °C (El Hadj-Ali et al., 2007). Additionally, its amylases and lipases have been engineered to perform under oxidative conditions and have been utilized in commercial formulations like Duramyl® and Purafect OxAm®. Notably, *B. licheniformis* also demonstrates strong enzyme yields when cultivated on proteinaceous wastes such as chicken feathers, enabling cost-effective production on otherwise low-value substrates.

Bacillus megaterium is recognized for its exceptional morphological and metabolic versatility. It secretes a wide range of detergent-relevant enzymes, including thermostable serine proteases, neutral and alkaline proteases, α-amylases, and lipases. These enzymes maintain activity over a broad pH range (7–11) and elevated temperatures (up to 70 °C), with some strains—such as *B. megaterium* TK1—retaining functionality after exposure to commercial laundry detergents like Surf Excel and Tide (Manavalan et al., 2020). Lipases from strains AKG-1 and F25, isolated from diverse habitats including soil and insect gut, exhibit high stability under alkaline and thermal stress, ideal for oil-based stain degradation. Moreover, this species thrives in submerged fermentation using agro-industrial residues like palm oil press fibers, validating its adaptability to complex, waste-derived media.

Bacillus amyloliquefaciens, originally isolated for its starch-liquefying capability, is extensively used in the commercial production of α-amylases and alkaline proteases. Its enzymes demonstrate high thermal and alkaline tolerance and are widely adopted in detergent formulations to improve the removal of starchy and proteinaceous stains. The BAN™ and

Optimase® product lines exemplify the application of its enzymes in laundry products. Furthermore, strain EGY3 was reported to produce a keratinolytic protease with significant stability in the presence of commercial detergent ingredients, highlighting its potential for sustainable enzyme production under harsh conditions (Nour et al., 2024). *B. amyloliquefaciens* is also frequently cultivated on low-cost substrates, including feather waste and starch-based by-products, supporting its role in circular biomanufacturing.

Collectively, these *Bacillus* species offer significant advantages for detergent enzyme production:

(1) secretion of functionally resilient enzymes active in alkaline, oxidative, and high-temperature environments;

(2) robust growth and enzyme production on industrial and agricultural wastes, reducing dependency on synthetic media;

(3) established industrial relevance, with numerous strains already contributing to commercial formulations across the detergent, leather, and food sectors.

These features align with the broader goals of sustainable bioprocessing and resource valorization, positioning *Bacillus licheniformis*, *B. megaterium*, and *B. amyloliquefaciens* as essential contributors to the next generation of eco-efficient detergent technologies.

7.3 Screening of industrial wastewaters as fermentation media by using commercial strains

To assess the feasibility of industrial wastewaters as substrates for microbial enzyme production, four distinct types were selected: starch industry wastewater (SIW), beverage wastewater (BW), pulp and paper mill sludge (PPMS), and food industry wastewater (FIW). All samples were retrieved directly from the effluent outlets prior to tertiary treatment, subjected to coarse filtration to remove large debris, stored in sterile containers at 4 °C, and processed within 24 hours of collection. Upon arrival at the lab, the samples were analyzed for pH (ranging from 2.45 to 6.65) and either used immediately or stored at 4 °C for no longer than 72 hours. Before fermentation, all wastewaters were sterilized at 121 °C for 15 minutes and adjusted to pH 7 using sodium hydroxide. This minimal pretreatment preserved the chemical identity of each substrate while ensuring microbial compatibility and experimental consistency.

The nutritional characteristics of the wastewaters—particularly their carbon-to-nitrogen (C/N) ratios, sugar composition, and mineral content—played a central role in shaping both microbial growth and enzyme biosynthesis. Substrates with readily metabolizable sugars and balanced C/N ratios, such as SIW, promoted strong bacterial proliferation and high amylase production. In contrast, mineral-rich media like PPMS were more conducive to protease expression, especially in strains such as *Bacillus megaterium* and *B. licheniformis*. On the other hand, carbon-dense but nitrogen-deficient wastewaters like BW led to more limited and strain-dependent enzyme yields. Meanwhile, nutrient-impoverished media such as FIW presented clear barriers to microbial activity, underlining the importance of both macronutrient and micronutrient availability in shaping bioprocess performance.

SIW demonstrated the most favorable overall profile, with high sugar concentrations (31.9 g/L glucose, 11.4 g/L fructose, 9.1 g/L lactose), moderate total solids (13.2 g/L), and a balanced C/N ratio (~11.8), all supporting efficient microbial growth. All three *Bacillus* strains achieved cell densities comparable to those in synthetic media, and *B. amyloliquefaciens* notably produced the highest amylase activity (4.26 U/mL). Protease was also detected across all strains, although no lipase activity was observed. SIW's high sugar content and overall nutrient balance made it the best substrate for amylase production.

BW presented a different nutritional profile, with the highest total solids (69 g/L), elevated TOC (345 g C/L), but a less favorable C/N ratio of ~31.4, indicating nitrogen limitation. Although its exact sugar composition was unknown, it likely contained residual fermentable compounds. All three strains grew on BW, but cell densities and enzyme yields were lower than those on SIW. *B. amyloliquefaciens* showed gradual adaptation over time, and moderate protease activity was observed in all strains. Amylase production was strain-specific, occurring only in *B. amyloliquefaciens*, and lipase was absent. These results suggest BW is a moderately supportive medium, but its performance could be improved with targeted nitrogen supplementation.

PPMS offered a contrasting set of advantages. Despite relatively low TOC (14 g C/L) and total solids (10.3 g/L), it featured a near-optimal C/N ratio (~10) and was enriched in calcium (22 g/L), iron (0.4 g/L), and sodium (10.2 g/L)—minerals known to stabilize and enhance protease activity. Although devoid of simple sugars, PPMS effectively supported growth of all three strains, with

enzyme profiles dominated by protease production. *B. megaterium* and *B. licheniformis* achieved the highest protease yields (53.12 and 49.59 U/mL, respectively), and low levels of lipase activity (0.01 U/mL) were also observed. Amylase activity was negligible. PPMS thus emerged as the most suitable substrate for protease-focused applications.

FIW, by contrast, posed significant challenges. Despite its extremely high TOC (387 g C/L), it had undetectable total solids and was critically deficient in nitrogen (0.339 g N/L) and phosphorus (0.039 g/L), resulting in an unmanageable C/N ratio (~1,142). Its acidic pH (2.45) and lack of buffering capacity further inhibited microbial viability. As a result, none of the strains showed measurable growth or enzyme activity in FIW. This underscores that even carbon-rich wastewaters may be unsuitable for fermentation unless they provide a more complete nutrient profile.

From a comparative standpoint, these wastewaters illustrate both shared and distinct limitations. While SIW and PPMS supported growth and enzyme production effectively, they did so through different nutrient mechanisms—SIW via sugar availability and PPMS via mineral support. BW, although rich in organic carbon, was hindered by nitrogen limitation, and FIW represented an extreme case of nutrient imbalance. This diversity highlights the dual challenge of matching microbial physiology with substrate composition and tailoring fermentation conditions accordingly.

Interestingly, the performance of commercial *Bacillus* strains in these real-world wastewaters closely mirrored their reported behavior in synthetic or agro-industrial media, reinforcing the robustness and versatility of these organisms. *B. amyloliquefaciens*, specialized for carbohydrate utilization, excelled in the sugar-rich SIW, while *B. megaterium* and *B. licheniformis*, both known for protease secretion, thrived in the mineral-rich but sugar-deficient PPMS. However, the strain-specific responses in BW and the total growth failure in FIW also emphasize the need for preliminary substrate characterization and potential supplementation when applying these strains in variable waste matrices.

Based on the integrated analysis of physicochemical parameters, microbial growth profiles, and enzyme production outcomes, two optimal strain–substrate pairings were identified. *Bacillus amyloliquefaciens* was selected for amylase production on SIW due to its rapid growth and high

enzymatic output on this sugar-rich medium. *Bacillus megaterium* was designated for protease and lipase production on PPMS, where it exhibited superior activity in a mineral-supported, sugar-poor environment. While BW remains a candidate for moderate enzyme production pending nutrient enhancement, FIW is unsuitable without substantial pretreatment. These results underscore the value of a tailored substrate–strain approach and demonstrate the potential of minimally processed industrial wastewaters as viable resources for detergent enzyme production.

7.4 Enzyme optimization

To identify efficient and scalable conditions for enzyme production from industrial wastewaters, response surface methodology (RSM) models were developed for *Bacillus megaterium* and *Bacillus amyloliquefaciens* cultivated on PPMS and SIW, respectively. Three key parameters—temperature, total solids, and inoculum size—were selected for optimization based on their operational relevance and practical impact on microbial performance. The optimization process was guided by specific constraints imposed on the models, as summarized in Table 7.1.

Table 7.1 Optimization constraints imposed on the models

Erreur ! Source du renvoi introuvable.

Criteria	Unit	Limit	Goal	Relative importance (1 to 5)
Temperature	°C	30– 37	Minimize	++ (2)
Total solids	g/L	15 – 25	Minimize	+++ (3)
Inoculum size	v/v	2 – 5	Minimize	+ + (2)
Enzyme	U/mL		Maximize	+++++ (5)

For SIW, the total solids level was later extended to 40 g/L based on model-guided extrapolation.

The goal of minimizing temperature and inoculum size reflected a desire to reduce energy input and resource usage, while maximizing enzyme yield remained the highest priority. Although total solids were not minimized, their importance lay in the need for careful tuning—since solids content significantly affected yield and tolerance varied between substrates.

For *B. megaterium* cultivated on PPMS, protease production was positively influenced by total solids and inoculum size, with no significant effect from temperature. The optimal conditions were identified as 30 °C, 25 g/L total solids, and 2% inoculum size. When 1% Tween 20 was added to the same setup to induce lipase production, the model response changed: total solids and temperature showed only minor effects, and inoculum size had no significant role. Accordingly, temperature and solids were maintained, while inoculum size was reduced from 3.5% (v/v) to 2% (v/v). This contrast—same strain, same substrate, but different enzyme—demonstrated the crucial role of inducers in selectively activating metabolic pathways. It also confirmed that surfactants like Tween 20 can redirect enzymatic outputs even under otherwise identical conditions.

In the case of *B. amyloliquefaciens* grown on starch wastewater, all three variables (temperature, total solids, and inoculum size) and their interactions significantly influenced α-amylase production. The model showed a strong positive linear effect of total solids, suggesting that increasing concentration initially enhanced yield. Although the model was built on a 15–25 g/L range, the absence of inhibitory effects within this window prompted further exploration. A subsequent experiment at 40 g/L total solids—guided by the model's linear trend—confirmed enhanced amylase production, highlighting starch wastewater's high substrate tolerance. The final optimized conditions were 25 °C, 40 g/L total solids, and 2% inoculum size (v/v).

The contrast in solids tolerance between starch wastewater and PPMS reflects their differing chemical complexities. Starch wastewater, composed mainly of readily degradable carbohydrates and low in inhibitors, supported higher substrate loading. Conversely, PPMS contains lignocellulosic residues, pulping chemicals, and trace metals, which can disrupt microbial metabolism at higher concentrations—limiting optimal solids to around 25 g/L for *B. megaterium*.

Together, these results emphasize that successful enzyme production from industrial wastewaters is highly context specific. Even when using a shared optimization framework, the interplay between substrate composition, microbial physiology, and enzyme target requires tailored strategies. Moreover, the ability to shift enzymatic profiles by introducing inducers (e.g., Tween 20) or adjusting solids concentration illustrates that industrial wastewaters are not rigid systems—

they can function as tunable fermentation platforms, where small interventions enable diverse and valuable biotechnological outputs.

7.5 Isolation, screening, and identification of indigenous enzyme-producing strains

Although the optimization of enzyme production using *Bacillus megaterium* on PPMS demonstrated only limited success—resulting in moderate protease levels and negligible lipase activity even after inducer supplementation—it underscored the challenges of utilizing PPMS as a fermentation substrate. Nevertheless, these outcomes also revealed that the sludge supported microbial growth under selective conditions, suggesting the presence of native strains with adaptive capabilities. This led to the second stage of the study, which aimed to isolate and characterize indigenous microorganisms from PPMS capable of producing detergent-relevant enzymes and tolerating harsh environmental conditions.

Isolation was carried out using selective media. On nutrient agar (NA) supplemented with 1% casein, four morphologically distinct colonies—designated P1 through P4—were isolated for protease screening. Similarly, NA supplemented with 1% Tween 20 was used for lipase enrichment, yielding four unique isolates, designated L1 through L4. All isolates were purified and stored in 10% glycerol at –20 °C for further analysis.

Protease screening in liquid culture revealed substantial variation among the casein-derived isolates. Of these, P1 and P4 showed the highest activities. When cultivated in shake flasks, P4 produced ~134 U/mL of protease after 48 hours—more than triple the yield of *B. megaterium* (39.6 ± 3.53 U/mL) and significantly higher than P1. Based on this exceptional performance, P4 was selected for detailed characterization. It formed ivory-yellow, circular colonies with a creamy texture and demonstrated broad growth tolerance across 15–45 °C and pH 6–9, indicating robust adaptability to environmental fluctuations.

Lipase screening revealed a different standout performer. Although all Tween 20-derived isolates produced visible precipitation on agar—indicating lipase activity—only L3 exhibited a substantial increase in activity over time, reaching 0.386 U/mL at 48 hours. This was nearly ninefold higher than its 24-hour activity and eight times greater than the next best performer, L4. L3 formed pale-yellow, glossy colonies approximately 0.1 mm in diameter and thrived between 15 °C and 40 °C,

with optimal growth between 30–37 °C. Unlike P4, L3 displayed limited pH tolerance, growing only at pH 7–9, suggesting a narrower ecological niche suited to mildly alkaline environments.

Molecular identification was conducted using 16S rDNA sequencing. The 1543 bp sequence of P4 showed 98.62% similarity with several species in the *Bacillus cereus* group, including *B. tropicus*, *B. paramycoides*, and *B. nitratireducens*. Based on morphological and functional alignment with literature, P4 was identified as *Bacillus tropicus*. This species is known for producing thermostable and industrially relevant enzymes, including proteases and keratinases, and has also been implicated in waste degradation and heavy metal detoxification.

L3's 16S rDNA sequence (1005 bp) showed 100% identity with *Acinetobacter tandoii* DSM 14970. This species is commonly found in activated sludge and wastewater environments, supporting its origin from PPMS. While *A. tandoii* is known for pollutant degradation—including nitrogen removal and phenol breakdown—this study is the first to report lipase production by this species, expanding its known biotechnological capabilities.

Together, these findings highlight the untapped enzymatic potential of indigenous wastewater-derived microbiota. The exceptional protease yield of *B. tropicus* P4 and the time-dependent lipase expression of *A. tandoii* L3 position these strains as strong candidates for future applications in detergent formulation or waste bioprocessing. Furthermore, this stage reinforces the broader conclusion that industrial wastewaters not only serve as fermentation substrates but also as reservoirs of specialized microbial functions—supporting both substrate valorization and novel biocatalyst discovery.

7.6 Role of inducers in enzyme production

This study highlights the enzyme- and strain-specific roles of inducers in modulating protease and lipase production in *Bacillus tropicus* P4 and *Acinetobacter tandoii* L3, respectively. All tested inducers positively influenced protease secretion by *B. tropicus*, with the most pronounced effect observed for 1% Tween 80 (285.78% increase), followed closely by casein at both 0.5% and 1% concentrations. These results suggest that non-ionic surfactants such as Tween 80 enhance enzyme release, likely by increasing cell membrane permeability and oxygen transfer, while proteinaceous substrates like casein may act as both nutrient sources and gene expression stimulators. In

contrast, wheat bran, though cost-effective and rich in complex nutrients, showed a weaker and dose-sensitive effect—possibly due to substrate inhibition or limited bioavailability at higher concentrations.

In the case of lipase production by *A. tandoii* L3, the native sludge medium supported the highest activity, peaking at 24 hours and slightly declining thereafter. The addition of Tween 20 and Tween 80 did not enhance activity beyond the control and, in fact, showed signs of temporary or delayed induction followed by inhibition. These results suggest that the intrinsic composition of PPMS already provides sufficient induction signals for lipase expression in this strain, and that surfactants may not be necessary—or may even suppress activity at higher concentrations—under these specific conditions.

Collectively, these findings underscore the importance of selecting inducers based on enzyme type, microbial physiology, and substrate context. While established surfactants like Tween 80 are effective in stimulating protease production in *Bacillus*, their role in lipase induction, particularly for *Acinetobacter*, may be limited or counterproductive depending on concentration and timing. This reinforces the broader conclusion that inducer selection must be empirically validated for each system, and that optimization strategies must consider not only economic feasibility but also the dynamic regulatory mechanisms underlying microbial enzyme synthesis.

7.7 Bioreactor and scale-up Performance

To validate enzyme production under scalable and controlled conditions, a series of 5 L and 150 L bioreactor experiments were conducted using optimized parameters derived from earlier flask-scale studies. The focus was on evaluating amylase, protease, and lipase production using native and commercial strains on minimally pretreated wastewater substrates.

7.7.1 Amylase production by *Bacillus amyloliquefaciens*

Two 5 L bioreactor trials were carried out on starch wastewater under optimized conditions (40 g/L total solids, 30 °C, 2% inoculum). In both batches, amylase secretion initiated during the exponential phase and peaked at 48 h (5.74 and 5.72 U/mL, respectively), followed by a slight decline by 72 h. The enzyme production appeared to be growth-associated, with activity

decreasing after 48 h, likely due to substrate depletion and the cessation of active amylase synthesis once nutrient availability diminished. The near-identical kinetic profiles confirmed process reproducibility and indicated that extended fermentation beyond 48 h offered no additional benefit. These results demonstrate that controlled bioreactor conditions (pH, aeration and agitation) significantly enhance amylase productivity compared to flask cultures and reinforce the scalability of the process.

7.7.2 Protease production by *Bacillus megaterium*

Using PPMS (25 g/L total solids, pH 7, 1% Tween 80), *B. megaterium* was cultured in 5 L bioreactors. Protease activity peaked at 24 h in both batches (52.91 and 57.91 U/mL) before declining, likely due to product instability or nutrient depletion. While yields were moderate compared to some literature, they were consistent with the basic cultivation setup. Variations with higher-reported yields (e.g., *Bacillus megaterium* RRM2 (Rajkumar et al., 2011)) can be attributed to richer media, elevated temperatures, and alkaline pH—conditions not used here in order to maintain process simplicity and compatibility with wastewater-based substrates.

7.7.3 Lipase production by *Acinetobacter tandoii* L3

Lipase production by *Acinetobacter tandoii* L3 was assessed in 5 L bioreactors using PPMS-based conditions. In both batches, peak activity (0.56 and 0.55 U/mL) was reached at 9 hours, followed by a sharp decline. In contrast, the 150 L reactor achieved a slightly higher peak (0.62 U/mL at 9 h), with no sharp drop observed. Instead, lipase activity remained above 0.53 U/mL for an extended period, lasting up to 48 hours.

Compared to flask-scale experiments, enzyme production was accelerated at both reactor scales, likely due to improved substrate accessibility or oxygen transfer. The broader activity window in the 150 L reactor reduces the urgency for precise harvest timing, offering greater operational flexibility. These findings underscore the potential for cost savings through shorter fermentation durations and minimal medium supplementation while maintaining enzyme stability at scale.

7.7.4 Protease production by *Bacillus tropicus* P4

Given the superior performance of the indigenous *B. tropicus* P4 in earlier screening, its protease production was scaled from 5 L to a 150 L bioreactor under similar conditions. The enzyme titer increased rapidly during the first 24 h (peaking at 847.64 U/L), then declined slightly to 710.58 U/L at 48 h, while cell density plateaued. The stable protease profile beyond the exponential phase indicates a well-balanced large-scale process, where key parameters such as pH, agitation, and oxygen transfer likely supported both cell viability and enzyme retention. These results confirm the feasibility of upscaling protease production using wastewater-based media and indigenous strains.

7.8 Discussion

7.8.1 Comparison between commercial and indigenous enzyme producers

The enzyme production profiles of the commercial strain *Bacillus megaterium* and the indigenous isolate *Bacillus tropicus* P4 and *Acinetobacter tandoii* L3 reveal notable differences in enzymatic potential under comparable conditions. In flask experiments, *B. megaterium* produced 39.6 ± 3.53 U/mL after 48 h on PPMS supplemented with 1% Tween 80, whereas P4 achieved approximately 134 U/mL without the need for enriched medium. This represents a more than threefold increase in protease activity, reaffirming the advantage of sourcing strains directly from their environmental origin. Likewise, for lipase, the commercial strain exhibited minimal activity, whereas *A. tandoii* L3 reached 0.386 U/mL at 48 h, over eight times higher than the next best strain. The superior performance of P4 and L3 highlights the adaptive traits of native microorganisms to complex substrates like PPMS, where chemical inhibitors and nutrient imbalances may limit the effectiveness of commercial strains. Indigenous isolates are often physiologically attuned to the conditions of their original habitat, enabling high enzyme yields with minimal supplementation. These findings reinforce the strategy of targeted strain isolation from challenging waste environments to unlock biocatalytic potential otherwise inaccessible to standard strains.

7.8.2 Role of commercial strains in substrate-specific applications

However, the relevance of commercial strains remains significant, particularly for wastewaters with physicochemical conditions unsuitable for most native isolates. For instance, starch industry wastewater (SIW) exhibited a strongly acidic pH (~3.99) that may inhibit the growth of many sludge-residing microbes. In contrast, *B. amyloliquefaciens*—a well-characterized commercial strain—was able to grow and efficiently produce amylase after a basic pretreatment step (pH neutralization and sterilization), reaching 5.73 ± 0.01 U/mL in 5 L bioreactors. This highlights the robustness and reliability of commercial strains in cases where native adaptation is limited or uncertain. Therefore, a dual strategy combining commercial strain deployment for accessible substrates and indigenous strain isolation for complex or inhibitory wastewaters offers a more comprehensive approach to enzyme bioprocess development.

7.8.3 Time-course comparison and evaluation of scalability

The transition from flask to 5 L and 150 L bioreactors revealed valuable insights into process reproducibility and kinetic behavior. For amylase production by *B. amyloliquefaciens*, peak activity occurred consistently at 48 h in both 5 L batches, confirming the scalability of optimized conditions and demonstrating the positive impact of bioreactor control systems (e.g., aeration, agitation) on enzyme yields compared to flasks.

In protease production, *B. megaterium* achieved higher titers in 5 L reactors than in shake flasks, but the indigenous isolate *B. tropicus* P4 outperformed both at larger scale. In 150 L bioreactors, P4 reached 847.64 U/L at 24 h, with only a slight decline at 48 h, indicating good enzyme stability and retention. This reflects an effective scale-up where environmental conditions supported both biomass and extracellular enzyme maintenance.

Lipase production by *Acinetobacter tandoii* L3 in 5 L and 150 L reactors exhibited early peak kinetics (9 h), with the highest activity reaching 0.62 U/mL in the 150 L scale—suggesting improved substrate accessibility and accelerated enzyme turnover. This narrow production window highlights the strain's suitability for fast-cycle, cost-effective lipase processes.

In the 150 L experiments, the effect of cell density (CFU/mL) on enzyme production was clearly evident, particularly in the case of *Bacillus tropicus* P4. Protease activity remained high after

peaking and was maintained at stable levels up to 48 h, corresponding to a relatively constant CFU/mL between 24 h and 48 h. This suggests that protease production is closely associated with active cell growth. In contrast, *Acinetobacter tandoii* L3 showed a sharp decline in CFU/mL over the same period. However, despite this reduction in biomass, lipase activity in the 150 L reactor remained high up to 48 h. This sustained activity is likely due to enhanced enzyme stability at larger scale rather than continued synthesis. Compared to the 5 L setup, the 150 L system offers more consistent environmental control, improved mixing, and potentially reduced enzyme degradation or inactivation. Nevertheless, it is also possible that *A. tandoii* L3 follows a partially growth-independent mechanism under certain conditions, contributing to lipase accumulation even as biomass declines. These findings emphasize the role of scale in preserving enzyme activity and underscore that, even under identical medium conditions, different strains exhibit distinct nutrient requirements, growth dynamics, and enzyme production profiles.

7.8.4 Implications for wastewater valorization

Collectively, the results demonstrate that industrial wastewaters—when matched with compatible strains and tuned conditions—can support scalable enzyme production. The integration of both commercial and native strains provides flexibility across substrates with diverse chemical compositions. Commercial strains like *B. amyloliquefaciens* offer predictability and robustness on moderately challenging media, while indigenous isolates like *B. tropicus* P4 and *Acinetobacter tandoii* L3 reveal untapped potential for complex and recalcitrant waste streams. Ultimately, combining substrate-specific pretreatment, strain selection, and bioreactor optimization enables an adaptable strategy for enzyme production within a sustainable circular bioeconomy framework.

7.9 Conclusion

This study demonstrated the feasibility of producing detergent-relevant enzymes—amylase, protease, and lipase—using industrial wastewaters as fermentation media, supported by both commercial and indigenous bacterial strains. Among the four tested wastewaters, starch industry wastewater (SIW) and pulp and paper mill sludge (PPMS) emerged as the most promising

substrates, each supporting strong microbial growth and enzymatic activity under minimal pretreatment.

Commercial strains such as *Bacillus amyloliquefaciens* and *Bacillus megaterium* proved effective on substrates like SIW and PPMS, particularly when fermentation conditions were optimized through response surface methodology. Notably, *B. amyloliquefaciens* achieved high amylase yields (5.73 ± 0.01 U/mL) in 5 L bioreactors using SIW, demonstrating the scalability of the process under defined conditions. In contrast, *B. megaterium* produced moderate protease levels on PPMS, with added Tween 80 improving yield but not significantly enhancing lipase output.

Recognizing the limitations of commercial strains in complex wastewaters, native microbial isolates were screened directly from PPMS. This approach led to the identification of *Bacillus tropicus* P4, which produced more than triple the protease activity of *B. megaterium* in flasks, and *Acinetobacter tandoii* L3, which exhibited rapid and high lipase production within 9 hours. The superior performance of *B. tropicus* P4 was confirmed through scale-up to a 150 L bioreactor, where protease titers exceeded 840 U/L and remained stable beyond the exponential growth phase, demonstrating the strain's robustness and industrial potential.

The combined results underscore several important conclusions:

- **Wastewater substrates are viable for enzyme production** when their chemical characteristics are properly matched with compatible strains.
- **Strain selection is critical**—commercial strains offer reliability in moderately challenging substrates, while native strains can unlock performance on chemically complex wastewaters.
- **Bioprocess scalability is achievable**, but enzyme-specific kinetics and substrate composition must guide harvest timing and operational strategies.
- **Targeted inducers and process tuning**—such as surfactant addition or solids adjustment—can significantly alter enzyme yields, but their effectiveness is strain- and enzyme-dependent.

Altogether, this work supports an integrated approach to enzyme production that leverages both strain engineering and substrate valorization, offering a pathway to more sustainable, low-cost, and flexible bioprocesses for detergent and environmental biotechnology applications.

7.10 Recommendations

- **Develop substrate-strain matching protocols**

  Given the diverse chemical profiles of industrial wastewaters, a decision-making framework should be established to match specific wastewaters with suitable microbial strains (commercial or indigenous) based on pH tolerance, nutrient profile, and inhibitor content.

- **Expand bioprospecting efforts in challenging waste matrices**

  The success of *Bacillus tropicus* P4 and *Acinetobacter tandoii* L3 highlights the untapped potential of native microbial communities in complex substrates like PPMS. Further exploration using high-throughput screening or metagenomic tools could identify additional robust strains with unique enzymatic traits.

- **Explore metabolic engineering of native isolates**

  Strains like *B. tropicus* P4, which already show high natural enzyme yields, could benefit from genetic modification or CRISPR-based tuning to enhance enzyme specificity, stability, or secretion—combining native resilience with engineered precision.

- **Optimize fermentation timing and harvesting strategies**

  Enzymes such as lipases from *A. tandoii* L3 show narrow activity windows. Future process design should incorporate real-time monitoring and controlled feeding or harvesting strategies to capture peak yields and minimize product degradation.

- **Evaluate synergistic co-culture systems**

  Combining commercial and indigenous strains in co-culture fermentation may allow for multi-enzyme production (e.g., amylase + protease) or improved substrate utilization, especially on mixed waste streams.

- **Conduct techno-economic and life cycle assessments**

  To translate lab-scale success into industrial feasibility, cost-benefit analyses and environmental impact studies should be integrated into future research. This would help identify the most viable configurations for detergent enzyme production in real-world applications.

- **Investigate enzyme formulation and stability in detergent systems**

To bridge fermentation with product development, downstream work should test enzyme compatibility, activity retention, and synergism with common detergent ingredients (e.g., surfactants, builders, bleaching agents) under real-use conditions (temperature, pH, water hardness).

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