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DÉVELOPPEMENT DE FORMULATIONS EFFICACES ET ÉCONOMIQUES DE *SINORHIZOBIUM MELILOTI* CULTIVÉ DANS LES EAUX USÉES DE L'INDUSTRIE D'AMIDON

Par

TAREK ROUISSI

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Examinateur externe et président de jury	Roger Lalande Agriculture et Agroalimentaire Canada
Examinateur interne	Patrick Drogui – INRS-ETE
Directeur de recherche	Rajeshwar D. Tyagi – INRS-ETE
Codirecteurs de recherche	Satinder K. Brar – INRS-ETE Danielle Prévost – INRS-ETE
Examinateur externe	Don Smith – Université McGill

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RÉSUMÉ

Ce travail a pour objectif de développer des formulations efficaces et à faible coût de *Sinorhizobium meliloti*, cultivé dans les eaux usées d'industries d'extraction d'amidon. Il a pour objectif également de créer de nouveaux volets de valorisation des rejets industriels à travers l'élaboration des biofertilisants, et des bioproduits en toute généralité. La récolte des cellules par centrifugation est la première étape qui se situe juste avant la formulation. En industrie, la récupération maximale de cellules est indispensable pour parvenir à formuler des produits concentrés en cellules. C'est ainsi que ce procédé de séparation (centrifugation) a fait l'objet de l'étude en premier lieu dans cette thèse. L'optimisation des conditions de récolte par centrifugation par la méthode de réponse de surface a montré que le maximum de récolte (>99%) est atteint à 8000g, pendant 20 min, à pH 7 en utilisant le rotor à gadgets mobiles. L'augmentation des volumes centrifugés 20 fois réduit le pourcentage de récupération des cellules de 90% à 87% pour le rotor à angle fixe lorsque la centrifugation a été faite à 8000g pendant 20 min.

Les formulations liquides de *Sinorhizobium meliloti* sont les plus adaptées pour les équipements agricoles et les plus faciles à appliquer. En effet, il est presque toujours recommandé de mettre les formulations en suspensions avant de les appliquer sur les grains de semence. Les faits relatifs à cet objectif ont été abordés dans cette étude. Les additifs, tels que le saccharose, le sorbitol, le polyvinylpyrrolidone (10000) (PVP), le polyéthylène glycol (8000) (PEG), les alginates de sodium, et les mélanges de PVP avec : le saccharose, le sorbitol et les alginates, et de PEG avec : le saccharose, le sorbitol et les alginates, ont été évalués pour leur capacité à protéger le *Sinorhizobium meliloti* au cours de 13 semaines de stockage réfrigéré. Les résultats montrent que toutes les formulations ont préservé la viabilité des cellules à des concentrations supérieures à 10^9CFU/ml. Le saccharose à 10% w/v était le meilleur additif avec une demi-vie de 83 semaines. Le suivi de la viabilité des cellules (formulations fraiches (FLF) et formulations conservées pendant 13 semaines (OLF) après application sur les semences de luzerne a montré que les formulations FLF de PEG et de PEG-Sorb ont maintenu des concentrations supérieures à 10^3CFU/grain (normes canadiennes) pour 4 mois de conservation. Parmi les formulations OLF, le PVP-sorb était le plus efficace, aboutissant ainsi au meilleur rendement en matière sèche de la luzerne (146 mg). La microscopie électronique à balayage a montré que les cellules de rhizobium sont réparties différemment à la surface des grains (comme agrégats ou répartie uniformément) selon les additifs utilisés.

En plus de la viabilité des cellules, la stabilité physique des bioinoculants liquides (décantation, floculation) est un critère à considérer au cours de l'évaluation de la qualité des produits. Une nouvelle méthode de caractérisation de la suspendibilité a été développée dans cette thèse comme alternative aux techniques de mesures classiques (basées sur la décantation). Les modèles, basés sur la régression linéaire multiple (avec: le pH, le potentiel zêta, la taille des particules et la concentration en sorbitol comme variables indépendantes) ont montré l'existence de relations significatives entre ces variables (p <0,001, R^2 = 0,98). La régression sigmoïdale a révélé une relation significative entre le potentiel zêta et la suspendibilité avec une probabilité p = 0,007 et R^2 = 0,86; et entre la viscosité et la suspendibilité (valeur p <0,0001 et R^2 = 0,9823). Ainsi, ces corrélations établies permettent de proposer le potentiel zêta et la viscosité comme paramètres pour décrire la stabilité physique des formulations liquides.

Les formulations en poudres de *Sinorhizobium meliloti* présentent plusieurs avantages que ce soit au cours de la conservation, du transport ou de l'application. Dans ce travail, les formulations en poudres mouillables ont été développées par atomisation. Le séchage par atomisation a été optimisé en utilisant la méthodologie de réponse de surface. Les paramètres étudiés sont : la température de séchage, la pression de pulvérisation, le débit d'air de séchage et le débit d'alimentation. Le lactosérum à 10% w / v, le sorbitol à 1% w/v et le saccharose à 1% w/v ont été ajoutés au bouillon fermenté concentré avant séchage. Les réponses étudiées étaient la viabilité des cellules, l'humidité et la température à la sortie. Dans les conditions optimales (température de séchage de 105°C, un débit d'air de séchage de 0.56m3/min, une pression d'atomisation de 0.07 MPa et un débit d'alimentation de 8 ml/min) qui ont permis d'atteindre le maximum de désirabilité (0,81), la viabilité des cellules obtenue était de $6.8x10^9$CFU /g, l'humidité était de 11,4% et la température de sortie de 42°C. La viabilité des cellules dans la poudre séchée a été maintenue supérieure à10^9 UFC / g au cours de 4 mois de conservation réfrigérée. Les formulations séchées ont donné un rendement en matière sèche de la luzerne (144.6 mg) ainsi qu'un indice de nodulation (15) similaire au control (cellules fraîches cultivées dans l'YMB (131 mg, 14) (p=0.23 pour le rendement en matière sèche; p=0.18 pour l'indice nodulaire). Les micrographes ont montré que les cellules de rhizobium étaient incorporées dans le mélange d'additifs (lactosérum-saccharose-sorbitol) qui les enrobait comme une matrice protectrice.

Dans le but d'améliorer la qualité des formulations de *Sinorhizobium meliloti*, la co-inoculation *Azospirillum* -*Sinorhizobium* a été étudiée dans ce travail. L'*Azospirillum brasiliense* ATCC29710 a été produit dans les eaux usées d'amidon (SIW), des suppléments de croissance à savoir le gluconate à 0.5%w/v, l'extrait de levure à 0.5% et le mélange (extrait de levure 0.5%w/v + gluconate 0.5%w/v) ont été étudiés, la comparaison a été réalisée avec le milieu Bashan et le SIW. Les résultats ont montré que l'ajout de l'extrait de levure ou de gluconate améliore significativement le taux de croissance de $0.266h^{-1}$ (pour SIW) à respectivement $0.315h^{-1}$ et $0.316h^{-1}$. Ces taux étaient statistiquement comparables au milieu Bashan ($0.324h^{-1}$). La méthodologie de réponse de surface a été appliquée afin d'optimiser la quantité d'extrait de levure ajoutée et la température de croissance d'*Azospirillum brasiliense* dans le milieu SIW. Le Modèle obtenu (R^2 = 0,92) a montré que la production maximale de cellules était à 34 °C et avec 0.28% w/v d'extrait de levure. Un test de confirmation « check point » a été effectué : *Azospirillum brasiliense* a été produit dans le fermenteur 7.5l. La concentration atteinte était de ≈ $0.90x10^9$CFU/ml, valeur située dans l'intervalle de confiance de la variable prédite. La comparaison entre les rendements en matière sèche des plantes de luzerne co-inoculées avec : *Azospirillum Brasiliense* (10^4,10^5et 10^6CFU/plante) et *Sinorhizobium meliloti* (à 10^5CFU/plante) a montré que la combinaison d'*Azospirillum* à 10^5 ou 10^6CFU /plante avec *Sinorhizobium meliloti* à 10^5CFU/plante a permis d'atteindre le plus haut rendement en matière sèche (augmentation respectives de 20 et 17%, p=0.037912) comparativement aux plantes de luzerne inoculées avec *Sinorhizobium meliloti* seul à10^5 CFU/plante.

ABSTRACT

This study is focused on developing series of formulations of *Sinorhizobium meliloti* by cultivation in starch industry waste water (SIW) with regard to the new recycling practices of industrial waste for the elaboration of biofertilizers as value added byproducts. For this purpose, harvesting of cells by centrifugation is recognized as the first pre- step in formulation and maximum recovery of cells required for the development of effective formulation (concentrated cells). Experimental optimization was carried out using surface response methodology. The recovery of cells was maximum at 8000 *g*, 20 °C, pH= 7 for 20 min using swinging-bucket type rotor (>99 %) compared to fixed-angle type rotor (90 %). Thus, a concomitant decrease in recovery from 90 to 87% was observed increasing the broth volume 20 times at 8000 *g*, for 20 min at 20 °C.

Liquid formulations of *Sinorhizobium meliloti* are the most suitable for agricultural equipment and are easier to apply on seeds. Indeed, it is mainly recommended to suspend formulations in water and apply them to seeds before sowing. For this objective, a study was conducted in the second part of this thesis to develop efficient and low cost liquid formulations. Additives, such as sucrose, sorbitol, polyvinylpyrrolidone (10000)(PVP), polyethylene glycol (8000)(PEG), sodium alginates, mixtures of PVP (with sucrose, sorbitol and alginates) and PEG (with sucrose, sorbitol and alginates) were evaluated for their capacity to maintain viability of *Sinorhizobium meliloti* during 13 weeks of refrigerated storage. All formulations allowed cell viability higher than 10^9CFU/ml. Sucrose at 10%w/v was the best additive to promote survival of cells as shown by the half life which was 83 weeks. Evaluation of liquid formulation (fresh and 13 weeks old) applied to alfalfa seeds showed that fresh formulation of PEG and PEG-sorbitol maintained cell viability at 10^3CFU/seed (Canadian standards) for 4 months of seed storage at ambient temperature. PVP-sorbitol formulation stored for 13 weeks was the most efficient, allowing the highest alfalfa shoot dry matter. Micrographs showed that rhizobial cells were distributed differently on the seed surface (as aggregates or uniformly distributed) according to the additives used.

In addition to cell viability, physical stability including sedimentation and flocculation of liquid bio-inoculants; was regarded as a crucial criterion to be considered in assessing final quality of the formulated product. A new method of characterization suspendibility was developed in this work as an alternative to traditional measurement techniques (based on settling). A Model based on multiple linear regression, zeta potential, average particle size and sorbitol concentration

concluded a significant relationship ($p<0.001$ and $R^2=0.98$). A Sigmoid regression revealed a significant relation between zeta potential and suspendibility with p value=0.007 and R^2 =0.86, and between viscosity and suspendibility (p value <0.0001 and R^2= 0.98).

The powder formulations of *Sinorhizobium meliloti* developed by spray drying have several advantages such as long storage period, low cost of transport and easy application. Spray was optimized via response surface methodology for the development of powder formulation in starch industry waste water. Process parameters including drying temperature, air drying rate, spray pressure and feed sample rate were studied. The role of cheese whey powder, sorbitol and sucrose in concentrations of 10% w/v, 1 % w/v and 1% w/v, respectively, were added as carriers to the concentrated fermented broth before drying. Responses studied comprised cell viability, moisture and outlet temperature. Under optimal conditions (drying temperature of 105°C, air drying rate of 0.56m^3/min, pressure of atomization 0.07 MPa and feed flow rate of 8 mL/min), desirability reached a maximum of 0.81, cell viability 6.8x10^9CFU/g, 11.4% of moisture and 42°C as outlet temperature. During 4 months of refrigerated storage, viability was maintained higher than 1x10^9 CFU/g. Analysis of inoculated alfalfa plants showed that spray dried formulation induced similar shoot yield, on a dry basis (144mg) (p=0.23) and nodulation index (15) (p=0.18) compared to fresh cultured cells produced in standard media (shoot yield and nodulation index were 131mg and 14, respectively). Micrographs showed that rhizobial cells were embedded in the mixture of additives (whey-sucrose-sorbitol) which acted as a matrix.

In order to improve the formulations of *Sinorhizobium meliloti*, co-inoculation with *Sinorhizobium – Azospirillum* was applied. *Azospirillum brasiliense* ATCC29710 was first produced in starch waste water (SIW). To increase its production, gluconate, yeast extract and combined (yeast extract+ gluconate) were evaluated in comparison with standard Bashan medium and SIW. Results showed that the addition of gluconate or yeast extract significantly improved the growth rate from 0.266h^{-1} (for SIW) to 0.315h^{-1} and 0.316h^{-1}, respectively, compared to 0.324h^{-1} for the standard Bashan medium. In addition, response surface methodology was applied in order to optimize the amount of yeast extract added and the growth temperature of *Azospirillum brasiliense* in SIW. A Statistical model obtained with a coefficient of R^2=0.92, and which showed that the optimal temperature and yeast extract amount for cell production was 34°C and 0.28% w/v, respectively. A checkpoint analysis carried out by culturing cells in a 7.5 L fermenter showed a concentration of ≈0.90x10^9CFU/ml, which was included in the confidence interval of the predicted variable. Comparison of co-inoculated alfalfa plants with *Azospirillum brasilense* (at 10^4, 10^5 and 10^6CFU/plant) and *Sinorhizobium meliloti* (at

10^5CFU/plant) showed that combination10^5 or 10^6CFU of *Azospirillum* /plant with 10^5CFU of *Sinorhizobium meliloti* plant allowed the highest plant yield on dry matter (increase of 20 and 17%, respectively, p=0.03) compared to inoculated alfalfa plants with *Sinorhizobium meliloti* at 10^5CFU/plant.

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a: particle radius

A_{131} : Hamaker constant

D: global desirability function

D_{43} : average particle size

$d_{\text{cell viability}}$: desirability function of cell viability

d_{moisture} :desirability function of moisture

DO: dissolved oxygen

$d_{T\ \text{outlet}}$: desirability function of temperature outlet

E : electric field

f(P) : retardation factor

FLF : fresh liquid formulation

H : distance separating the surfaces the two interacting particles

k : inverse Debye length

KV : kilovolt

M_w : average molecular weight

OLF: old liquid formulation

P: Pressure of atomization (MPa)

PEG : polyethylene glycol

PVP : polyvinyl pyrrolidine

RSM : response surface methodology

[Sorb] : sorbitol concentration

SIW: starch industrial waste water

Sorb : sorbitol

Susp : *suspendibility*

T drying : temperature inlet of air drying

t (1/2) : half life time

T_{oulet} : temperature outlet of air drying

Va: van der Waals attractive energy and

Vr: electrical double layer energy

V_{TE} : total interaction energy

w/v: weight per volum

Zp : zeta potential

ε : dielectric constant of water

ε_0 : permittivity of free space

ε_r : dielectric constant of the suspension

η :viscosity

λ : wavelength of the intrinsic electronic oscillations

υ: velocity of the particles

INTRODUCTION GÉNÉRALE

L'utilisation des rhizobiums a considérablement contribué au développement et à l'amélioration des rendements agricoles des légumineuses (Kloepper *et al.*, 1978; Hilali *et al.*, 2001). Ces bactéries sont capables de fixer l'azote atmosphérique grâce aux nodules qu'elles créent au niveau des racines, ce qui permet aux plantes d'avoir de l'azote sous forme assimilable. Elles sont capables aussi d'améliorer la croissance de plusieurs plantes non légumineuses : les rhizobiums présents dans la rhizosphère peuvent favoriser la solubilisation du phosphore, inhiber la prolifération des champignons phytopathogènes et produire des phytohormones (Wiehe *et al.*1995; Chabot *et al.*, 1996; Terouchi *et al.*,1990).

Un aperçu sur l'histoire des techniques agricoles révèle que c'est à la fin du xix^{ième} siècle que *Hellriegel* et *Wilfarth* ont découvert que la fixation de N_2 est reliée à la formation des nodules au niveau des racines, suite à l'action de certains agents infectieux. L'identification de ces agents parvient plus tard grâce aux travaux de *Beijerinck* qui ont permis d'isoler et de décrire ces bactéries (Wilson *et al.*, 1932). À cette époque, les agriculteurs aux États-Unis savaient déjà qu'il était bénéfique pour augmenter les rendements des légumineuses d'inoculer au début de la saison, avec de la terre provenant des champs déjà cultivés avec ces mêmes plantes (Smith, 1992). La commercialisation de bio-inoculants à base de rhizobium a commencé juste au début du XX^{ième} siècle par certaines petites entreprises (Yoav, 1998).

Le début du vingtième siècle a coïncidé également avec l'exploitation excessive des ressources naturelles. Le recours aux engrais chimiques était fréquent partout dans le monde, dans le but d'augmenter les rendements agricoles. La conséquence des ces pratiques était l'augmentation de la pollution des eaux souterraines. De plus, avec le recours aux industries chimiques pour la synthèse des fertilisants azotés et l'évolution des activités industrielles, une autre problématique est apparue concernant le traitement et la gestion des rejets industriels.

Dans ce travail, nous avons essayé de trouver une alternative économique qui permet de remédier à l'utilisation des fertilisants chimiques et en même temps valoriser des rejets agroindustriels (eaux usées d'industries d'amidon). Il s'agit d'une alternative biologique non polluante : le développement de bio-inoculants de *Sinorhizobium meliloti.* L'intérêt porté à cette bactérie revient à l'importance de la plante qu'elle nodule : la luzerne. La luzerne est une légumineuse agricole utilisée dans l'alimentation du bétail au Québec et partout dans le monde.

Plusieurs inoculants commerciaux sont déjà sur le marché. Le but de notre projet est le développement de formulations efficaces et à faible coût.

Un autre objectif serait de réduire les problèmes de pollution engendrés par les rejets agroindustriels en utilisant les eaux usées provenant de l'industrie d'amidon comme support de croissance pour cette bactérie. En effet, ces rejets sont très abondants dans le monde (Rajbhandari et Annachhatre, 2004). En parallèle, plusieurs travaux ont démontré la capacité de ces rejets à supporter la croissance de plusieurs bactéries comme *Bacillus mucilaginosus, Bacillus thuringiensis et Trichoderma viride* (Deng *et al.*, 2003, Brar *et al.*, 2005, Verma *et al.*, 2007)

Le premier chapitre de cette thèse présente une synthèse bibliographique sur les rhizobiums (le *Sinorhizobium meliloti*) et sur la formulation des bio-inoculants, ainsi que la problématique et les objectifs de cette étude. Les chapitres de 2 à 6 représentent les articles scientifiques issus de ces travaux de doctorat. Le chapitre 7 est une conclusion générale issue de ces travaux.

CHAPITRE 1

SYNTHÈSE

1. Revue de littérature

1.1 Généralités sur les rhizobiums

Les rhizobiums sont des bactéries du sol, aérobies strictes, capables de fixer l'azote atmosphérique. Elles font partie des protéo-bactéries aérobies, des gram-négatives non sporulantes, appartenant à la famille des *Rhizobiaceae*. La plupart de ces bactéries appartiennent à la catégorie des alpha-protéobactéries. Jusqu'aux débuts des années 90, on comptait 4 genres : *Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, et *Sinorhizobium* (Riedacker, 1993). Actuellement, la liste des genres et des espèces s'élargit au fur et à mesure de l'avancement des recherches sur ce sujet. Le développement de nouvelles techniques de caractérisation de l'ADN et l'ARN cellulaire a permis la découverte des genres *Allorhizobium,* et *Mesorhizobium* (Sebbane, 2006).

Les rhizobiums sont aussi classés selon leur vitesse de croissance dans un milieu synthétique à base de glucose et d'extrait de levure. On distingue les rhizobiums à croissance rapide (se caractérisant par un temps de génération inférieur à 6 heures) et les rhizobiums à croissance lente (le temps de génération est supérieur à 6 heures). Les rhizobiums à croissance rapide sont capables de dégrader une large gamme de sucres (fructose, glucose, maltose, ...). Les rhizobiums à croissance lente sont plus spécifiques, ils peuvent utiliser le gluconate et certains sucre-alcools comme le mannitol et le glycérol. Les sources de carbone préférées par les rhizobiums à croissance rapide sont le sucrose et le mannitol, tandis que pour les rhizobiums à croissance lente, la source de carbone préférée est l'arabinose (Stowers, 1985; Lie *et al.*, 1992; Allen *et al.*, 1950)

Certains métaux comme le Zn, le Mn et le Fe se sont avèrés indispensables pour la croissance des rhizobiums (Willson and Reisenauer, 1970). Le calcium favorise la croissance des rhizobiums et améliore leur survie dans les milieux acides (Howieson *et al.*, 1992). L'abscence du Ca dans le milieu conduit à la perte de l'intégrité de la paroi cellulaire (Vincent et Humphrey, 1963). Le nickel est un élément nécessaire pour la synthèse de l'hydrogénase, l'enzyme intervenant dans le cycle de l'hydrogène (Partridge and Yates, 1982). La présence du phosphore est indispensable pour la croissance de plusieurs souches de rhizobium : à des concentrations inférieures à 0.06 µM, certaines souches de *Sinorhizobium meliloti* sont inhibées (Klucas *et al.*, 1983). La présence de soufre dans le milieu est nécessaire pour la croissance de ces bactéries, à des concentrations inférieures à 20 µM, des travaux ont mentionné que la

croissance des *Bradyrhizobium japonicum et* des *Bradyrhizobium sp.* est ralentie (O'Hara *et al.*, 1988). La présence des ions cobalt est nécessaire puisqu'ils interviennent comme composant dans la vitamine B12 chez les *Sinorhizobium meliloti* (Inukai *et al.*, 1977).

Les besoins en vitamines varient en fonction de l'espèce, l'addition de biotine favorise la croissance des rhizobiums à croissance rapide (Allen *et al.*, 1950). La biotine est aussi une source de vitamines satisfaisante pour la croissance de la plupart des rhizobiums à croissance lente (Stowers et Elkan, 1984). La vitamine B12 joue un rôle important dans la multiplication cellulaire (Inukai et al, 1977). Watson *et al.* (2001) en étudiant vingt-sept souches de *Sinorhizobium meliloti* ont démontré que la biotine, le cobalt et la méthionine sont des facteurs de croissance indispensables pour la croissance de toutes les souches étudiées.

Les travaux de Ben Rabah *et al.* (2001) ont montré que l'addition de nutriments (sources d'azote et de vitamines) par l'intermédiaire des extraits de levure (à 0.5, 1,2 et 4g/l), aux boues secondaires de la communauté urbaine de Québec (choisies comme milieu de culture dans ces études) a permis d'améliorer la croissance de *Sinorhizobium meliloti*. L'addition de glycérol à 7.5 g/l a permis d'augmenter la concentration des cellules par un facteur de 1.6. Skinner *et al.* (1977) ont montré que des concentrations en extrait de levure supérieures à 0.35% sont capables de provoquer la distorsion des cellules.

Les rhizobiums à croissance rapide sont des bactéries acidifiantes : une diminution du pH du milieu de culture a été observée à la fin de la fermentation. Les rhizobiums à croissance lente sont alcalinisants, ils sont à l'origine d'une augmentation du pH. Les études relatives à ce sujet ont révélé l'importance de ce facteur surtout si le milieu de culture ne dispose pas de propriété tampon. L'évolution du pH et sa valeur finale sont affectées par la composition du milieu de culture (Stowers and Elkan., 1984; Allen *et al.*, 1950)

Graham *et al.* (1994), partant d'une étude sur 45 souches de *Rhizobium*, *Bradyrhizobium*, et *Mesorhizobium*, et en faisant varier le pH du milieu de croissance entre 4 et 5.5, ont découvert que seuls les : *Rhizobium tropici*, *Mesorhizobium loti*, et *Bradyrhizobium* sp. étaient capables de se proliférer à un pH de 4.5. Ils ont noté également que les *Sinorhizobium meliloti* étaient particulièrement sensibles aux pH acides. Brockwell *et al.* (1991) ont mis aussi en évidence la sensibilité des *Sinorhizobium meliloti* envers les milieux acides. Leurs travaux ont montré que ces bactéries étaient capables de se multiplier pour atteindre 8.9 $x10^4$ cellules par gramme dans des sols à pH supérieur à 7. Mais lorsque le pH était au dessous de 6, leur nombre se limitait à 37 par gramme.

La température de croissance optimale pour les rhizobiums se situe entre 29 et 31°C. Allen *et al.* (1950) ont reporté que le *Sinorhizobium meliloti* est un cas particulier, avec une température optimale de croissance de 35°C. Les travaux Ben Rebah (2001;2002a, b, c, d) et ceux de Dufresne (2004) ont montré que la température 30°C était optimale pour la croissance de plusieurs bactéries rhizobiums, notamment le *Sinorhizobium meliloti.*

1.2 La nodulation

Les rhizobiums sont des bactéries aérobies connues par leur capacité à former des nodules avec les plantes légumineuses (Jordan, 1984). En effet, la survie de ces plantes est sujette à la présence de ces rhizobiums. Les exsudats qu'elles génèrent au niveau de la rhizosphère (sucres, acides aminés et autres...) offrent un environnement adéquat pour la prolifération des rhizobiums. Ces populations bactériennes, profitant de ces sources d'énergie et de nutriments, leurs procurent leurs besoins en azote en conditions défavorables. Un dialogue à l'échelle moléculaire a lieu dans ce cas entre la bactérie et sa plante hôte. La plante commence d'abord par la sécrétion de ses inducteurs propres (iso-flavonoïdes), substances reconnaissables par les rhizobiums qui lui sont compatibles. Bien que chaque plante soit reconnue par une bactérie rhizobium qui lui est spécifique, des chercheurs ont signalé que certaines espèces de rhizobium possèdent la faculté de s'associer avec plusieurs plantes à la fois, cette propriété a été démontrée par exemple chez les *Rhizobium tropici* CIAT899 (Hernandez *et al.*, 1995).

Les bactéries cibles sont ainsi attirées et on assiste à l'expression des gènes de nodulation par la synthèse des facteurs nod (appelés encore LCOs :lipo-chitoseoligosaccharides) (Esseling *et al.*, 2004; Riely *et al.*, 2004; Mulder *et al.*, 2005; Oldroyd *et al.*, 2005). Ces facteurs de nodulation jouent le rôle de signaux entre la bactérie et sa plante hôte (Larouge *et al.*, 1990). Ils sont à l'origine de la mobilisation et l'association de ces microorganismes au niveau des racines.

Les rhizobiums infectent les racines au niveau de l'épiderme à des zones bien déterminées et pénètrent à l'intérieur du cytoplasme. Les cellules végétales se divisent en conséquence aboutissant à la déformation, la courbure, et la ramification des poils absorbants : l'organogenèse des nodules commence à ce stade par la formation de nodules primaires (Long, 1989; Van Rhijn and Vanderleyden, 1995). Les nodules primaires se différencient pour former les nodules matures tandis que les bactéries se regroupent dans des vésicules pour former des bactéroïdes endosymbiotiques. La synthèse de la dinitrogénase, l'enzyme qui catalyse la

réduction de l'azote en ammoniaque, se déclenche au niveau de ces bactéroïdes (Mylona *et al.*, 1995).

1.3 Production de rhizobium dans les rejets industriels et boues d'épuration

Le milieu de croissance couramment utilisé pour la production des rhizobiums est l'YMB (yeast mannitol broth) (Stephens and Rask, 2000; Ahmad and Smith, 1985; Vincent, 1970). Plusieurs travaux ont été réalisés pour évaluer la possibilité de produire les rhizobiums à partir des rejets industriels et des boues d'épuration. Bissonnette *et al.* (1986) ont étudié la production de *Sinorhizobium meliloti* dans le lactosérum (dans les fermenteurs de 5L) : ils ont reporté qu'une durée d'incubation de 48 h a permis d'atteindre des concentrations en cellules de l'ordre de 4.7 10^9UFC/ml. Les rejets d'industries de bière se sont avérés capables de supporter la croissance de *Sinorhizobium meliloti* (en fiole et en fermenteur de 5L) à des concentrations en cellules supérieures à 5.10^9UFC/ml (Boiardi and Ertola, 1985). Des essais de production de *Rhizobium leguminosarum bv. trifolii* dans des milieux à base de cosse de pois et de mélasse (en fermenteurs 25 et 135 l) ont permis d'atteindre des concentrations en cellules supérieures à $1.75x10^{10}$ UFC/ml (Gulati, 1979).

Les travaux de Ben Rebah (2001,2002a, b, c, d, e) et ceux de Dufresne (2004) ont mis en évidence l'intérêt de l'utilisation des boues d'épuration pour la production de plusieurs souches de rhizobiums. Les boues d'épurations primaires (station Black Lake, Québec) étaient capables de supporter la croissance de *Sinorhizobium meliloti*, de *Rhizobium leguminosarum bv. viciae*, et de *Bradhrhizobium japonicum* jusqu'à des concentrations finales en cellules respectivement de l'ordre de 1.12 10^9UFC/ml, 3.10 10^9 UFC/ml, et 0.67 10^9 UFC/ml. Les boues secondaires provenant de la même station d'épuration ont permis d'atteindre une concentration de *Sinorhizobium meliloti* de 2.2 10^9 UFC/ml, de *Rhizobium leguminosarum bv. viciae* de 2.96 10^9 UFC/ml, de *Bradhrhizobium japonicum* de 0.47 10^9 UFC/ml, et de *Bradhrhizobium elkanii* de 0.50 10^9 UFC/ml. Les mélanges des boues d'épuration provenant de la station d'épuration de la ville de Québec (boues d'épuration primaires clarifiées + boues d'épuration secondaire obtenues après bio-filtration) ont permis d'atteindre une concentration en *Sinorhizobium meliloti* de $0.85x10^9$UFC/ml.

1.4 La formulation des inoculants

Beaucoup de progrès ont été observés ces dernières années au niveau du développement des formulations de bioinoculants, et ce pour répondre à la demande croissante en terme de production des légumineuses. En effet, le besoin de conserver des agents biologiques de divers facteurs extérieurs (contamination, dessiccation, déshydratation, rayonnement...) pour des durées assez longues et d'assurer leur survie tout en maintenant leur efficacité, ne peut être atteint par le simple recours aux techniques de conservation classiques (froid, séchage,...).

La formulation est une solution développée pour surmonter ces contraintes. Elle fait appel à l'utilisation de plusieurs ingrédients (additifs) et techniques qui permettent de réaliser les objectifs visés au départ. La qualité d'une formulation de bioinoculants est déterminée à travers plusieurs critères qui doivent être satisfaits. L'efficacité de la souche sélectionnée et son adaptation aux différents procédés utilisés, ainsi que le bon choix des conditions de culture des contribuent en grande partie au maintien de l'efficacité de la formulation (Fages, 1990; Mary *et al.*, 1985; Paul *et al.*, 1993). En parallèle, l'absence de contaminant est un autre critère qui devrait être respecté pour garantir la commercialisation des bioinoculants. La formulation devrait répondre aussi aux normes qui impose un nombre minimal de rhizobiums qui devrait se trouver au moment du semis: il s'agit couramment de 10^3, 10^4 et 10^5 cellules par grain pour respectivement les semences de: petites, moyennes et grandes tailles (Agence Canadienne de l'inspection des aliments ACIA; Lupwayi *et al.*2000).

Actuellement, les formulations se divisent en solides, liquides, et formulations de grains pré-enrobés. En fonction de son mode d'application et de la forme sous la laquelle se présente une formulation, les additifs utilisés varient en quantités ainsi qu'en fonction (anti-dessiccation, cryo-protection...). Dans cette section, nous allons décrire ces différentes formulations ainsi que les additifs utilisés.

1.4.1 Formulation solide

Selon les techniques utilisées, les formulations solides sont classées en poudre et en granules. Dans le présent paragraphe, nous allons décrire les formulations les plus utilisées dans les activités agricoles. Nous tenons à souligner que peu de travaux ont été publiés, où ont décrit les procédés de formulation de rhizobiums, la plupart des recherches dans cette discipline sont brevetées.

Formulation en poudre

Dans les applications agricoles, deux types de poudres peuvent être définis selon le degré d'humidité : les poudres sèches mouillables, et les poudres humides. Les poudres sèches mouillables sont en général appliquées directement sur les grains à ensemencer. L'adhésion de ces poudres aux grains est d'autant meilleure qu'elles soient de faibles tailles. En général, les tailles standard varient de 0.075 à 0.25 mm, une quantité entre 200 et 300 g est utilisée par hectare (Smith, 1997). Les poudres peuvent être aussi mises en suspension dans l'eau ou mélangées avec d'autres vecteurs pour être pulvérisées au niveau du sol (Paau, 1998).

Les poudres sèches mouillables peuvent être obtenues par lyophilisation ou par atomisation des cultures bactériennes, mais pour des applications industrielles à grande échelle, c'est l'atomisation qui est couramment utilisée. Les additifs utilisés sont des dispersants, du support, et des surfactants. D'autres ingrédients comme les stabilisateurs de membranes et les colorants sont généralement ajoutés. Certaines conditions sont exigées au niveau du choix de l'agent de remplissage qui doit être hydrophile et inerte pour assurer une meilleure dissolution et mélange avec l'eau (Paau, 1998; keith *et al.*, 1998). Le rôle du dispersant est de neutraliser les forces d'interaction attractive entre les différentes particules afin d'assurer une uniformité lors de la dissolution dans l'eau. L'addition de l'agent de remplissage a pour but d'éviter les agglomérations des particules hydrophobes pendant le stockage ce qui pourrait bien réduire la mouillabilité, l'ajout de l'agent de remplissage assure aussi la fluidité lors des mélanges au cours de la préparation et l'utilisation.

Le surfactant a pour rôle de faciliter le mélange des ingrédients avec l'eau ou les autres liquides ajoutés avant le séchage. Un agent de mouillage peut être incorporé pour permettre aux poudres l'absorption de l'humidité une fois introduites au sol, ce qui va faciliter l'activation des rhizobiums. L'utilisation du support est essentielle vu la protection qu'il garantit à la biomasse inoculante pendant et avant le séchage, en particulier contre la formation de croûte et d'agrégats (Paau, 1998; Burges *et al.*, 1998).

Les poudres peuvent être obtenues autrement en partant des granules mouillables ou des agrégats de grandes tailles. Le broyage permet ainsi d'obtenir une poudre fine. Cependant, cette opération mécanique n'est pas très souhaitable pour les bactéries, sensibles à de telle force. Les formulations en poudres humides sont réalisables pour les cultures des microorganismes non filamenteux, cette propriété permet la récolte des biomasses qui peuvent être mélangées avec différents ingrédients pour donner des produits applicables, soit

pulvérisées, pour un mélange avec les graines de semence, ou encore pour une décharge directe au sol. (Paau, 1998).

Formulation en granules

La granulation est l'opération par laquelle des particules (poudres) sont agglomérées pour former des entités plus grandes. Généralement, un dispositif de pression est utilisé ce qui permet d'exercer une force de compression sur les particules afin d'assurer leur coalescence et transformation en granules. Elle est utilisée dans plusieurs applications, en particulier dans l'industrie pharmaceutique pour la préparation des tablettes. L'objectif de ce procédé est d'accroitre la taille des particules, de les agglomérer, ce qui permet de faciliter l'écoulement des produits tout en améliorant leur dissolution et leur densité (Tardos, 2001). Dans l'agriculture, ce genre de formulation a été développé pour les inoculants depuis les années soixante et a commencé à être appliqué depuis (Brockwell *et al.*, 1980).

La granulation commence par l'introduction de l'agent d'adhésion et de son mélange avec le reste des ingrédients (le support et les bactéries). L'ajout direct de la totalité de l'agent d'adhésion peut être aussi réalisé. La cohésion est assurée par l'intervention des forces de capillarité et des forces de viscosité entre les particules solides et l'agent d'adhésion. La deuxième phase consiste en un brassage mouillant, au cours de laquelle les particules augmentent de taille, s'accolent, s'agglomèrent et se consolident pour donner des granules.

Au niveau industriel, deux types de granules peuvent être définis selon le degré d'humidité : sec mouillable et humide. La granulation sèche mouillable peut être réalisée avec l'utilisation soit d'un lit fluidisant, un mélangeur à tambour, ou un mixeur à haute vitesse. L'application de ces techniques de transformation de poudre en granules humides est limitée par la fragilité des bactéries et leur possible endommagement suite à une agitation mécanique prononcée comme le cas dans ce procédé. La taille des granules varie généralement de 0.35 à 1.18 mm (Bashan, 1998).

1.4.2 Formulation liquide

Une formulation liquide est définie comme étant une suspension ou une émulsion. Dans le cas d'une suspension, les rhizobiums sont dispersés dans l'eau, un agent de suspension ou un dispersant est généralement ajouté. Dans le cas d'une émulsion, les rhizobiums sont dispersés dans l'huile : ce mélange est stabilisé par ajout d'agents émulsifiants.

Les formulations liquide par émulsion ont pour avantage de réduire les problèmes liés à la sédimentation des particules pendant le stockage (Keith *et al.*, 1998). Les huiles d'arachides et de soja se sont avérées capables de protéger les *Bradyrhizobium japonicum* une fois enrobés sur les grains de semence à des températures de 4, 28 et 34°C (Hoben *et al.*, 1991).

Les inoculants liquides sont utilisés pour l'enrobage des grains de semences au semis ou appliqués directement au niveau du sol par pulvérisation. L'intérêt de cette technique de formulation revient en grande partie à sa simplicité si on la compare aux formulations solides : elle ne fait pas intervenir plusieurs procédés au cours de sa préparation. Concernant le mode d'application, l'administration directe des inoculants liquides au niveau des sillons offre de meilleures opportunités pour la survie des rhizobiums si on la compare par rapport aux autres techniques (Smith, 1995). Les effets relatifs aux pesticides et fongicides sont évités ainsi avec ce mode d'application (Deaker *et al.*, 2004).

Cependant, plusieurs critiques ont été reportées à cette technique de formulation, en effet, bien qu'elle soit simple en terme d'application et de préparation, la survie des rhizobiums est jugée faible vu l'absence de protection contre les effets des facteurs environnementaux après enrobage sur les grains (température, dessiccation). Les bactéries sont exposées à plusieurs facteurs qui affectent leur nombre et leur efficacité (Yoav, 1998; Singleton *et al.*, 2002; Tittabutr *et al.*, 2007).

La microencapsulation est une nouvelle alternative pour la formulation des rhizobiums qui peut être inscrite sous la catégorie des bionoculants liquides. Il s'agit d'un procédé par lequel l'élément actif (les bactéries) est entouré par un film continu de polymères. Peu de travaux ont été consacrés à l'évaluation de la faisabilité de cette nouvelle technique pour la formulation des bio-inoculants. La limitation de l'application de cette technologie est liée principalement à son coût élevé.

Les objectifs généraux de la microencapsulation sont compatibles avec la formulation des rhizobiums puisqu'on vise toujours la protection, et la stabilisation de ces bactéries. Bashan (1998) a spécifié que deux points importants sont à respecter pour la formulation par microencapsulation. Premièrement, la formulation devrait protéger temporairement les rhizobiums des différents facteurs qui peuvent toucher à leur survie une fois ensemencés dans le sol (acidité, compétition par les autres bactéries). L'autre point concerne la libération progressive des rhizobiums vers les racines pour commencer la nodulation. Le polymère le plus couramment utilisé pour la microencapsulation des microorganismes est l'alginate, polymère

biodégradable qui garantit une libération progressive des bactéries dans le sol (Bashan, 1998, Young *et al.*, 2006).

1.4.3 Inoculation des grains de semence

L'enrobage des grains permet d'acheminer les rhizobiums directement aux racines des plantes. Avec cette application, il est possible d'ensemencer les grains avec les bactéries tout en limitant les effets liés aux facteurs environnementaux (Mark *et al.*, 1998). Vu sa simplicité, cette pratique est largement utilisée par les agriculteurs: les compagnies offrent depuis plusieurs années sur le marché des semences pré-inoculés, ce qui évite à l'agriculteur de procéder à l'inoculation de ses semences. Le principe consiste à mélanger les grains de semence avec les bactéries en suspension et ensuite à laisser sécher le mélange. Les rhizobiums seront en contact avec les grains et par suite avec les racines.

Mark *et al.*(1998) ont décrit deux procédures différentes : l'enrobage simple, et l'enrobage par granulation des grains de semences. L'enrobage simple consiste à mettre en solution un agent adhésif auquel on ajoute les grains, le support et les rhizobiums, le mélange est ensuite agité jusqu'à l'apparition d'une couche enrobant les grains, un séchage à froid est réalisé après pour permettre l'obtention à la fin des grains enrobés. La granulation des grains de semence est réalisée de la même façon sauf que les grains sont enroulés après par un film fin, ceci étant suite à l'ajout dés le départ de carbonate de calcium, du phosphate naturel ou de l'argile. Une autre méthode est aussi appliquée, et qui consiste à ajouter les inoculants en poudres ou en microgranules déjà formulés aux grains de semences.

Certaines critiques ont été reportées concernant la faible survie des bactéries avec ce mode de formulation (Salema *et al.*, 1982; Gemmell *et al.*, 2002). La diminution de la population des flores inoculantes a été observée surtout si les conditions environnementales sont non favorables (Brockwell *et al.*, 1987). En plus, les exsudats de certaines variétés de légumineuses peuvent être d'une certaine toxicité pour les rhizobiums, ce qui contribue d'avantage à la réduction des activités nodulantes de ces bactéries (Materon and Weaver, 1984). Le séchage des grains enrobés ainsi que l'addition de fertilisant lors des semences contribuent également à la réduction de la flore inoculante (Salema *et al.*, 1982; Kremer *et al.*, 1982). Il en est de même à propos de l'emploi des insecticides et des fongicides sur les grains avant l'enrobage (Mark *et al.*, 1998).

1.4.4 Additifs relatifs à la formulation

Le recours à l'addition de divers additifs est indispensable pour garantir la qualité du bio-inoculant, notamment avec la multitude des facteurs qui touchent à la survie des bactéries au cours de la conservation et de la formulation.

Support pour les formulations solides

Le support est la fraction volumique la plus importante qui intervient dans la constitution d'un inoculant. Il permet la protection des rhizobiums durant la période de conservation : cette protection leur garantit un milieu de survie même après l'ensemencement dans le sol. Ceci étant en réduisant l'influence des variations au niveau de la température et de l'humidité du milieu extérieur. Le support doit répondre aussi à certaines exigences relatives aux procédés utilisés et aux besoins des bactéries inoculées. Le choix du support repose principalement sur son aptitude à maintenir en vie et en bon état le maximum de bactéries inoculantes (Bashan, 1986, 1991 ; Fages, 1990; Smith, 1992; Trevors, 1992). Un support dédié pour une production à l'échelle industrielle devrait avoir une bonne capacité de rétention d'eau en plus de la stabilité et l'uniformité au niveau de ses propriétés chimiques et physiques. Il devrait être exempt de tout composé toxique qui peut mettre en cause la survie des rhizobiums ou qui peut être source de pollution pour le sol. Vu que le support est la fraction volumique la plus importante, il devrait permettre l'obtention d'inoculant facilement maniable dans les activités agricoles, qui s'adaptent aux techniques courantes utilisées par les agriculteurs (Yoav, 1998).

Le support choisi devrait avoir aussi un pH qui se situe prés de la zone de neutralité ou facilement ajustable. Une adaptabilité pour tous les rhizobiums est exigée avec la disponibilité et le faible coût (Stephens and Rask, 2000; Ferreira and Castro, 2005).

Les supports utilisés jusqu'à ces jours sont diverses et multiples. À ce sujet, Yoav (1998) a proposé une classification en se basant sur leurs origines, c'est ainsi qu'il les a subdivisés en quatre grandes classes. Il a rassemblé la tourbe, le charbon et l'argile sous la même classe : les supports dont l'origine est le sol. La deuxième classe l'a attribuée aux matériaux inertes (la vermiculite, la perlite, l'alginate, le sulfate calcium, le phosphate naturel, et les gels de polyacrylamide). La troisième classe concerne les cultures microbiennes lyophilisées, la quatrième classe englobe les effluents des industries de transformation, ou la matière première est végétale.

La tourbe est le support de choix pour les rhizobiums, elle a été largement utilisée et étudiée pour la formulation des PGPR (Plant Growth Promoting Rhizobacteria) et des agents de biocontrôle (Williams, 1984; Vidhyasekaran and Muthamilan, 1995; Kishore *et al.*, 2005; Albareda *et al.*, 2008).

Divers autres supports ont été testés, Albareda *et al.* (2008) ont étudié les supports à base de fibres de canne à sucre, de fibres de liège, de l'attapulgite, de la sépiolite, de la perlite et du silicate amorphe : ils ont observés que la survie des rhizobiums était comparable pour tous ces différents supports avec la tourbe inoculée.

D'autres travaux réalisés par Ferreira and Castro. (2005) ont montré que les supports à base de des rejets de l'industrie du liège permettaient de maintenir des densités de rhizobiums assez importantes, ces supports étaient pratique pour les manipulations agricoles, de forte capacité de rétention d'eau, et sans aucune toxicité.

Daza *et al.* (2000) ont réalisé une comparaison entre les inoculants formulés à base de perlite et de tourbe, les résultats ont montré que le nombre de nodules formés, les teneurs en matières sèches des nodules, les rendements des récoltes et les teneurs en azote étaient comparables.

D'autres supports ont été élaborés à partir des mélanges de perlite à 25%, avec soit de la cosse de riz, des résidus de malt, des résidus des canne à sucre, ou du charbon. La survie des rhizobiums (dans cette étude, la souche choisie était *Bradyrhizobium japonicium* CB 1809) a été maintenue à des concentrations supérieures à $1x10^9$ UFC/g pour une période de conservation de 6 mois, à 4°C, sauf pour les supports contenant de la cosse de riz ($5x10^8$ UFC/g) (Khavazi, *et al.*, 2007).

Les boues d'épuration ont été étudiées dans le but de remédier aux problèmes de pollution et en même temps, trouver un support disponible et à faible coût. Ben Rebah *et al.* (2002) ont mis en évidence l'intérêt de cette alternative en comparant des supports à base de tourbe avec de la boue déshydratée, et avec des supports formulés à partir du mélange des deux (boues déshydratées + tourbe). Au cours de la conservation à -20 C, les boues désydratées ont garanti la meilleure survie. Les formulations inoculées à base de tourbe et de boues étaient comparables lorsqu'elles ont été conservées à 4°C et 25°C.

Le son de blé a été également étudié. Mohamed *et al.* (2001) ont évalué son efficacité dans la formulation des inoculants, la comparaison a été faite avec de la tourbe et des fibres de canne à sucre. Les inoculants utilisés étaient des mélanges de *Bradyrhizobium* + *Aspergillus niger.*

Les meilleurs résultats (en termes de viabilité) ont été obtenus avec, successivement, la tourbe, le son de blé, et les fibres de cane à sucre.

D'autres supports à base de granules de bentonite, d'ilite, de smectite, et du silica ont été élaborés. L'ajout du glycérol et du glutamate à ces supports a permis d'améliorer la survie des cellules, et les teneurs en azote dans les plantes de soja (Fouilleux.*et al.*, 1996). Le talc a été également étudié et proposé par Kloepper and Schroth (1981) comme un support efficace pour la formulation des rhizobiums.

Les osmo-protectants, agent anti-dessiccation

La dessiccation est le phénomène qui affecte le plus la survie des rhizobiums. Il a été démontré que les cellules sont capables de résister aux endommagements dus à la dessiccation si elles parviennent à accumuler des substances capables de maintenir un équilibre osmotique de part et d'autres de la membranes cellulaire. La liste des osmoprotectants comporte : le glutamate, la glutamine, la proline, les amines quaternaires (glycine et bétaine), les sucres comme le tréhalose, le saccharose, glucosylglycerol; et les ions potassium (Deaker *et al.*, 2004).

Les agents adhésifs

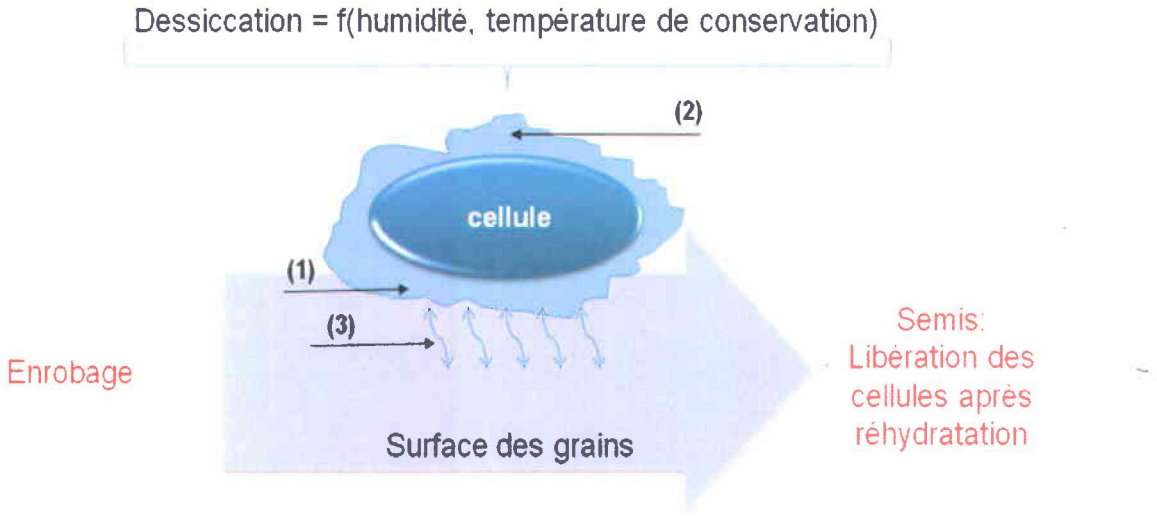
Les agents adhésifs sont utilisés pour assurer l'agglomération des particules solides. Il s'agit de polymères à haut poids moléculaires solubles dans l'eau. Ces polymères sont généralement ajoutés sous forme de solution au cours de la formulation. L'effet liant de ces additifs disparait une fois les inoculants sont ensemencés grâce à l'humidité du sol, pour permettre un meilleur contact des rhizobiums avec les racines (Paau, 1998; Deaker *et al.*, 2004). Les agents adhésifs sont également ajoutés au cours de l'enrobage des grains de semence, ces additifs ont pour rôle de protéger les rhizobiums contre la dessiccation une fois enrobés sur les grains de semences et assurer leurs adhésions au cours des manipulations ultérieures (Fig 1.1).

Plusieurs travaux ont été réalisés dans le but de sélectionner des agents adhésifs efficaces pour la formulation des inoculants :

- La gomme arabique : extraite de la plante *Acacia*, elle est largement utilisée dans l'enrobage des grains. L'utilisation de la gomme arabique a permis d'améliorer la survie des rhizobiums, principalement grâce à la protection qu'elle permet aux cellules contre la dessiccation (Vincent *et al.*, 1962).
- Le méthylcellulose, les sels de caséinate, le polyvinyl-acétate, la gélatine, la gomme de l'acacia, les dextrines, et les hydroxyropylmethylcelluloses sont également utilisés

comme des liants. Certains supports comme les alginates peuvent jouer ce rôle grâce à leurs structures (Paau, 1998).

- le pyrollivinyl-pyrrolidone et le polyvinyl alcohol ont été proposés vu leur capacité de rétention d'eau et leur stabilité (Deaker *et al.*, 2004; 2007)
- Les co-polymères de vinyl-pyrrolidone se sont avérés capables de garantir la protection des *Sinorhizobium meliloti* une fois enrobés sur les grains de semence (Williams, 1992).
- Brockwell (1977) a noté que l'utilisation des sucres est prometteuse pour l'enrobage grâce à leurs fortes capacités de rétention d'eau. Cependant, les risques de contamination par les autres bactéries sont augmentés dans ce cas. L'efficacité des huiles de soja et d'arachides s'est avérée comparable à celle de la gomme arabique en termes d'adhésion et aussi de protection contre la dessiccation pour le cas de *Bradyrhizobium japonicum* (Hoben *et al.*, 1991).

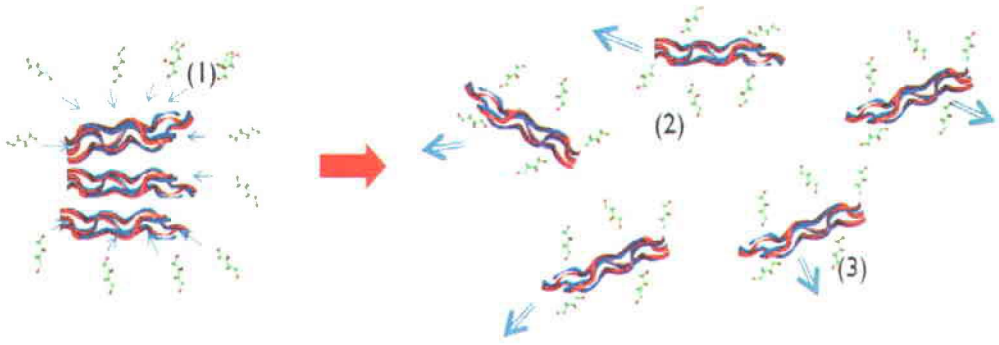


(1) Adhésion des cellules à la surface des grains
(2) Agent d'adhésion
(3) exsudats des grains

Figure 2.1 Mode d'action des agents adhésifs

Les agents de suspensions

Les agents de suspensions sont utilisés généralement dans les formulations liquides, mais ils peuvent être aussi incorporés durant l'enrobage pour homogénéiser la suspension formée et faciliter le mélanger avec les grains de semences. Ces additifs sont ajoutés pour maintenir les différents ingrédients de la formulation en état de suspension et éviter la sédimentation à cause de l'effet de pesanteur. Le principe d'action des additifs se résume au niveau de la réduction des forces attractives existantes entre les particules, ce qui se traduit en conséquence par l'augmentation des forces répulsives et de la distance inter-particulaire. Les agents de suspensions les plus couramment utilisés dans la formulation sont soit des sucres simples, sorbitol, gomme de xanthane (Yoav, 1998) (Fig 1.2)



(1) addition de l'agent de suspension
(2) Augmentation de la distance inter-particulaire
(3) Augmentation des forces répulsives inter-particulaire

Figure1.2 mode d'action des agents de suspension

Les cryoprotecteurs

Les cryo-protecteurs sont utilisés pour protéger les bactéries contre les endommagements dus aux abaissements de la température, notamment au cours de la lyophilisation et la congélation. Les disaccharides sont capables de protéger les cellules non seulement au cours de la lyophilisation mais aussi au cours du séchage. Leur mode d'action se résume au niveau de la stabilisation des membranes cellulaires (Crowe *et al.*, 1988). Le tréhalose est un agent de

protection efficace, vu sa faible température de transition vitreuse, sa présence dans le milieu réduit la formation des cristaux de glace. L'utilisation du tréhalose permet en conséquence de réduire les endommagements des cellules suite à la lyophilisation (Miller *et al.*, 1998). Le lait écrémé et les sucres alcools comme le mannitol, le sorbitol, et le glycérol sont également utilises comme des cryo-protecteurs. Plusieurs polymères comme l'éthylène glycol, le propylène glycol, le polyvinylpyrrolidone, le polyéthylène glycol, l'oxyde de polyéthylène et le polyvinyl alcool ont des propriétés cryo-protectrices des microorganismes. (Hubalek, 2003).

Tableau 2.1Tableau récapitulatif des différents additifs utilisés dans la formulation des bioinoculants

additifs	Mode d'action	Exemples
Supports solides	• Protection des rhizobiums durant la période de conservation. • Réduit l'influence des variations au niveau de la température et de l'humidité du milieu extérieur sur les cellules.	• La tourbe la vermiculite, la perlite, l'alginate, le sulfate de calcium, le phosphate naturel, gels de polyacrylamide (Yoav, 1998). • fibres de cannes à sucre, fibres de liège, de l'attapulgite, de la sépiolite, de la perlite et du silicate amorphe (Albareda *et al.*, 2008) • boues d'épuration (Ben Rebah *et al.*, 2002) • granules de bentonite, d'ilite, de smectite, et du silica (Fouilleux.*et al.*, 1996)
Adhésifs	• Assurer l'agglomération des particules solides • L'effet liant de ces additifs disparait une fois les inoculants sont ensemencés grâce à l'humidité du sol, pour permettre un meilleur contact des rhizobiums avec les racines • Protéger les rhizobiums contre la dessiccation une fois enrobés sur les grains de semences et assurer leurs adhésions au cours des manipulations ultérieures.	• La gomme arabique (Vincent *et al.*, 1962). • Le méthylcellulose, les sels de caséinate, le polyvinyl-acétate, la gélatine, la gomme de l'acacia, les dextrines, et les hydroxyropylmethylcelluloses (Paau, 1998). • le pyrollivinyl-pyrrolidone et le polyvinyl alcohol (Deaker *et al.*, 2004; 2007) • Les co-polymères de vinyl-pyrrolidone (Williams, 1992). • Les sucres (Hoben *et al.*, 1991).
Agents de Suspension	• Maintenir les différents ingrédients de la formulation en suspension (éviter la décantation ou la floculation)	• Sorbitol, sucrose, gomme de xanthane, gomme arabiqu (Yoav, 1998).
Cryo-protecteurs	• Protéger les bactéries contre les endommagements dus aux abaissements de la température par Stabilisation des membranes cellulaires	• Polyéthylène glycol, propylène glycol, polyvinylpyrrolidone, polyéthylène glycol, l'oxyde de polyéthylène et le polyvinyle alcool (Hubalek, 2003).
Osmo-protectants, agents anti-dessiccation	• Maintenir un équilibre osmotique de part et d'autres de la membrane cellulaire.	• Glutamate, la glutamine, la proline, les amines quaternaires (glycine et bétaine), les sucres comme le tréhalose, le saccharose, glucosylglycerol; et les ions potassium (Deaker *et al.*, 2004).

1.5 Procédé en amont de la formulation

1.5.1 La centrifugation

La centrifugation est l'opération unitaire de choix qui permet la concentration des cellules et la réduction des volumes de bouillon fermenté, avec le minimum d'endommagement des cellules. La centrifugation s'est avérée capable d'augmenter la concentration des cellules de *Sinorhizobium meliloti* plus de 20 fois (Rouissi *et al.*, 2010). La plupart des milieux de culture utilisés et jugés efficaces, qu'il s'agisse du milieu synthétique (YMB) ou de rejets étudiés dans la littérature permettent d'atteindre des concentrations en cellules maximales autour de 6.10^9UFC/ml pour le *Sinorhizobium meliloti* (Ben Rabah *et al.*, 2007). L'efficacité d'une formulation est dépendante de sa concentration en cellules viables : une concentration initiale élevée en cellules augmente la durée de vie des produits sur le marché.

1.5.2 La lyophilisation

La lyophilisation est une technique de séchage qui permet d'obtenir des poudres concentrées en cellules. En se basant sur le principe de la sublimation, elle permet la déshydratation et la dessiccation sous vide à basse température. Grâce à la transition de l'état congelé à l'état déshydraté, les altérations généralement observées au cours des procédés de séchage classique sont réduites. L'utilisation des cryo-protecteurs au cours de ce procédé améliore la survie des rhizobiums et garantit une meilleure stabilité au cours de la conservation. La lyophilisation est décrite comme étant une alternative à coût abordable et facile à réaliser (Morgan *et al.*, 2006; Bozoglu *et al.*, 1987), mais non pratique pour une production à grande échelle.

1.5.3 L'atomisation

Le spray-drying ou encore atomisation est une technique de séchage qui permet d'obtenir des poudres de culture bactérienne (Kim *et al.*,1990; To *et al.*,1997 ;Wen *et al.*,2002). C'est la

méthode la plus utilisée pour les applications à l'échelle industrielle. Le principe consiste à pulvériser une suspension de cellules sous un certain débit à un flux d'air chaud. L'optimisation des températures et la pression de l'air chaud, du débit de la suspension à sécher et de diamètre de l'orifice de pulvérisation est indispensable pour maximiser la récolte des cellules viables. Les conditions de conservation et la réhydratation des poudres de cellules sont des facteurs importants qui peuvent affecter la stabilité des formulations ultérieures.

1.6 Conservation des formulations

La plupart des inoculants sont conservés à la température de réfrigération (4°C). Cependant, cette condition n'est pas toujours pratique pour les grains pré-inoculés au cours des manipulation dans les fermes (Deaker *et al.*, 2004). En plus de la température, l'humidité relative de l'air et la présence d'oxygène dans le milieu entourant les cellules affectent la survie des rhizobiums au cours de la conservation. Il s'est avéré que les bactéries à croissance lente tolèrent mieux les conditions extrêmes que les rhizobiums à croissance rapide (Mary *et al.*, 1998). La présence d'oxygène devient toxique lorsque l'humidité relative descend au dessous de 70%, ce qui provoque l'endommagement des protéines et des membranes cellulaires (Deaker *et al.*, 2004; Potts,1994).

La dessiccation est le phénomène qui affectent le plus la viabilité des cellules au cours de la conservation, en particulier dans le cas des formulations de grains enrobés et des formulations solides. Deux phases distinctes peuvent être décrites au cours de ce phénomène: une première déshydratation rapide ou autrement dit réduction de la teneur en eau est observée. La plus grande fraction de l'eau libre se trouvant dans les cellules est évaporée : on observe une diminution rapide du nombre des cellules viables à ce niveau. Dans la deuxième phase, la déshydratation continue des cellules conduit à l'état de dessiccation, la viabilité des cellules diminue en fonction des pertes d'eau, de l'humidité relative du milieu et de la présence d'agents anti-dessiccation (Deaker *et al.*, 2004).

1.7 Qualité d'une formulation et co-inoculation *Rhizobium-Azospirillum*

La co-inoculation des rhizobiums avec les bactéries *Azospirillum* est une nouvelle alternative qui permet d'améliorer la qualité des produits (formulation des rhizobiums) (Bashaln, 1990). La

coexistence de ces deux bactéries (*Rhizobium-Azospirillum*) au niveau des racines des plantes a été reportée bénéfique dans plusieurs travaux. Bashan *et al.*(2004) a énuméré plus d'une centaine de plantes auxquelles l'application des bactéries *Azospirillum* s'est avérée intéressante. Les études relatives aux légumineuses ont démontré que l'association *Rhizobium-Azospirillum* améliore la nodulation et augmente la croissance des plantes pour la luzerne (Hassouna *et al.*, 1994; Itzigsohn *et al.*,1990), le pois chiche (Parmar et Dadarwal,1999), le soya (Iruthayathas *et al.*, 1983) et le haricot (Burdman *et al.*,1997).

Les premières espèces d'*Azospirillum* ont été isolées pour la première fois par *Beijerinck* au Pays-Bas en 1925. Elles sont couramment décrites comme étant les bactéries les plus prédominantes dans la rhizosphère des plantes oléagineuses tropicales, céréales, et légumineuses (Bahsan, 1990). Les bactéries du genre *Azospirillum* sont des protéo-bactéries, diazotrophes, aérobies, gram-négatifs, parfois capable de fixer l'azote sous forme libre (Cohen *et al.*, 2008; Okon *et al.*, 1983). Le nombre des espèces identifiées de ce genre ne cesse de croître, jusqu'à la dernière étude publiée à ce sujet, on comptait 9 espèces : il s'agit de : *Azospirillum lipoferum, Azospirillum brasilense, Azospirillum amazonense, Azospirillum halopraeferens, Azospirillum irakense, Azospirillum largimobile, Azospirillum doebereinerae* et *Azospirillum oryzae* (Mehnaz *et al.*, 2007). Ces bactéries sont capables d'utiliser plusieurs sources de carbone qui se trouvent dans le sol, mais leur métabolisme varie selon l'espèce en question. À titre d'exemple, l'*Azospirillum brasilense* dégrade préférentiellement les acides organiques comme source principale de carbone, à savoir l'acide malique, l'acide succinique, gluconique, lactique et α-ketoglutarique (Döbereiner and Day 1976). *Azospirillum lipoferum* est capable de croitre en utilisant le glucose ou le fructose comme source de carbone (Goebel and Krieg, 1984). Vu que les milieux de culture utilisés pour la production des *Azospirillums* à l'échelle industrielle sont brevetés, peu de travaux sont publiés concernant la problématique de la culture et récolte de ces bactéries. Le milieu de croissance le plus utilisé est le NFB « nitrogen free media » (Bashan *et al.*, 1993; Bashan *et al.*, 2004), la plupart des cas modifié par ajout de supplément comme l'extrait de levure, le NH_4Cl ...etc. (Dobereiner and Pedrosa, 1987). De récents travaux de Bashan and de-Bashan (2011) ont permis de développer deux nouveaux milieux efficaces pour la culture d'*Azospirillum.*

1.7.1 Avantage de la co-inoculation *Rhizobium-Azospirillum*

Vu leur faculté à améliorer la croissance de plusieurs plantes, les bactéries *Azospirillum* sont décrites comme étant les inoculants universels (Bahsan and de-Bashan, 2010). Elles

interagissent avec les plantes à travers la sécrétion de plusieurs hormones (principalement l'acide indole-3- acétique et les gibbérellines), cytokinines, éthylène, polyamines et acides aminés. Ces hormones stimulent la croissance des plantes à travers l'altération de leurs métabolisme et morphologie (Spaepen *et al.*, 2007; Bottini *et al.*, 2004). En termes de morphologie, la prolifération améliorée des racines a été observée autour des zones qui sont physiologiquement actives pour l'absorption de l'eau et des nutriments dans plusieurs cultures à savoir le maïs, la tomate, le sorgo, l'orge, et le mil (Dobbelaere and Okon, 2007). Ces développements racinaires se traduisent par l'augmentation de la densité, du nombre et de la longueur des racines formées (Dobbelaere *et al.*, 1999), par le raccourcissement des durées de leur apparition (Okon and Kapulnik, 1986) et par l'expansion latérale des racines (Barbieri *et al.*, 1988). L'inoculation des plantes avec l'*Azospirillum* augmente leur capacité d'absorption de l'eau et de nutriments, leurs résistances aux stress de manque d'eau et d'excès de sel, et contribue à la solubilisation du phosphate dans le sol (Bahsan and de-Bashan, 2010).

La fixation d'azote est aussi un autre mécanisme par lequel les *Azospirillums* peuvent améliorer la croissance des plantes, ce qui contribue à la réduction des quantités de fertilisants chimiques appliqués. Ce fait a été mis en évidence dans plusieurs tests suite à l'augmentation de l'activité de la nitrogénase au niveau des racines (Bashan and Holguin, 1997; Doroshenko *et al.*, 2007; Eckert *et al.*, 2001). Plusieurs critiques ont été reportées à ce sujet « controversé » vu que d'autres essais n'ont pas aboutit à la même évidence. Bashan et de-Bashan (2010) confirment que même si la quantité d'azotes fixée est faible, l'association des divers mécanismes à travers lesquels les *Azospirillums* interagissent avec les plantes est capable d'améliorer leur croissance.

1.7.2 La nodulation et la co-inoculation

La fixation des rhizobiums au niveau des racines est favorisée par la co-inoculation : l'application des bactéries *Azospirillum* est responsable du développement du système racinaire ce qui augmente le nombre et la densité des sites susceptibles à la nodulation. En plus, les bactéries *Azospirillum* sont responsables du déclenchement du processus de sécrétion des flavonoïdes par les plantes, ce qui stimule l'infection des racines par les rhizobiums et favorise ses premiers stades (Iruthayathas *et al.*, 1983). Cet effet a été observé dans le cas de l'haricot inoculé par le *Rhizobium elti* (Dobbelaere et Okon, 2007). La nodulation précoce ainsi que l'augmentation du nombre total des nodules ont été observées lorsque l'*Azospirillum* a été coinoculé avec les rhizobiums dans le cas de : la luzerne, le soja, le pois chiche et le trèfle en

comparaison avec l'inoculation avec les rhizobiums seuls (Singh and Subba Rao, 1979; Plazinski *et al.*, 1984; Burdman *et al.*, 1997; Yahalom *et al.*,1990). Suite à la co-inoculation, la distribution des nodules au niveau des racines a été observée aussi changée: les nodules sont plus répartis au niveau des branchements racinaires latéraux, les plantes inoculées avec les rhizobiums seuls ont une grande densité de nodules au niveau de la racine principale (Iruthayathas *et al.*, 1983).

2. Problématique

Problématique de traitement et valorisation des rejets agroindustriels

Les rejets agroindustriels sont une menace continue pour l'environnement : à titre indicatif chaque année, 3.5 millions de tonnes de céréales sont transformées au Canada[1], une grande partie est destinée pour l'extraction en amidon. La valorisation de ces rejets et leur exploitation pour l'élaboration des produits de haute valeur ne peut avoir un impact direct positif sur la problématique de gestion des rejets et l'environnement que si :

- De grands marchés de consommation y seront directement concernés.
- Les procédés utilisés pour l'élaboration de ces produits sont extrapolables à l'échelle industrielle,
- La technologie développée est simple et permet d'assurer une rentabilité économique.
- Les produits développés sont efficaces, stables au cours de la conservation et simples lors de l'utilisation.

En effet, bien que plusieurs travaux ont démontré la faisabilité de l'utilisation de plusieurs rejets comme milieux de culture pour certains champignons et bactéries, ou pour l'extraction de certaines molécules. La formulation du produit fini demeure toujours un souci à considérer.

Problématique de bioinoculants commercialisés

Le *Sinorhizobium meliloti* est la bactérie qui nodule la luzerne : une des plantes légumineuses les plus cultivées au Canada. La luzerne intervient comme ingrédient principal dans l'alimentation du bétail, en particulier les bovins. Les bioinoculants associés et qui se trouvent sur le marché sont soit de la tourbe inoculée, des suspensions liquides ou des grains pré-enrobés. Ces produits sont développés à partir des cellules cultivées dans des milieux synthétiques, la pluspart des cas, du YMB (Vincent, 1970). Par conséquence, le coût de production et la disponibilité de la tourbe sont les principaux problèmes auxquels le développement de l'industrie des bioinoculants dans plusieurs pays fait face.

[1] http://investiraucanada.gc.ca/fra/publications/grain.aspx

3. Hypothèses, objectifs et originalité

3.1 Hypothèse de travail

L'objectif principal de cette étude de recherche, est le développement de formulations de *Sinorhizobium meliloti*, cultivé dans les eaux usées d'industrie d'amidon. L'optimisation des conditions de croissance a été déjà réalisée grâce aux travaux de Khan *et al.* (2008)(rapport confidentiel) qui ont montré la capacité de ces rejets à supporter la croissance de cette bactérie : l'utilisation des rejets des industries d'amidon est donc une alternative prometteuse pour le développement de formulation de *Sinorhizobium meliloti*. Ces rejets étaient capables de supporter la croissance de ces bactéries à des concentrations qui dépassent les 10^9UFC/ml, sans traitements préalables, ce qui est comparable à celles obtenues avec le milieu standard (YMB). Sachant que le coût engendré par les substrats dans la production conventionnelle représente 35 à 59% du coût total de production (Tirado-Montiel *et al.*, 1998 ; Sachveda *et al.*, 1999). On propose de développer de ce fait des formulations à faible coût (autour de 50% moins que les formulations standards), ce qui contribuera d'avantage à l'utilisation des biofertilisants comme alternative aux engrais chimiques, à la valorisation des rejets industriels, et à la réduction des volumes traités par les stations d'épuration.

Originalité globale : *développement des formulations nouvelles et efficaces de rhizobiums à partir des rejets agroalimentaires*.

3.2 Objectifs et originalité

1ier objectif

La récolte des bactéries par centrifugation est une opération clef dans l'acheminement des formulations. Le bon choix des paramètres relatifs à ce procédé permet de maximiser la récolte des cellules, et ainsi la possibilité d'augmenter les durées de conservations des formulations, c'est pourquoi on a procédé à cette optimisation en utilisant la méthode de surface de réponse.

- Originalité : c'est la première fois qu'on présente une optimisation de la centrifugation en utilisant la méthode de réponse de surface pour la récolte des *Sinorhizobium meliloti*. Cette approche peut être appliquée à toute autre bactérie cultivée dans des rejets industriels.

2ième objectif

Le deuxième objectif de ce travail a porté sur le développement des formulations en suspension. En effet, indépendamment de la formulation utilisée, qu'il s'agisse des formulations en poudres, ou liquides, on aura toujours recours à la mise en suspension pour l'enrobage des grains de semences à la fin. Le bon choix des agents de suspension et des agents adhésifs va permettre d'augmenter l'efficacité des formulations qui seront développées.

Pour la réalisation de cet objectif, nous avons utilisé le polyvinyle pyrrolidone (10000), le polyéthylène glycol(8000), l'alginate de sodium, le sucrose, le sorbitol ainsi que des combinaisons de polymère-sucrose et polymère-sorbitol. Nous avons étudié la stabilité de ces formulations pendant trois mois de conservation à 4°C. L'efficacité de ces formulations a été évaluée en termes de viabilité de *Sinorhizobium meliloti* une fois enrobé sur les grains (nombre de cellules viables par grain). Les grains enrobés ont été conservés à la température ambiante. Pour affiner le choix des formulations efficaces, une comparaison a été faite entre l'enrobage avec des formulations conservées et des formulations fraichement préparées. L'efficacité à la fin a été évaluée à travers les tests de nodulation en sachets de croissance et le rendement des plantes en matières sèches.

- Originalité :
  - Les formulations en suspension de *Sinorhizobium meliloti* cultivé dans les eaux usées d'amidon n'ont jamais été développées.
  - La combinaison de ces polymères-sucres n'a pas été étudiée.
  - L'effet de la conservation des formulations en suspension sur la viabilité ultérieure des cellules après enrobage des grains n'a pas été abordé jusqu'à présent.

3ième objectif

Le troisième objectif a été consacré à l'optimisation des conditions de séchage par atomisation des cultures de *Sinorhizobium meliloti* et sur le développement de formulation en poudre. Le premier objectif, déjà réalisé, contribue en grande partie à la réalisation de cette étape car il a permis d'atteindre une récolte maximale de cellules. Cette optimisation a été réalisée en utilisant la méthode de réponse de surface et en faisant intervenir les différents paramètres relatifs au séchage par atomisation (débit d'air, orifice de pulvérisation, débit de la suspension de la culture de *Sinorhizobium meliloti*, agent de protection).

- Originalité :
  - L'optimisation du séchage de *Sinorhizobium meliloti* cultivé dans les eaux usées d'industrie d'extraction d'amidon par la méthode de réponse de surface.
  - L'utilisation des rejets industriels comme agent de protection au cours de cette opération est une approche unique et écologique.

4ième objectif

Le quatrième objectif a porté sur l'évaluation de la co-inoculation *Sinorhizobium meliloti- Azospirillum brasiliense* sur les plantes de luzerne, en passant par l'optimisation des conditions de croissance des *Azospirillum brasiliense* dans les eaux usées d'amidon.

Originalité : production de l'*Azospirillum brasiliense* dans les eaux usées d'amidon et évaluation de la faisabilité de la co-inoculation *Sinorhizobium meliloti- Azospirillum brasiliense.*

4. Démarche méthodologique

Les démarches méthodologiques qui ont servi pour la réalisation des objectifs spécifiques cités si dessus ont été détaillées dans les différents chapitres qui lui sont correspondants.

4.1 Les eaux usées d'amidon

Le milieu de culture utilisé dans cette étude provient des rejets de l'industrie d'amidon. Ces eaux usées ont été récupérées à la fin de toute la chaîne d'extraction d'amidon (ADM Ogilvie; Candiac, Québec, Canada). Vu que le procédé utilisé par l'usine est basé sur des cycles d'extraction avec de l'eau chaude, suivi par concentration et séchage (un procédé d'extraction physique), les rejets sont exempts de toutes formes de composés chimiques qui peuvent inhiber la croissance des cellules ou affecter la qualité finale des formulations. La composition, les propriétés physiques et chimiques de ces rejets ainsi que la composition du milieu standard (YMB) sont présentés dans les annexes 1.1, 1.2 et 1.3.

2.2 Méthodologie d'optimisation utilisée

Les travaux d'optimisation présentés dans cette thèse ont été réalisés en utilisant la méthodologie de réponse de surface (MRS). La MRS peut être décrite comme étant la démarche statistique de choix pour l'optimisation des bioprocédés. Elle permet de modéliser statiquement le procédé en question avec le minimum d'essais expérimentaux. Le plan expérimental utilisé dans la MRS fait intervenir les plans factoriels partiels à deux niveaux combinés aux plans en étoiles ce qui permet de couvrir un large intervalle de valeurs : la combinaison de ces deux plan permet de tester les réponses à 5 niveaux différents (le centre de l'expérience, +1;-1, $+\alpha$; et $-\alpha$) pour chaque variable étudiée.

Pour monter un plan d'expérience (Fig1.3), la première étape à faire consiste à choisir les paramètres de l'étude et qui doivent être indépendants les uns des autres. La deuxième étape est de choisir les intervalles d'étude pour chaque variable, l'optimal recherché devrait être généralement inclus dans ce domaine expérimental. Une première série d'expérience est réalisée au début de chaque étude pour localiser un optimum provisoire autour duquel sera dressé le plan des expériences. Le choix des pas pour chaque variable étudiée est la troisième étape à réaliser et qui doit tenir compte des techniques de mesure utilisées, de la spécification du matériel utilisé et de la réponse étudiée.

Les résultats sont présentés sous formes de modèles polynomiales de second degré faisant intervenir les interactions entres les différentes variables.

$$Y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j$$

Où: Y, β_0, X_i and X_j, β_i, β_{ii}, et β_{ij} sont respectivement la réponse étudiée, constante, les variables indépendantes de l'étude, les coefficients de régression linéaire, les coefficients de régression quadratiques, les coefficients de régression de l'interaction entre chaque deux variables indépendantes.

Dans certains cas, on est obligé d'aborder des études où on fait affaire à l'optimisation de plusieurs réponses à la fois, avec diverses contraintes qui devront être imposées (en maximisant ou minimisant certaines réponses. L'étude de plusieurs réponses en parallèles est possible en utilisant les fonctions de désirabilité (Derringer and Suich, 1980). Il s'agit d'une transformation des réponses étudiées en une fonction de désirabilité globale D qui est la moyenne géométrique pondérée de plusieurs fonctions de désirabilité partielles.

$$D= (d_1 \, x d_2 x \ldots . d_k)^{1/k}$$

Avec d_i la désirabilité partielle associée à chaque réponse Y_i étudiée, par exemple si on cherche à maximiser la réponse, la désirabilité sera définie comme suit :

$d_i = 0$ si $Y_i \leq A$

$d_i = (Y_i - A)/(B-A)^r$ si $A \leq Y_i \leq B$

$d_i = 1$ si $Y_i \geq B$

$r = 1/k$

Les valeurs de A et B représentent les contraintes fixées pour la réponse en question.

Si on cherche à minimiser la réponse, la fonction désirabilité partielle sera définie comme suit :

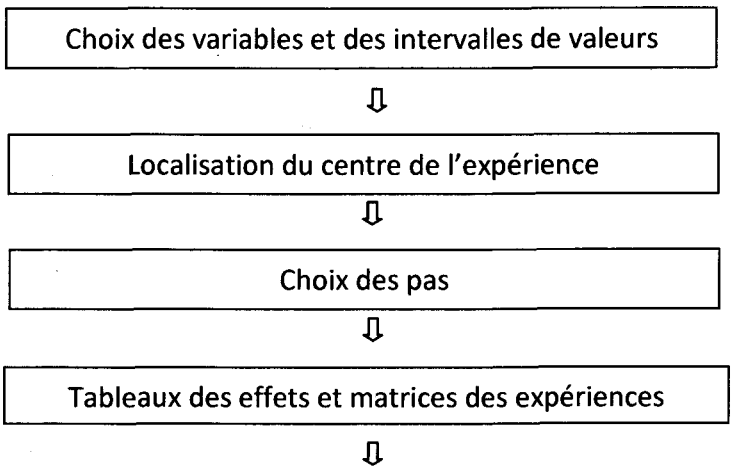
$d_i = 1$ si $Y_i \leq A$

$d_i = (Y_i - B)/(B-A)^r$ si $A \leq Y_i \leq B$

$d_i = 0$ si $Y_i \geq B$

La désirabilité globale sera donc définie comme une fonction répondant aux différentes conditions (contraintes) du problème traité et qui inclue les variables indépendantes étudiées. Ainsi

$$D = f(X_1, X_2, \ldots\ldots X_n);\ n \text{ nombre de variables étudiées}$$



Nombre de variables indépendantes k	Nombre de points			Total des expériences	α
	Extrémité 2^k	Étoiles	Centre		
2	4	4	5	13	1.414
3	8	6	6	20	1.682
4	16	8	7	31	2
5	32	10	6	48	2

Figure 1.3 méthodologie de l'optimisation utilisée

5. Résultats

5.1 Optimisation des conditions de récolte par centrifugation *de Sinorhizobium meliloti*

Les résultats issus de l'optimisation des paramètres relatifs à la centrifugation (température de centrifugation, pH, durée et force de centrifugation) ont montré qu'il est de multiplier par un facteur de 20 la récolte des cellules (rotor utilisé à godets mobiles). Le modèle obtenu à montré que seule l'interaction entre la durée et la force de centrifugation était statistiquement significative.

$$Y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j \qquad \text{(Eq.1)}$$

(*Y*, β_0, *Xi* et *Xj*, β_i, *βii,et βij* sont respectivement la réponse étudiée, valeur moyenne de la réponse étudiée, les variables indépendantes, les coefficients de régression linéaires, les coefficients de régression quadratiques, et coefficients de régression relatifs à l'interaction entre chaque deux variables indépendantes)

$$Y = 99.3258672 - 0.0000035109 \text{ Time x Centrifugal force} \qquad \text{(Eq.2)}$$

D'après les expériences réalisées et le modèle obtenu (Eq.1 et 2), la centrifugation à une vitesse de 8000g pendant 20 min, à 20°C, et pH =7 a permis de concentrer 20 fois de plus le bouillon fermenté. La centrifugation en utilisant les rotors à godets mobiles à permis de récolter plus de cellules que le cas des rotors à angles fixes (10% de plus de cellules). Ce fait est dû à l'augmentation de la distance entre rayon min à l'intérieur des bouteilles de centrifugation et le rayon max de celui de l'extérieur. L'orientation des godets de 90° durant la centrifugation par rapport aux rotors à angle fixe favorise la séparation de phase.

5.2 Développement de formulations liquides de *Sinorhizobium meliloti* et formulations de grains enrobés

Les résultats ont montré que toutes les formulations liquides développées dans ce travail ont permis de maintenir la survie de *Sinorhizobium meliloti* à des concentrations supérieures à 10^9UFC/ml après 13 semaines de conservation réfrigérées (la norme de qualité des inoculants étant de 10^7 à 10^9UFC/ml, selon la dose à appliquer sur les grains). Le calcul de demi vie a montré que les formulations les plus efficaces étaient successivement celles de : sucrose, alginates, sorbitol, PEG, PVP et finalement les formulations combinées : le meilleur résultat était avec le sucrose à 10% w/v (83 semaines, contrôle : 1.6 semaine). Le détail concernant la description de la protection des cellules par ces additifs est présenté dans le chapitre 3.

Les formulations de grains enrobés développées à partir de bouillon fermenté frais (FLF) ont permis de maintenir la viabilité des cellules pour des durées de conservations supérieures à celles à partir de formulations liquides conservées(OLF). La formulation combinée PEG-Sorb et la formulation PEG 1% ont maintenu une concentration en *Sinorhizobium meliloti* supérieure à 10^3CFU/grain pour plus de 120 jours.

L'évaluation du rendement des plantes a montré que la durée conservation affecte les résultats obtenus que ce soit pour les grains enrobés ou les formulations liquides avant application sur les grains : les formulations où les cellules sont fraichement produites et/ou fraichement enrobées ont permis d'atteindre les rendements les plus élevés en poids secs. En plus, elles ont maintenu la viabilité des cellules aux concentrations les plus élevées et pour les durées de conservation les plus longues.

5.3 Nouvelle méthode pour l'évaluation de la suspendibilité des bioinoculants

Durant la conservation des formulations liquides, la stabilité physique est un critère important pour la détermination de la qualité du bioinoculant. L'évaluation de cette stabilité revient en grande partie au suivi de sa suspendibilité. L'étude du potentiel zêta, de la taille des particules, du pH et de la viscosité des formulations développées à partir de bouillon fermenté de *Sinorhizobium meliloti* et du sorbitol (entre 0 et 10%w/v) a révélé que la suspendibilité est significativement corrélée au potentiel zêta et à la viscosité (Eq. 3 et 4).

Suspendibilité= 94.8666 / (1+exp(-(η - 18.8267)/ 1.8918));

p-value <0.0001; R2= 0.9823

(Eq.3)

Suspendibilité= 93.7495 / (1+exp(-(Zp+39.2603)/-1.1734));

p-value = 0.007, R2 =0.8625

(Eq.4)

La mesure du potentiel zêta et de la viscosité peut être utilisée comme une nouvelle alternative à la mesure classique de la suspendibilité (basée sur le calcul des volumes décantés après 12h). La stabilité des suspensions exige des forces répulsives entre les particules afin qu'une barrière énergétique limite leur approche. Le sorbitol a été démontré au cours de cette étude comme agent de suspension efficace : l'addition de sorbitol est à l'origine de l'augmentation des forces répulsives et par conséquent la réduction des interactions attractives entre les molécules (responsables de la décantation), ceci étant expliqué en se basant sur la théorie DLVO (Derjaguin, Landau, Verwey et Overbeek) et en corrélant la taille des particules à la concentration de sorbitol et le potentiel zêta mesuré. La description détaillée et les équations relatives à ces paramètres sont présentées dans le chapitre 4.

5.4 Optimisation du séchage par atomisation de *Sinorhizobium meliloti* et développement de formulation en poudre

Les résultats relevant de l'optimisation des paramètres de séchage étudiés (température de séchage, pression de pulvérisation, débit d'air de séchage, débit de la solution à pulvériser) et du plan d'expérience utilisé (plan composite centré) ont démontré la possibilité d'obtenir de la poudre formulée contenant une concentration en *Sinorhizobium meliloti* jusqu'à $6x10^9$CfU/g. Avant séchage, le bouillon fermenté a été concentré par centrifugation auquel a été ajouté: du sorbitol à 1%w/v, du sucrose à 1% w/v, et du lactosérum à 10% w/v : ces additifs jouent le rôle de protecteurs de cellules contre l'effet de la température élevée et la dessiccation au cours de la conservation. Ils favorisent en plus la solubilisation rapide de la poudre dans l'eau avant l'enrobage des grains.

La modélisation des résultats avec la méthode de réponse de surface a permis de développer un polynôme de second dégrée qui relie les paramètres de séchage à la viabilité des cellules selon l'équation5:

$$\textbf{Cells viability } *10^9 = -215.256 + 602.279 \text{ Air drying} - 625.914 \text{ Air drying}^2 - 0.009T^2_{inlet} + 1150.503 \text{ Air drying} * P$$

(Eq.5)

Ce modèle permet une bonne explication des résultats observés (R2=0.79). Le maximum de cellules viables (≈6x10^{9}CFU/g) est obtenu lorsque le séchage a été réalisé à une température de séchage de 111 °C, une pression de pulvérisation de 0.08 MPa, un débit d'air de séchage de 0.56 m^3/min et un débit de la solution à pulvériser de 8 ml/min.

L'optimisation simultanée de la viabilité des cellules, de l'humidité de la poudre et de la température à la sortie du séchoir(T_{outlet}) à travers l'introduction de la fonction désirabilité globale a montré que le séchage à 105°C, 0.07 MPa, 8ml/min et 0.56m^3/min permet de maximiser la récupération des cellules (≈6.18x10^9) tout en garantissant une humidité de poudre inférieure à 15%(11.6%), et en imposant comme contrainte de minimiser la température de sortie du séchoir (42°C).

Après 4 mois de conservation, la poudre formulée de *Sinorhizobium meliloti* a maintenu une concentration en cellules viables supérieure à 2x10^{9}CFU/g, la protection de la membrane cellulaire a été réalisée par l'effet combiné des protéines et du lactose provenant du lactosérum, et du sorbitol ajouté. Les photos prises avec le microscope électronique à balayage ainsi que la description détaillée des propriétés de la poudre sont présentés dans le chapitre 5.

L'évaluation de cette formulation à travers les tests de croissance en sachets et le calcul de l'indice nodulaire a montré que les cellules qui s'y trouvent étaient capables d'infecter les racines de la luzerne et de former de nodules. La comparaison par rapport au control (cellules cultivées dans l'YMB) a révélé que les *Sinorhizobium meliloti* formulé étaient comparables en termes de nodulation et rendement en poids secs.

5.5 Amélioration de la qualité des formulations de *Sinorhizobium meliloti* par co inoculation avec *Azospirillum brasiliense* : optimisation des conditions de production d'*Azospirillum brasiliense* dans les eaux usées d'amidon et études des doses appliquées

L'évaluation de l'ajout du gluconates, de l'extrait de levure et de ces deux suppléments à la fois au SIW a été réalisée en première partie. Les résultats ont démontré une amélioration significative au niveau du temps de génération et du taux de croissance de l'A*zospirillum brasiliense* en comparaison par rapport au milieu SIW ($p<0.05$). Le SIW supplémenté avec le l'extrait de levure à 0.5% w/v ou avec du gluconate à 0.5% w/v est capable de supporter la croissance de l'*Azospirillum brasiliense* autant que le milieu standard de Bashan ($p>0.05$). L'extrait de levure a été retenu comme supplément dans le reste de ce travail.

L'optimisation de la température de fermentation et la quantité d'extrait de levure ajoutée ont été abordées en seconde partie dans ce travail. Ces expériences ont été planifiées en suivant un plan composite à deux variables et 5 répétitions au centre. Les résultats ont été étudiés en utilisant la méthode de réponse de surface. Le modèle obtenue présente une bonne explication de la variabilité observée entre les mesures expérimentales, soit un R^2 de 92%, le maximum de cellule a été produit lorsque la fermentation a été réalisée à une température de 33°C et à une concentration en extrait de levure de 0.3%w/v, ce qui permet de réduire les quantités d'extrait de levure utilisé et par suite le coût de production.

Les tests de croissance en sachet ont été réalisés pour comparer l'effet de la concentration d'*Azospirillum brasiliense* à coinoculer avec 10^5 cellules de *Sinorhizobium meliloti* par plante de luzerne. Les résultats ont montré que les combinaisons 10^5 *Azospirillum brasiliense* + 10^5 *Sinorhizobium meliloti* et 10^6 *Azospirillum* + 10^5 *Sinorhizobium meliloti* ont permis d'atteindre des rendements en poids secs améliorés de 17 et 20% par rapport au control (plante inoculée avec 10^5 *Sinorhizobium meliloti*) ($p<0.05$). Ces résultats peuvent être expliqués par l'effet stimulateur exercé par les *Azospirillum brasiliense* sur les plantes. À travers la sécrétion de phytohormones, les *Azospirillum* stimule le développement des racines, ce qui augmente la surface de contact rhizobium-racine. En plus, elles produisent des flavonoïdes qui permettent la fixation des rhizobiums au niveau des racines et ainsi le début de la nodulation. Le traitement 10^4 *Azospirillum brasiliense* + 10^5 *Sinorhizobium meliloti* était comparable au contrôle ($p>0.05$). L'interprétation détaillée de cette section est présenté dans le chapitre 6.

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CHAPITRE 2

RÉCOLTE PAR CENTRIFUGATION DES CELLULES DE RHIZOBIUM FERMENTÉES DANS LES EAUX USÉES D'AMIDON ET DÉVELOPPMENT DE FORMULATIONS STABLES

CENTRIFUGAL RECOVERY OF RHIZOBIAL CELLS FROM FERMENTED STARCH INDUSTRY WASTEWATER AND DEVELOPMENT OF STABLE FORMULATION

RÉCOLTE PAR CENTRIFUGATION DES CELLULES DE RHIZOBIUM FERMENTÉES DANS LES EAUX USÉES D'AMIDON ET DÉVELOPPMENT DE FORMULATIONS STABLES

Tarek Rouissi[1], Rojan P. John[1], Satinder K. Brar[1], R.D. Tyagi[1]*, D. Prévost[2]

[1]INRS-ETE, Universite du Quebec, 490, Rue de la Couronne, Quebec, Canada G1K 9A9

[2] Agriculture et Agroalimentaire Canada, Sainte-Foy, QC, Canada G1V 2J3

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RÉSUMÉ

L'inoculation avec les rhizobiums est une pratique agricole qui a connu beaucoup de succès comme solution pour augmenter la croissance et le rendement des légumineuses. Le développement de culture concentrée de cellules est une étape essentielle pour l'avancement des formulations. Dans ce contexte, cette étude propose d'utiliser les eaux usées de l'industrie de l'amidon comme source de carbone pour la production de *Sinorhizobium meliloti*, ce qui a permis d'atteindre une concentration de 4 x 10^9 CFU/mL. La récupération des cellules par centrifugation a été optimisée en utilisant la méthodologie de réponse de surface. Le maximum a été atteint (> 99%) à une vitesse de 8000 g, une température de 20 ° C pendant 20 min, à pH égal à 7 et avec un rotor à gadgets mobiles. L'addition d'amidon à 2%w/v au bouillon fermenté permet de réduire les pertes en cellules. Une comparaison a été faite entre les rotors à gadgets mobiles et les rotors à angles fixes, les résultats montrent que la récolte était maximale avec le rotor à gadgets mobiles (> 99%) par rapport au rotor à angle fixe (90%). L'augmentation des volumes centrifugés 20 fois réduit le pourcentage de récupération des cellules de 90% à 87% pour le rotor à angle fixe lorsque la centrifugation a été faite à 8000g pendant 20 min à 20 ° C. Des formulations en suspensions à base d'alginates ont permis de maintenir la viabilité de *Sinorhizobium meliloti* à une concentration supérieure à 10^9 UFC/ml après 9 semaines de stockage. Cette étude a démontré la faisabilité de la récupération des cellules et du développement simultanée des formulations de *Sinorhizobium*. De nouveaux travaux d'optimisation avancée sont nécessaires pour le développement de formulations à partir des concentrées de cellules récupérées.

Mots clés: eaux usées d'amidon; récolte par centrifugation; *Sinorhizobium meliloti*; formulation en suspension

ABSTRACT

Application of rhizobial inoculant is a successful agricultural practice used to increase growth and yield of leguminous plants. Development of concentrated culture is an essential step for development of advanced formulations. In this context, this study comprised use of starch industry wastewater as a potent carbon source for the production of *Sinorhizobium meliloti* with a maximum cell count of 4 x 10^9 CFU/mL. The maximum recovery of cells (>99 %) during centrifugation was optimized to be 8000 *g*, temperature of 20 °C for 20 min., pH of 7 using swinging-bucket type rotor using surface response methodology. Out of the different centrifugal aids starch (2%, w/v) reduced the loss of microbial cells during recovery through the supernatant. A fixed-angle type rotor experiment was also carried out to judge the recovery differences between the two centrifugal configurations and it was found that the recovery was maximum while using swinging-bucket type rotor (>99 %) as compared to fixed-angle type rotor (90 %). There was a further decrease (from 90 to 87%) in recovery observed when there was a 20 times increase in the broth volume at 8000 *g*, for 20 min at 20 °C. Suspension formulation with alginate additive allowed cell viability of more than 10^9 CFU/mL after 9 weeks of storage. This study proved the feasibility of cell recovery and simultaneous formulation development of *Sinorhizobium*. Further investment in parameter optimization for the development of advanced formulation using recovered cells is needed.

Keywords: Starch industry wastewater; Centrifugal recovery; *Sinorhizobium meliloti*; suspension formulation

2.1. Introduction

Legume inoculation is an established agricultural practice used for decades. It has contributed to increased nitrogen fixation through symbiotic association with organisms such as, rhizobia and helps legume crops to yield better in situations where natural nitrogen fixation is not most favorable. It was estimated that approximately 2000 tons of inoculants are produced worldwide annually, a quantity sufficient to inoculate 20 million ha of legumes [1].

The standard medium yeast mannitol broth (YMB) used for laboratory scale production of legume inoculant is not suitable for industrial level production as it is not economically viable and can be modified by introduction of other carbon sources. Sucrose is the most commonly used carbon source, and mannitol and glycerol are sometimes used for slow-growing rhizobia [2]. Glycerol has the most beneficial effect on growth of slow growing rhizobia [3]. Numerous investigators have been searching for alternative ways of producing rhizobia by using low cost media. Many agro-industrial by-products have also been investigated as potential alternatives for refined carbon and nitrogen sources. The residues, such as cheese whey, a by-product of the cheese industry [4], malt sprouts, a by-product of the malt industry [5], agriculture wastes, such as pea husks, molasses, water hyacinth [6] and wastewater sludge [1,7] and others have been evaluated for cost effective production of media.

Currently, industrial waste treatment is a field of major concern. Industrial wastewaters can be treated by physical, chemical and/or biological treatment. Biotechnological conversion of starch industry wastewater into value-added product can be an alternative to the physical and chemical treatment. Fuel hydrogen [8], biocontrol agents such as *Trichoderma*, *Bacillus thuringiensis* [9-13] and others have been produced effectively from SIW. Starch industry wastewater can be economically utilized for the production of rhizobia and the inherent characteristics of suspended or soluble solids in the wastewater after fermentation can be helpful in the formulation of these microorganisms. Commercial inoculant formulations include powder or granular carriers, broth cultures or liquid formulations [14]. A good carrier for microorganisms should have properties such as, high water-holding capacity, chemical and physical uniformity, lack of toxicity to microbes in the formulation and other useful microbial strains in soil, environment-friendly and be locally present and economically viable. Peat is one of the best carriers for microbial inoculants, however, some countries lack natural peat deposits or the peat mines are located in preserved wetland ecosystems, so that its extraction is forbidden [15]. Liquid inoculants are economically

feasible for the production and reduction in cost but the survival of microorganisms are comparatively lower during storage and field application. It can be overcome with the application of suitable additives to suspensions or oils for emulsification.

A concentrated culture of inoculants which maintains constant viable numbers over long periods would be desirable for several reasons. Concentrated preparations of cells can be produced well in advance of seasonal demands and the cell counts can be standardized. Stocks of concentrated cells can be stored and easly transported from the fermentation plant to the site of inoculants manufacture or application site [16]. The bacteria can be produced and made to concentrate well in advance to meet the unprecedented seasonal demand and it can be used for preparation of any type of inoculants of required standards (either soil or seed application) just before use [17]. The concentrated culture can be obtained by centrifugation of the fermented broth. Hence, the current study focused on:

1) production of *Sinorhizobium meliloti* by using the effluent from the starch industry wastewater;

2) efficient recovery-optimization of *S. meliloti* from fermented broth by centrifugation for concentrated cultures and;

3) utilization of the broth for production of suspension and emulsion formulation.

2. Materials and methods

2.1 Microorganisms and inoculum preparation

The fast-growing *Sinorhizobium meliloti* strainA2 (Agriculture and Agri-food Canada, Sainte-Foy, QC, Canada) was used in the current study. Cultures were maintained at 4±1 °C on yeast mannitol agar (YMA) slants or plates. Cell production for inocula was carried out in standard liquid medium yeast mannitol broth (YMB) (MAT laboratories, Quebec). The YMB medium contained the following constituents in grams per liter: K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; yeast extract, 1.0; and mannitol, 10.0. The strains were grown at 30±1 °C for 48 h on a rotary shaker at 200 rpm and the cell counts were determined on YMA plate supplemented with Congo red (0.25% w/v) after a serial dilution on saline solution (NaCl, 0.85% w/v). Colonies were counted after incubation of plates for 72 h at 30±1 °C.

2.2 Preparation of production media and cultivation conditions

For the production of rhizobial cells, starch industry waste water containing ~1.3% w/v total solids (A.D.M. Ogilvie, 155, Iberia Avenue, Candiac, Quebec, J5R 3H1, Canada) was used as the sole carbon source. The starch wastewater pH was adjusted to 7.0 and supplemented with 0.1 %, w/v yeast extract for growth promotion. Sterilized (121±1 °C, for 15 min.) starch industry wastewater medium inoculated with 3%, v/v rhizobial culture containing 10^9 CFU/mL and incubated at 30±1 °C in a rotary shaker (200 rpm). Fermentation was carried out for 48 h and cell concentration was estimated as CFU by the plating method.

2.3 Optimization of centrifugal recovery and statistical analysis

A Central composite design was used to study interactions and select optimum conditions for centrifugal recovery, Data were subjected to statistical analysis of variance (ANOVA), regression analysis and the response surface methodology were performed with STATISTICA trial version 8 (Statsoft.com). The confidence level fixed for this study was 0.05. Four parameters were selected for the recovery experiment: temperature, centrifugation duration (time), centrifugal force and pH of the broth. Each variable studied has 5 levels in different combinations. The levels of parameters and combination of different levels are shown in Table 2.1 and Table 2.2.

Centrifugation was conducted on the basis of design and CFU were tested in the supernatant and centrifugate. A total of 31 experiments were conducted based on the model and those were performed in triplicate.

Three rotors were used to perform centrifugation of various working volumes: swinging bucket 25 mL (HB-6, 27622 x g), fixed angle 25 mL (SA- 600, 30453 x g) and fixed angle 25 mL (SA-600, 30453 x g) and fixed angle 500 mL (GS-3, 13679 x g).

2.4 Viscosity

Viscosity of starch industry wastewater, fermented broth and formulation was determined by using a rotational viscometer Brookefield DVII PRO+ (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA) equipped with Rheocalc32 software. Two different spindles, namely, SC-34 (small sample adaptor) and ultralow centipoise adapter, were used with sample cup volumes of 15 mL and 50mL (spindle dependent).

2.5 Effect of pH on centrifugation

Recovery was carried out at various pH of the fermented broth, from 4 to 7 at an interval of 0.5 units for the other optimised parameters (temperature, centrifugal force) as obtained from the response surface methodology. This experiment was conducted for validation and to check the most effective pH for minimal cell loss.

2.6 Influence of additives during centrifugation

An experiment was conducted to reduce the loss of microbial biomass during the recovery process from the supernatant. The effect of various additives, such as ghatti gum, phytagel, soy flour, carboxy methyl cellulose, corn starch and corn flour were tested at different concentrations (0.2, 0.8, 2%, w/v). Carboxy methyl cellulose and phytagel were tested upto 0.2 and 0.8%, w/v respectively due to their solidifying nature, which might have resulted in erroneous findings. The additives were to the fermented broth and centrifugation was conducted under optimal conditions of 8000 *x g*, at a temperature of 20 °C for 20 min at a pH of 7. The loss of cells in the supernatant was determined by plating on YMB-agar after serial dilution.

2.7 Suspension formulation of *Sinorhizobium*

Suspension formulation was developed using the fermented broth at pH 8.5 after adding an antimicrobial agent sorbic acid (0.2 %, w/v) and the pH was further adjusted to 7.2 by using sulphuric acid. The suspension contained 4 x 10^9 CFU/mL. Three different additives, such as sorbitol, sodium alginate and sodium monophosphate (1, 0.5 and 1 % w/v, respectively) were evaluated. As survival of bacteria was maximum in alginate amended suspensions, different concentrations was tested to determine the actual effect. All suspensions were kept at 4 °C and stability was assessed based on the survival of bacteria after 9 weeks.

All the experiments were conducted in triplicate and the standard errors were always ±5%.

3. Results and discussion

3.1 Production of *Sinorhizobium* using starch industry wastewater

Starch industry waste water contains nearly 1.3%, w/v total solids and which constitutes more than 50 %, w/v carbon and a lower ammount of nitrogen. The viscosity of the starch industry wastewater was 1.3597 mPas. The cell count in the final fermented broth was 4 x 10^9 CFU/mL with addition of yeast extract to SIW. The effect of yeast extract on rhizobial strain production has been already reported to increase cell concentrations [4,16]. Raw whey supplemented with 1.0 g of yeast extract per liter yielded 1.25 times more cells than the ones obtained with mannitol or sucrose broth and the yield increased 2.5-fold with a higher concentration of yeast extract at 5.0 g/L [4]. Meade *et al.* [16] reported that recovery of *Rhizobium leguminosurum* cells by centrifugation after growth in an industrial fermenter was 100-fold higher when cells were grown on yeast extract (5 gL^{-1}) as sole source of carbon and nitrogen when compared with the yields when cells were grown in standard mannitol-yeast extract medium. The fermented broth had a viscosity of 1.9995 mPas and confirmed the growth of *S. meliloti* in the medium as there was an increase from the raw substrate, which may be attributed to the formation of extracellular polymeric substances.

3.2 Centrifugal recovery of *Sinorhizobium* from fermented wastewater: Optimization by Response Surface Methodology

The selected factors were tested for the efficient recovery and all of them resulted in higher percentage of recovery (Table 2.2). The evaluation of statistical significance of the factors studied and their interaction was based on probability (p) values (p=0.05). The statistical analysis showed that only two factors (centrifugal force and centrifugal time) have major influence as independently as well as in interactions. The Prob>F values are used as a tool to check the significance of each of the variables. The smaller the magnitude of the Prob>F, the most significant is the corresponding model term. The variables Prob>F less than 0.05 were considered to be significant in influencing the desired response. Hence, these variables were significant in enhancing the centrifugation. All other variables were having Prob>F values above 0.05 and were therefore insignificant. P-values are summarized in the Pareto chart (Fig. 2.1). The positive sign of the effect of tested variables indicated that the influence of this variable on

centrifugation was greater at a high level. When the sign of the effect of tested variable was negative, the influence of that variable on recovery was greater at low level.

Based on the design the predicted response of recovery can be represented by Equation 1 (Eq. 1).

$$Y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j \qquad \text{(Eq.1)}$$

(Y, β0, Xi and Xj, βi, βii, and βij are the predicted responses of the dependent variable, second-order reaction constant, the independent variables, linear regression coefficient, the quadratic regression coefficient, and regression coefficient of interactions between two independent variables, respectively)

According to the generated data, the general model presented in (Eq.1) can be simplified after excluding insignificant regression coefficients ($p < 0.05$) by using the interaction of time and centrifugal force (Eq.2).

Y = 99.3258672 – 0.0000035109 Time x Centrifugal force (Eq.2)

The magnitude of the effect of each variable indicated the intensity of its significance on the studied response. The greater the magnitude of the effect of the tested variable, the higher was the significance of that variable. As shown in Fig. 2.2, maximum recovery can be achieved by mainly two ranges. The maximum recovery can be achieved by mainly two sets of conditions when considering the two most important variables. Lower centrifugal force would require longer time and higher centrifugal force would need less time. However, maximum recovery was obtained at higher centrifugal force and longer centrifugal time. Brar *et al.* [10] reported that 9000 g is the most suitable for the commercial scale recovery and in their laboratory, recovery of bacteria (95%) from fermented starch industry wastewater was obtained at 48000 g for 30 minutes. Hence, the current centrifugal force and time are most reliable for commercial application.

Temperature and pH interact as shown in Fig. 2.3. When the temperature increased, the pH increased upto a certain point with almost constant recovery of the bacterial cells. Although, the best indicated pH in statistical optimization was 7, another experiment was conducted with change in pH as a lower pH would ensure less contamination problems [18]. pH 6.5 (loss of cells in supernatant was 2.3×10^5 CFU/mL) had almost same effect as pH 7 (loss of cells in supernatant was 2.4×10^5 CFU/mL) and all other had slightly negative effects. The loss of cells from the supernatant were 10.08×10^6, 10.1×10^6, 9.28×10^6, 1.95×10^6, 5.4×10^5 CFU/mL at

pH 4, 4.5, 5, 5.5 and 6, respectively. Usually acidic pH conditions were found to be better, as the sedimentation velocity would have been enhanced due to neutralization of negative charges on the protein surface, which promoted the interaction between charges and particle size increments [19]. With increasing pH values, the floc system will be increasingly negatively charged and similar charges in the floc structure would cause repulsion. This will lower the possibility of adsorption of cells on the flocs in the broth and cause suspension resulting in poor harvest [10]. Further, acidic pH lowers the viscosity due to compactness of the floc structure [20]. The lower viscosity would enhance sedimentation rate (according to Stoke's law, viscosity and sedimentation velocity are inversely related) [10]. Hence, the influence of starch additive (corn starch in centrifugal aids) may be the reason for change in the recovery at lower pH.

3.3 Rotor configuration

The types of rotor used for the centrifugal recovery also played a significant role in cell recovery. Swinging-bucket and fixed-angle rotors were tested in the current experiments as they are most common styles for bench top, low-speed, and high-speed floor-model centrifuge applications. Vertical rotors are primarily used in ultracentrifugation. The recovery in the former one, swinging-bucket, (which has been used in statistical optimization analysis) gave maximum recovery of more than 99% total viable bacteria. In fact, the fixed angle rotor affected the recovery which decreased to 90% viable cells. Swinging-bucket type rotor can support two types of separations such as rate-zonal (i.e., based on mass or size) or isopycnic (i.e., based on density). For rate-zonal separations, a swinging-bucket rotor is advantageous as the path length of the gradient, i.e., distance from R_{min} (inside top of meniscus) to R_{max} (outside bottom of tube) is long enough for separation to occur. Additionally, as the buckets are positioned at 90° during the run and return to a vertical position at the end of the run, the sample retains its orientation, which minimizes any pellet or band disturbance[21]. As one goes to industrial scale recovery, there is a lower recovery. The decrease in recovery (from 90 to 87%) was observed when broth volume increased 20 times (from 25 mL to 500 mL) at 8000 *xg*, for 20 min at 20 °C. At this centrifugal force (best for commercial scale), the fixed angle rotor and higher volume increased shearing effect on flocculated particles, and the degree of difficulty in scrolling the settling solids, due to increasing slippage forces [21]. The difference in recovery efficiency can also be explained by the calculation of time of centrifugation. To do the same, Stoke's law explaining the resulting settling velocity (or terminal velocity) was used as given by:

$$V_s = \frac{2}{9}\frac{(\rho_p - \rho_f)}{\mu} gR^2 \quad \text{(Eq. 3)}$$

Where; V_s is the particles' settling velocity (m s^{-1}) (vertically downwards, if $\rho_p > \rho_f$, upwards if $\rho_p < \rho_f$), g is the gravitational acceleration (m s^{-2}), ρ_p is the mass density of the particles (kg m^{-3}), and ρ_f is the mass density of the fluid (kg m^{-3}); R is the radius of the spherical object (in m) and μ is the fluid's dynamic viscosity (in Pa s). The sedimentation velocity was later used to calculate the sedimentation coefficient which is defined as the ratio of particle's sedimentation velocity to the acceleration that is applied to it (causing the sedimentation) and represented as:

$$s = \frac{v_s}{a} \quad \text{(Eq. 4)}$$

Where; s = sedimentation coefficeint of a particle; v_s (in ms^{-1}) = sedimentation velocity also known as the terminal velocity; a (in ms^{-2}) = applied acceleration can be either the gravitational acceleration g, or more commonly the centrifugal acceleration $\omega^2 r$. In the latter case, ω is the angular velocity of the rotor and r is the distance of a particle to the rotor axis (radius). Angular velocity was chosen to be the maximum value that can be given by the rotor as manufacturer manuals provided precise value. Finally, the sedimentation coefficient will give the corresponding centrifugation time as given by Equation 5:

$$t = \frac{k}{s} \quad \text{(Eq.5)}$$

Where; t = centrifugation time (hours); k = clearing factor expressed in hour-Svedbergs and s = sedimentation coefficient of a particle in Svedberg units. Keeping in conformity with angular velocity values used in Equation 4, k values will also be derived from manufacturer tables provided with the centrifuge and denoted for maximum speeds. On performance of these calculations, it was found that the centrifugation time for the swinging bucket rotor was 23.76 seconds and that for the fixed angle rotor was 12 960 seconds explaining the high recovery (≥ 99%) of rhizobial cells for the former configuration.

3.4 Development of liquid formulations

Evaluation of the survival of *S. meliloti* under storage over a period of time is essential to test the suitability of the formulation. Different suspending agents must be checked for their appropriateness in the medium. As a preliminary step, alginates, sodium mono-phosphate and sorbitol were tested and the viable cell count was carried out over 9 weeks of storage. Maximum survival was observed in alginate or sorbitol (10^9 $CFUmL^{-1}$). As the maximum positive effect was in the case of alginate, different concentrations of sodium alginate were tested with the maximum concentration of 0.5% w/v.

Sodium alginate is a natural polymers commonly used for cell encapsulation and as adhesive compounds with solid based carriers to be applied to seed [24, 25]. It is a heteropolymer with large molecular weight, composed of D-mannuronic acid and L-guluronic acid [26]. The main advantages of this polymer are its non-toxic and biodegradable nature [14, 27]. Addition of alginates in suspension increased viscosity of each formulation in comparison with the control. The viscosity of 0.2, 0.3 and 0.5%, w/v alginates were 6.48, 6.72 and 8.56 mPas respectively while that of the control was only 2 mPas. This viscous nature might be induced in the present formulation because it reduces water activity in the formulation and resulting in microbial stability of each formulation [28]. Moreover, this viscous nature will improve the survival of cells on seed after application by restraining heat transfer during the desiccation process of the inoculants [29]. The survival of cells in suspension containing different concentration of alginate is shown in Figure 4. The alginate added suspensions enhanced the survival of cells to more than 10^9 CFU/mL at all concentrations, where as in the control there were nearly no viable cells after nine weeks of storage. The survival of bacteria was almost same in 0.3 and 0.5%, w/v of alginates after 9 weeks of storage. The immobilized cell showed tolerance to alcohols [30] and was mainly due to enhanced modification of cell membrane [31]. The fatty acid impurities in alginate probably result in a modified fatty acid pattern for immobilized cells compared with free cells [32]. Similar effect on tolerance against phenol in *E. coli* was reported by Keweloh *et al.* [33] and it was due to the uptake of saturated fatty acids present in commercial alginates, and incorporation into membrane lipids [31].

4. Conclusions

The recovery of *S. meliloti* cells from the fermented broth is necessary to make highly concentrated liquid formulations such as emulsion, suspension etc. or other powder or spray dried solid formulation and to reduce the cost of transportation and storage volume. It can be concluded that cells can be recovered efficiently using a swinging bucket type rotor with less loss in centrifugate at lower volumes at 2000 to 4000 *g* for 20 to 25 minutes or 8000 to 10000 *g* for 5-10 minutes. Fixed angle rotors can be used for large volume recovery, even though lesser recovery was observed with it than the swinging bucket type rotor. Additives or pH can be selected for the recovery based on either type of soil application (acidic or alkaline soil), or type of formulation (emulsion, suspension, lyophilization, microencapsulation or spray drying) which is used for the storage. Based on the preliminary experiments, suspension formulations are effective for the storage of recovered bacteria. Further, screening of additives and their concentrations, conditions for formulation development are the best formulation.

5. References

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Table 2. 1 Independent variables used in the optimization of centrifugal recovery

	Codes and values of independent variables of experimental designs for screening using response surface methodology				
Levels	-2	-1	0	+1	+2
Temperature (°C)	5	10	15	20	25
Time (min.)	5	10	15	20	25
Centrifugal force (g)	2000	4000	6000	8000	10000
pH	4	5	6	7	8

Table 2.2 Design showing the set of each run and their recovery percentage, (p =0.05)

Sl. No.	Temperature (°C)	Time (min.)	Centrifugal force (g)	pH	CFU/mL (10^6)	% of recovery
1	10	10	4000	5	6.52	99.837
2	10	10	4000	7	5.72	99.857
3	10	10	8000	5	1.72	99.957
4	10	10	8000	7	1.44	99.964
5	10	20	4000	5	1.94	99.9515
6	10	20	4000	7	1.6	99.96
7	10	20	8000	5	0.32	99.992
8	10	20	8000	7	0.26	99.9935
9	20	10	4000	5	7.2	99.82
10	20	10	4000	7	6.82	99.8295
11	20	10	8000	5	2	99.95
12	20	10	8000	7	1.6	99.96
13	20	20	4000	5	1.36	99.966
14	20	20	4000	7	2.1	99.9475
15	20	20	8000	5	9.16	99.771
16	20	20	8000	7	0.23	99.99425
17	5	15	6000	6	0.31	99.99225
18	25	15	6000	6	1.88	99.953
19	15	5	6000	6	3.28	99.918
20	15	25	6000	6	0.43	99.98925
21	15	15	2000	6	2.54	99.9365
22	15	15	10000	6	0.36	99.991
23	15	15	6000	4	1.46	99.9635
24	15	15	6000	8	3.52	99.912
25	15	15	6000	6	1.3	99.9675

Table 2.2 Design showing the set of each run and their recovery percentage, (p =0.05) (continued)

26	15	15	6000	6	1.34	99.9665
27	15	15	6000	6	1.32	99.967
28	15	15	6000	6	1.39	99.96525
29	15	15	6000	6	1.35	99.96625
30	15	15	6000	6	1.38	99.9655
31	15	15	6000	6	1.3	99.9675

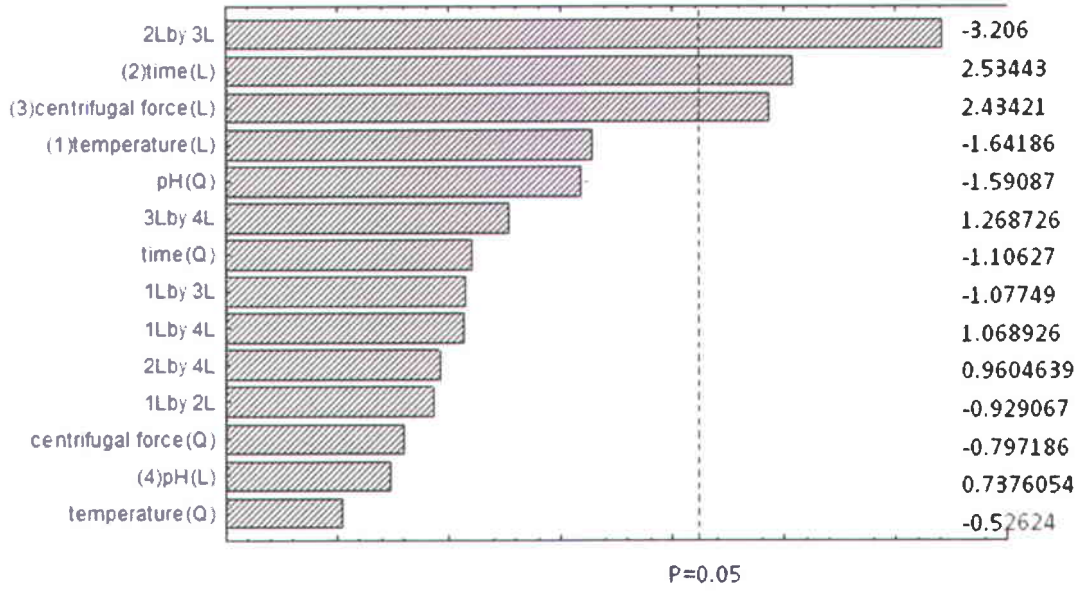

Figure 2. 1 Pareto chart showing the significance of parameters.

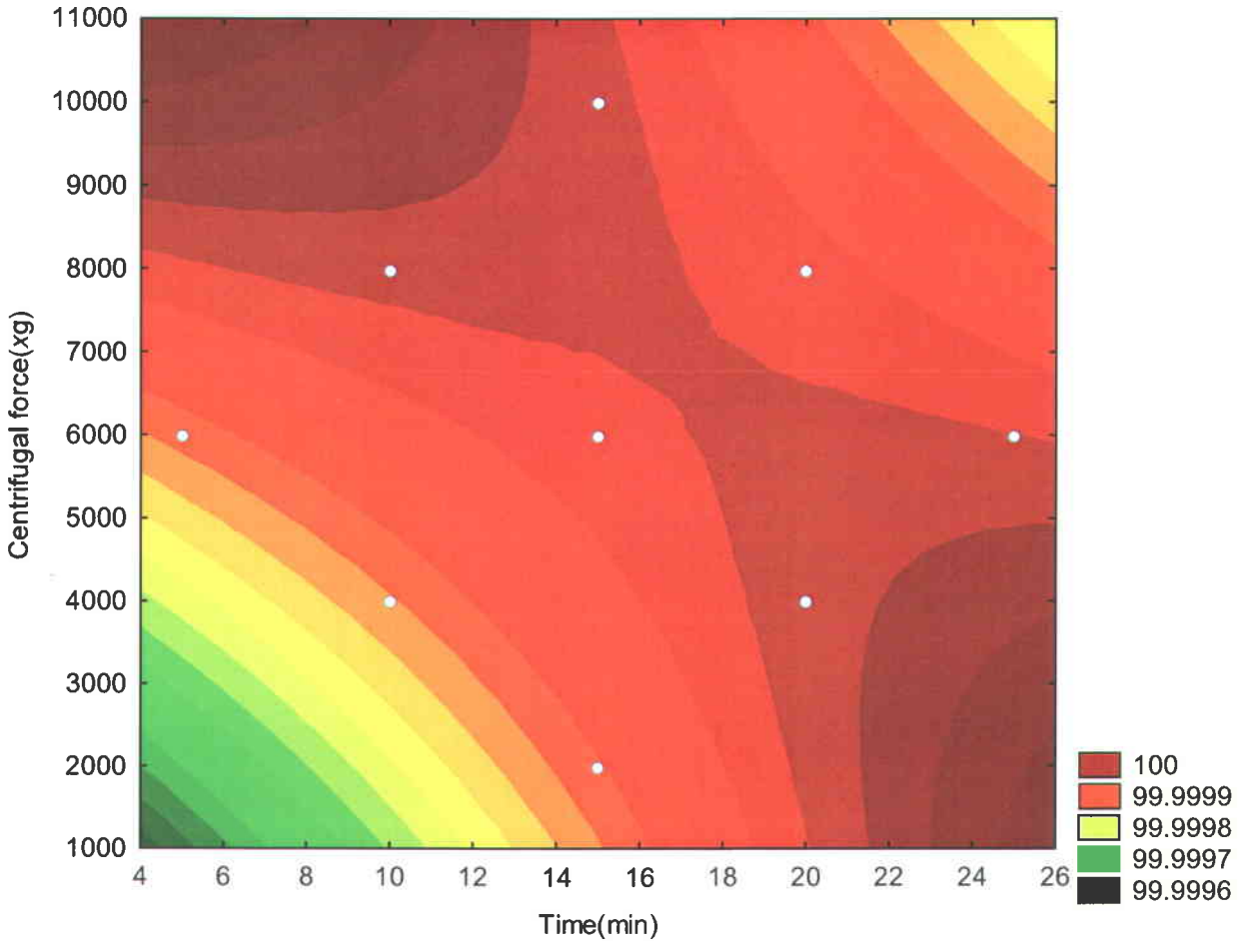

Figure 2.2 Interaction of centrifugal force (*g*) and centrifugal time (min.) on recovery of *S. meliloti* from fermented starch industry wastewater.

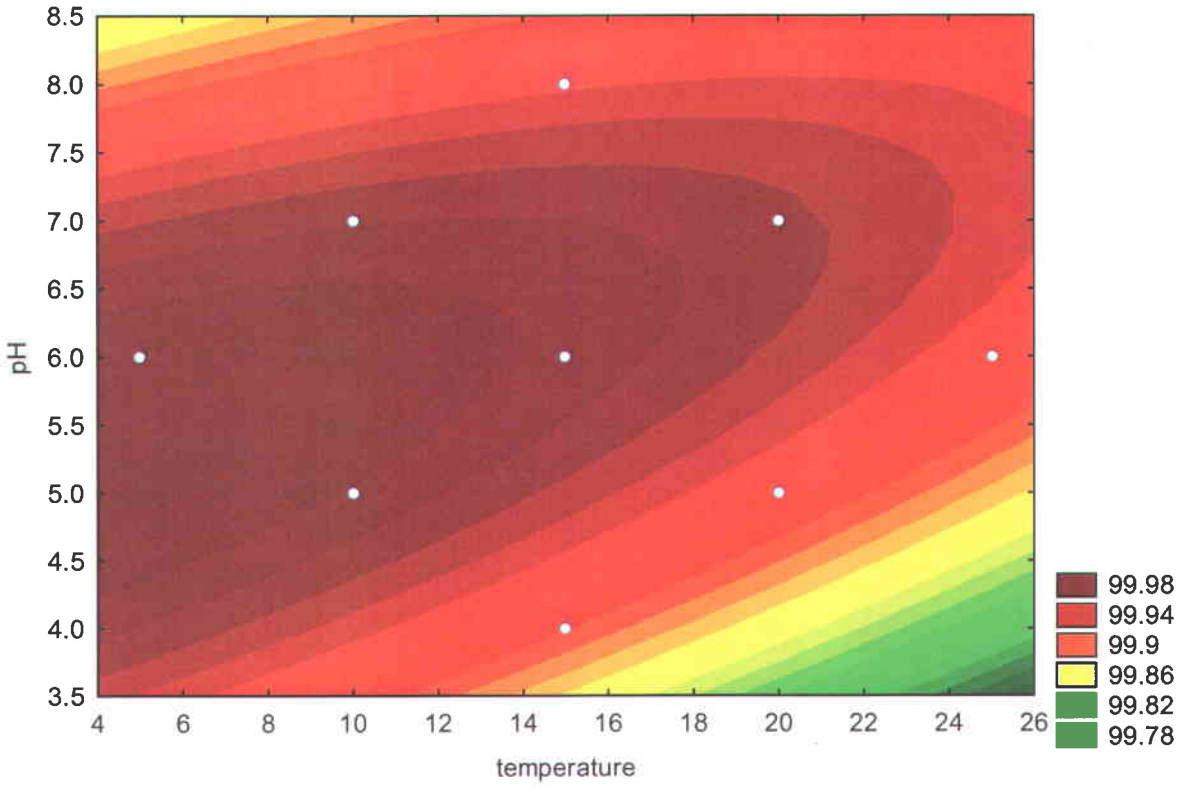

Figure.2.3 Interaction of temperature and pH on recovery of *S. meliloti* from fermented starch industry wastewater

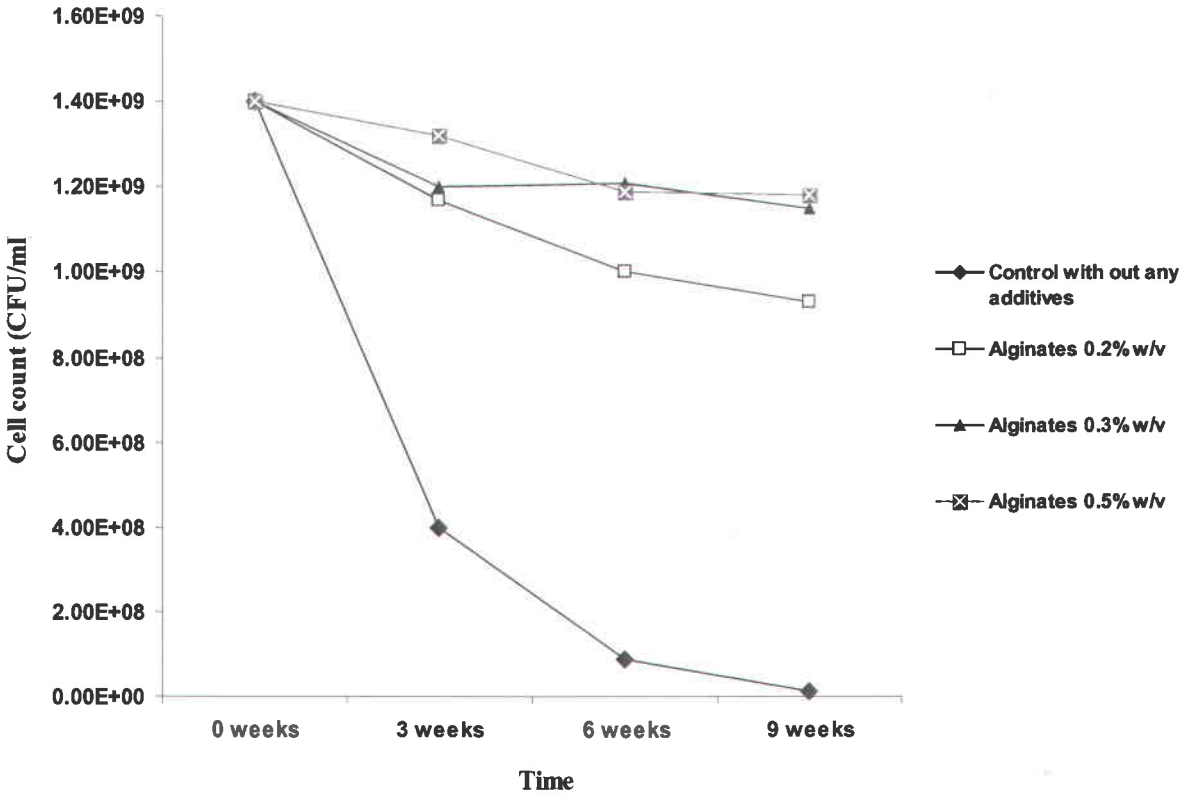

Figure 2.4 Influence of alginate concentration on suspension formulation of *S. meliloti*

CHAPITRE 3

ÉLABORATION D'UNE MÉTHODE SIMPLE ET EFFICACE POUR LA DÉTERMINATION DE LA SUSPENDIBILITÉ DES BIO-INOCULANTS LIQUIDES

EFFICIENT AND SIMPLE METHOD FOR DETERMINATION OF SUSPENDIBILITY OF BIO-INOCULANT SUSPENSIONS

ÉLABORATION D'UNE MÉTHODE SIMPLE ET EFFICACE POUR LA DÉTERMINATION DE LA SUSPENDIBILITÉ DES BIO-INOCULANTS LIQUIDES

T. Rouissi[1], R.D. Tyagi[1]*, S. K. Brar[1], D. Prevost[2], R P. John[1], R Y Surampalli[3]

[1]INRS-ETE, Université du Québec, 490, rue de la Couronne, Québec,

Canada G1K 9A9

[2]Agriculture et Agroalimentaire Canada, Québec, QC, Canada G1V 2J3

[3]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

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RÉSUMÉ

Ce travail présente une nouvelle méthode de caractérisation de la suspendibilité des formulations de *Sinorhizobium meliloti*, cultivée dans les eaux usées de l'industrie d'amidon. Le sorbitol a été utilisé comme agent de suspension dans cette étude. Les modèles, basés sur la régression linéaire multiple (avec: le pH, le potentiel zêta, la taille des particules et la concentration en sorbitol comme variables indépendantes) ont montré l'existence de relations significatives entre ces variables ($p < 0,001$, $R^2 = 0,98$). La régression sigmoïdale a révélé une relation significative entre le potentiel zêta et la suspendibilité avec une probabilité $p = 0,007$ et $R^2 = 0,86$; et entre la viscosité et la suspendibilité (valeur $p < 0,0001$ et $R^2 = 0,9823$). Ainsi, ces corrélations établies permettent de proposer le potentiel zêta et la viscosité comme paramètres pour décrire la stabilité physique des formulations liquides.

Mots clés : *Sinorhizobium meliloti,* stabilité, suspension, suspendibilité, viscosité, potentiel zêta

ABSTRACT

This study assessed the utilization of viscosity and zeta potential as a novel method to evaluate suspendibility of formulations of *Sinorhizobium meliloti* grown in starch industry wastewater (SIW) for use as bio-inoculants. For this objective, sorbitol was used as a suspending agent at concentrations of 0 to 10% w/v. A model, based on multiple linear regression (with pH as dependant variable, and zeta potential, average particle size and sorbitol concentration as independent variables) demonstrated an important relationship ($p<0.001$, $R^2=0.98$). Sigmoid regression revealed a significant relationship between zeta potential and suspendibility with p value=0.007 and R-squared =0.86, and between viscosity and suspendibility (p value <0.0001 and R squared= 0.9823).These direct correlations allowed the lowering of measurement time from 12 h to 5 min.

Key words: *Sinorhizobium meliloti,* stability, suspension, suspendibility, viscosity, zeta potential

1. Introduction

Bio-inoculant industries have seen rapid progress in recent decades. An estimate revealed that 2000 tonnes of inoculants (solids, powders and liquids) are produced worldwide annually, corresponding to a quantity sufficient to inoculate 20 million ha of legumes (Rebah *et al.*, 2007). Bio-inoculant suspension is one a liquid formulation largely applied to seeds and agricultural land. However, its stability has not been investigated in the literature. The research to date in this field mainly focused on the cell viability, while physical parameters play an important role in phenomena controlling the biological activity during storage and application. Cell suspension formulations are usually turbid, containing colloidal material and particle aggregates. Decreases in stability are usually related to reduction of cell viability and increased sedimentation (Narong and James, 2006). Thus, suspendibility could be defined as an indicator of mixture stability and homogeneity. Traditional methods used for the measurement of suspendibility, such as measure of turbidity, decanted or suspended volume are non-repetitive, long, usually imprecise, especially in the case of commercial product formulations and prone to personal analytical artifacts.

Many researchers have focused on the study of suspensions and tried to establish a relationship between stability and pH, particle size, zeta potential, viscosity and/or solids content (Chang and Chang, 2002; Narong and James, 2006). A correlation has been demonstrated between ζ-potential and suspension in the case of *Saccharomyces cerevisiae* suspensions (Chang and Chang, 2002). Gustafsson et al (2003) demonstrated that rheological proprieties follow the net interaction between particles in the case of anatase suspensions.

In this work, a novel and rapid method has been presented to characterize the rhizobial cells in suspension state. Based on the correlation to be established between suspendibility and viscosity and between suspendibility and zeta potential in the case of bio-inoculant formulation, this research investigated a simple and precise alternative method. This correlation was investigated by studying the relation between the intervening parameters, such as average particle size, pH and viscosity along with zeta potential and suspendibility.

2. Materials and Methods

2.1 Microorganism and media

Bacteria used to produce fermented broth for this study was *Sinorhizobium meliloti* A2 (Agriculture and Agri-Food Canada, Sainte-Foy, QC, Canada). Starch industry waste water (A.D.M. Ogilvie Candiac, Quebec, Canada, SIW, total solids ≈1.3% w/v) was used as medium. Cells were produced using a method described by Rouissi *et al.* (2010).

2.2 Suspension formulation

Suspension formulation of *Sinorhizobium meliloti* was prepared by adding sorbitol as a suspending agent to the fermented broth at the following concentrations: 0, 1, 2, 3, 5, 7, 8 and 10 % w/v. The initial pH of the fermented broth was adjusted from 8.6 to 7 by using sulphuric acid (98%). The suspension contained ≈ 5 x 10^9 CFU/mL of *Sinorhizobium meliloti* cells.

2.3 Viscosity and Particle size

Viscosity was measured using a rotational viscometer Brookefield DVII PRO+ (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA) equipped with Rheocalc32 software. Characterization of particle size was performed by determination of volume mean diameter, D_{43} (referred to as average particle size). Measurement was performed using a Fritsch laser particle sizer analyzette 22, the operation of which is based on diffraction laser principle; stirrer and pump speed used in this experiment were 250 rpm and 500, respectively. Analysis was carried out in triplicates for each sorbitol concentration.

2.4 Zeta-potential

Zeta potential was determined after dilution of samples with filtered deionized water at room temperature (250 µL of each sample in 250 mL of deionized water). All measurements were carried out using zetaphoremeter (Zetaphoremeter IV and Zetacompact Z8000, CAD Instrumentation, Les Essarts le Roi, France) and zeta potential values were calculated using

Smoluckowski Equation (Liao *et al.*, 2001). The zeta potential values were obtained from the average of 10 measurements.

2.5 Measurement of suspendibility

Suspendibility was evaluated by determining the suspended and settled volume of the suspension at room temperature. The fermented broth (70 ml) was fortified with sorbitol at concentrations of 0, 1, 2, 3, 5, 7, 8 and 10 % w/v. Suspensions were thoroughly stirred for 2 min and left for decantation in separating funnels for 12h. The final settled volume was measured using a graduated cylinder and the percent suspendibility was calculated using Eq.1. Each measurement was carried out in triplicates.

$$\textit{Suspendibility (\%)} = [(V_{initial} - V_{settled}) / V_{initial}] \times 100 \qquad (1)$$

2.6 Statistical analyses

Statistical analyses were performed using R version 2.10.1 (Copyright (C) 2009, The R Foundation for Statistical Computing ISBN 3-900051-07-0). Simple and multiple linear regressions were utilized to evaluate relation between the parameters studied in this work. The regression was considered significant at $p < 0.05$ (α was fixed to 0.05). The error bars on the Figures indicate the mean standard deviations for the data points.

3. Results and discussion

3.1 Zeta potential and average particle size

Figures 3.1(a) and (b) show zeta potential, average particle size and suspendibility as a function of sorbitol concentration. Zeta potential decreased with increase of sorbitol concentration. The behaviour can be traced in three different phases in relation to the quantity of sorbitol added. The three phases were : a) a rapid decrease in zeta potential was observed with sorbitol at concentrations ranging from 0 to 2% w/v; b) from 2 to 5% w/v (or 150% increase of sorbitol concentration), zeta potential was relatively stable (at 2 and 5% w/v sorbitol concentration, the zeta potential values were -40.5625 ± 0.2678 and -40.7225 ± 0.4328 mV, respectively); and c) a faster decrease of zeta potential with sorbitol concentrations of 5 to 10% w/v; zeta potential reached the value of -43.6380 ± 0.4884 mV at 10 %w/v. Statistical analysis using sigmoid regression indicated a correlation between zeta potential (Zp) and sorbitol concentration [Sorb] (Eq.2)

$$Zp = -389.20 / [1+ exp(-([Sorb]-211.3)/96.63)]; R^2=0.899 \quad (2)$$

In the first phase, zeta potential decreased rapidly due to dispersion of bacterial cells (negatively charged) in suspension. In the second phase, relatively stable behaviour was observed due to the fact that the quantity of sorbitol added was not enough to increase the repulsive forces (by cell dispersion from agglomerates) to a point that it could affect the zeta potential. The third phase rapid decrease could be attributed, again, to the same phenomenon as observed in the first phase. The suspensions studied in this work were highly concentrated in cells of *Sinorhizobium meliloti* (5.1 ± 0.27 x 10^9 CFU/ ml), possessing negative charges. Increase in repulsive forces, increase in suspendibility and a decreased zeta potential were thus promoted by the high sorbitol concentrations.

Variation of particle size with sorbitol concentration is presented in Figure3.1b. Increase in sorbitol concentration and decreased value of zeta potential exhibited dominating effects on the average particle size. Statistical analysis using sigmoid regression indicated a correlation between D43 (the volum mean diameter) and sorbitol concentration.

$$D43= 138.4130/ (1+exp (-([Sorb]- 0.2682)/ 0.6794)); R^2=0.98, p value= <0.0001 \quad (3)$$

Two different phases described this relationship: the first phase was related to a linear rapid increase of the average particle size (from 83.28 ± 5.12 to 138.72 ± 6.22 μm) in the concentration range from 0 to 2% w/v (Figure 3.1(b)). With the addition of sorbitol, particles tend to open and thus become more dispersed. The average size increased because of the adsorption of sorbitol to the surface. The increase was 67% at a sorbitol concentration of 2 % w/v in comparison to the control. Sorbitol in this case acts as a suspending agent by reducing the attractive forces and promoting the repulsive interactions between the macromolecules present. Petersen *et al.* (2004) demonstrated that use of sorbitol was able to reduce aggregation between proteins and thus increased suspendibility. In the second phase, increase in sorbitol concentration from 2 to 10% w/v did not increase the average particle size. This could be due to the possibility that all sites capable of adsorbing sorbitol on the particle surfaces were saturated by sorbitol concentration of 2% w/v.

3.2 pH

A substantial increase in pH was observed from 3 to 10% w/v sorbitol concentration (Figure 1b). Maximum pH of 5.84 was reached at a sorbitol concentration of 10% w/v.

Multivariate regression analysis was carried out by using pH as the dependant variable and zeta potential, sorbitol concentration and average particle size as independent variables (Eq.4). Model presented was significant with a *p-value*= 0.0001944<0.05 and an adjusted R^2=0.9822. Results related to estimated effects are presented in Table 1.

$$pH = 4.8395307 - 0.0077431 x Zp + 0.0011993 x D_{43} + 0.0092808 x [Sorb.] \quad (4)$$

It has been already mentioned that an increase of pH was associated with a decrease in zeta potential value, in the interval 2-8 (Kirby *et al.*, 2004). Chang and Chang (2002) also observed the same phenomenon while studying the variation of zeta potential versus pH for suspensions of *Saccharomyces cerevisiae* at different concentrations of NaCl. pH increased with the average particle size. Two different mechanisms were responsible for this dependence: a) adsorption of sorbitol to particles resulted in volume increase and; b) repulsive forces were induced between aggregates of particles, thereby preventing the bonding phenomena, as discussed earlier. The increase of repulsive interactions will lead to enhanced surface contact of bacteria with water and decreasing the zeta potential (particle charge).

To explain the mechanism of polyols suspension stabilization effect, researchers proposed effects of polyols on the structure of water in the case of protein suspension. Vagenende *et al.* (2009) demonstrated that glycerol prevented protein aggregation by inhibiting protein unfolding and by stabilizing aggregation prone intermediates through preferential interactions with hydrophobic surface regions that favored amphiphilic interface orientations of glycerol. The increased pH, as observed in Figure1b, was a consequence of the neutralization of H+ ions by the negatively charged bacterial cells released (from the aggregates) due to increase in suspendibility.

3.3 Viscosity

A gradual increase in viscosity was seen over all sorbitol concentration ranges studied (Figure 1b). Depending on the concentration of sorbitol, viscosity (η) increased from 18.20 ±0.20 to 21.27± 0.31 mPas.s for 0 and 3 % w/v sorbitol, respectively. The final viscosity value reached was 24.29± 0.70 mPa.s at 10%w/v concentration of sorbitol, which meant an increase of 33% in comparison to the control (0% w/v of sorbitol). Multiple linear regression was carried out considering viscosity as dependant variable while zeta potential, average particle size (D43), pH and sorbitol concentration as independent variables (Eq.5).

$$\eta = 18.964698 \times pH + 0.332472 \times [Sorb.] + 0.174299 \times Zp - 0.002054 \times D43 \qquad (5)$$

The global model (Eq. 5) was statistically significant, with p *value* of 0.002313 <0.05 and adjusted R^2 of 0.9905. Analyses of estimate coefficients related to this model are presented in Table 1. Earlier research focused on the study of relationship between rheology of suspensions (especially, viscosity) and particle interactions clearly demonstrated a dependence relation (Gustafsson *et al.*, 2003; Hang *et al.*, 2009). Benitez *et al.* (2009) mentioned that an increase in particle volume fraction increased specific viscosity, this was specifically observed in the case of different glucose concentrations, which is also confirmed by our study. Average particle size increased in the sorbitol concentration interval of 0 to 5% w/v from 83.28 ± 5.12 to 140.96 ± 5.6 μm, respectively, leading to an increase in viscosity.

Zeta potential was demonstrated to be related to the viscosity of the solution by the Helmholtz–Smoluchowski equation (Narong and James, 2006) (Eq. 6)

$$Zp = \eta u / \varepsilon_r \varepsilon_0 E \qquad (6)$$

Where Zp u, E, η, ε_r, ε_0 are the Zeta potential, the velocity of the particles, the electric field, the viscosity of the solution, the dielectric constant of the suspension and the permittivity of free space, respectively. According to this equation, an increase of Zp resulted in an increase of viscosity which could probably explain the positive coefficient observed in Eq. 6. Likewise, other researchers studied the effect of sorbitol on the starch system and observed an anti-plastic behaviour, so that viscosity increased with the sorbitol amount added to the starch solution (Gaudin *et al.*, 2000). The same viscosity behaviour was observed in the current study so that continuous addition of sorbitol from 0 to 10% w/v resulted in a gradual increase of viscosity. These results are in concordance with the published literature. Gaudin *et al.* (2000) related this phenomena to the creation of more links between sorbitol and starch in the media.

3.4 Suspendibility

To investigate the stability of suspensions, suspendibility was defined as percentage of suspended volume versus total volume after 12h of settling (Eq. 1). The increase in viscosity resulted in increase in suspendibility and in fact the correlation followed a significant sigmoidal regression (Eq. 7). In the same manner, when relation between suspendibility and zeta potential was studied, a significant sigmoidal regression was also observed (Eq.8).

$$Susp.= 94.8666 / (1+exp(-(\eta - 18.8267)/ 1.8918)); \text{ p-value } <0.0001; R^2= 0.9823 \quad (7)$$

$$Susp.= 93.7495 / (1+exp(-(Zp+39.2603)/-1.1734)); \text{ p-value } = 0.007, R^2 =0.8625 \quad (8)$$

It is known that the rate of suspension stability depends mainly on the colloid particle interactions. Interpretation of these phenomena is usually assessed in terms of DLVO theory, which defined total interaction energy (V_{TE}) as a function of the separation of two particles. V_{TE}, is expressed as sum of the van der Waals attractive energy (Va) and the electrical double layer (Vr):

$$V_{TE}= Va + Vr \quad (9)$$

With the general consideration of spherical particles, Va and Vr can be expressed as described by (Narong and James.2006):

$$Va= -a A_{131} f(P) /2H \quad (10)$$

$$Vr= \varepsilon a Zp^2 ln(1+ exp(-kH))/2 \quad (11)$$

Where **ε** is the dielectric constant of water, **a** is the particle radius, **Zp** is the zeta potential of the particle, **k** is the inverse Debye length, **H** the distance separating the surfaces the two interacting particles, $\mathbf{A_{131}}$ the Hamaker constant and **f(P)** is the retardation factor.

Where: $f(P)= 1/(1+1.77P)$, for $P \leq 0.5$ and **(12)**

$$f(P)= 2.45/5P - 2.17/15P^2 + 0.59/35P^3, \quad \text{for } P > 0.5 \quad \textbf{(13)}$$

$P= 2\pi H/\lambda$, λ is the wavelength of the intrinsic electronic oscillations.

Flocculation or suspended state occurs depending on the value of V_{TE}, when it is negative, particles will be attracted to each other. When V_{TE} is positive and increasing, but inferior to certain maximum, suspension state occurs. When V_{TE} decreases after this maximum, flocculation will occur.

$$V_{TE}= -a A_{131} f(P) /2H + \varepsilon a \psi^2_s ln(1+ exp(-kH))/2 \quad \textbf{(14)}$$

When sorbitol concentration increased, Zp in absolute value and average particle size followed the same behaviour (Figure 1a, 1b). With the consideration that A_{131} is constant, f(P) is a decreasing function since H, the distance separating the surfaces of the two interacting particles is increasing, due to the increased of mutual charge |Zp|.

When considering two sorbitol concentration (C1) and (C2) in the interval 0- 2 % w/v, C1>C2: $a_1 < a_2$; $|Zp_1| < |Zp_2|$; $H_1 < H_2$, consequently, $V_{TE\,1} < V_{TE\,2}$.

When sorbitol concentrations are between 2 and 10%: C1>C2, $a_1 \approx a_2 = a$, $|Zp_1| < |Zp_2|$, $H_1 < H_2$, $VT_{E\,1} = Va + Vr_1 < V_{TE\,2} = Va + Vr_2$, consequently: $Vr_1 < Vr_2$

Total interaction energy increases with the increase of sorbitol concentration. When the amount added exceeded 2 % w/v, this increase is mainly due to the contribution of the repulsive energy (Vr).

In the literature, stability of suspensions was largely studied, but usually defined by the terms of solubility, turbidity, flocculation, or sedimentation, depending on the study (Chang and Chang, 2002; Narong and James, 2006). In the case of cell suspensions, the intervening phenomena are the same and considered as short range and long range forces. Short-range forces

concerned hydration interactions, which can be either repulsive or attractive, depending on the nature of the surfaces involved (hydrophilic or hydrophobic).

The increase in suspendibility with the viscosity may be related to the fact that as sorbitol concentration was increased in the medium, the particle size increased to certain extent (Figure 1b), and then further addition of sorbitol did not produce any change in particle size. Meanwhile, the viscosity kept on increasing, which may be due to the fact that the individual particles created by the addition of sorbitol remain in suspended state, which may also be supported by the theory of hydrogen bonding and van der Waals interaction between sorbitol and the bacterial cells which are negatively charged. The increase in viscosity may be attributed to increased due to the presence of various particles in the suspension.

The stability state is related to equilibrium occurring between these phenomena. The increase in electrostatic repulsion of surface charges opposes particles coming closer and forming agglomerates. Increasing the surface charge of particle density in absolute value could generate a strong repulsive force, which plays major role in the stabilizing mechanism by increasing total interaction energy (V_{TE}) (Singh *et al.*, 2005).

The method can be easily adapted to other similar applications, such as biopesticides, biocontrol agents, and pharmaceutical products, among others. Furthermore, viscosity is also an indicator of the stability of cell suspensions with regard to storage and application.

4. Conclusion

The stability of *Sinorhizobium meliloti* suspensions was related to interactions between particles. Viscosity and zeta potential were related proportionally to the suspendibility, confirming a sigmoidal regression. Sorbitol was also demonstrated to be an efficient suspending agent for cells; recommended concentration was 3% w/v. Suspendibility correlated with viscosity or zeta potential resulting in a simple and faster method of measurement of suspendibility for bioinoculant formulations.

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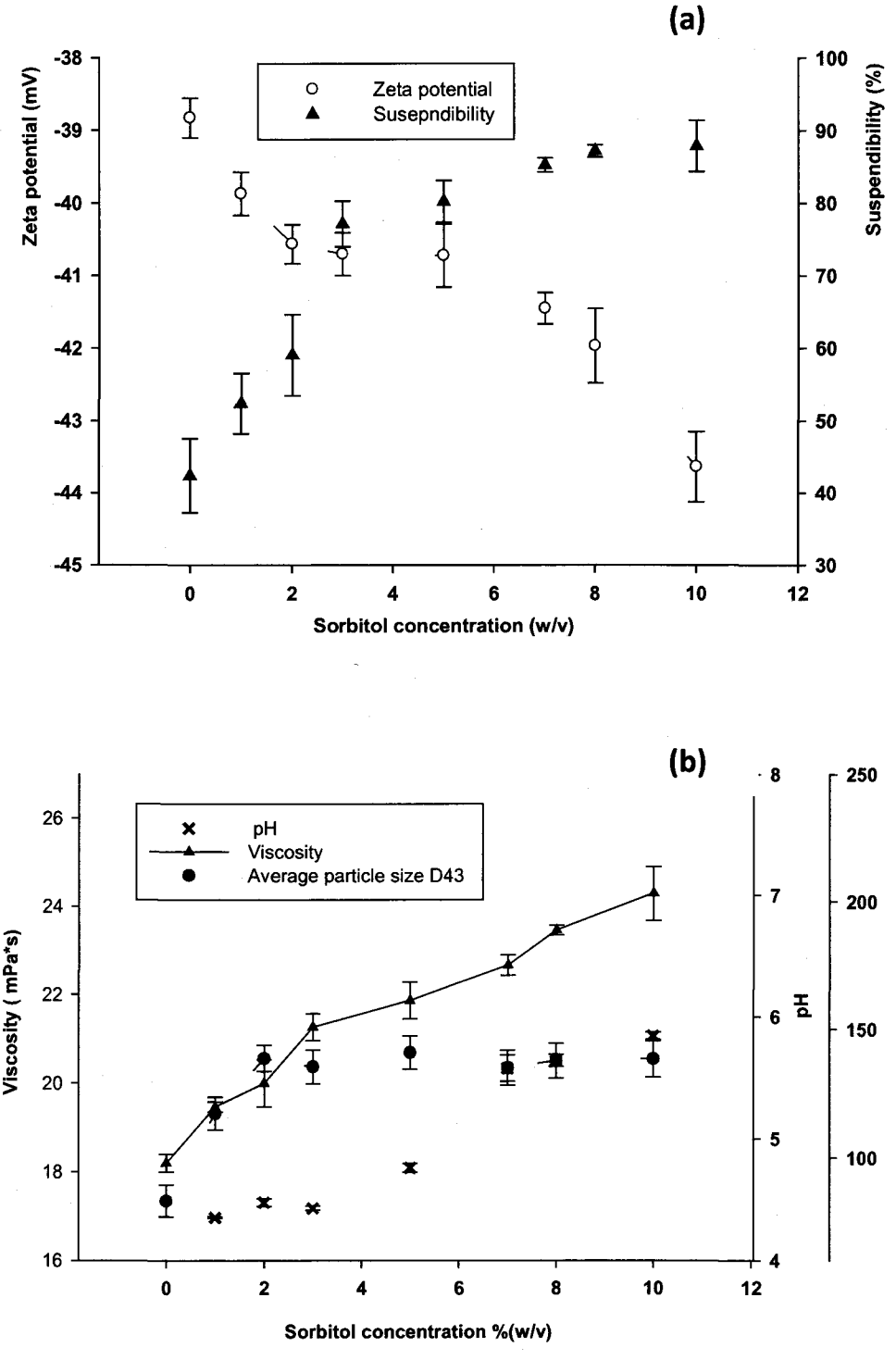

Figure3.1 Profiles of studied parameters: (a) zeta potential, and suspendibility = f (concentration); (b) viscosity, pH and suspendibility = f(concentration)

Table 3.1 Results related to estimated effects

Equation		Estimate	Std. Error	t value	Pr(>|t|)
Eq.4:pH = f(zeta potential,sorbitol concentration,average particle size) **Multiple R^2: 0.989,** **Adjusted R^2: 0.982** **F-statistic: 129.4 on 3 and 4 DF, *p*-value: 0.0001944**	**(Intercept)**	4.8395307	0.2833690	17.079	6.9e-05
	Zeta potentiel	-0.0077431	0.0074473	-1.040	0.3572
	Average particle size	0.0011993	0.0002274	5.274	0.0062
	Sorbitol concentration	0.0092808	0.0027636	3.358	0.0284
Eq.5:Viscosity = f (pH, Sorbitol concentration, zeta potential, average particle size) **Multiple R^2: 0.990,** **Adjusted R^2: 0.977** **F-statistic: 77.94 on 4 and 3 DF, p-value: 0.002313**	**(Intercept)**	-74.197871	90.649036	-0.819	0.473
	Sorbitol concentration	0.332472	0.200960	1.654	0.197
	Zeta potentiel	0.174299	0.312303	0.558	0.616
	Average particle size	-0.002054	0.023863	-0.086	0.937
	pH	18.964698	18.603826	1.019	0.383

CHAPITRE 4

DÉVELOPMENT DE FORMULATION EN DE SUSPENSION EFFICACE DE *SINORHIZOBIUM MELILOTI* CULTIVÉ DANS LES EAUX USÉES D'AMIDON

DEVELOPMENT OF AN EFFICIENT SUSPENSION FORMULATION OF STARCH INDUSTRY WASTEWATER GROWN *SINORHIZOBIUM MELILOTI*

DÉVELOPEMENT DE FORMULATION EN SUSPENSION EFFICACE DE *SINORHIZOBIUM MELILOTI* CULTIVÉ DANS LES EAUX USÉES D'AMIDON

T. Rouissi[1], R. P. John[1], R.D. Tyagi[1]*, D. Prévost[2], S. K. Brar[1], R Y Surampalli[3]

[1]INRS-ETE, University of Quebec, 490, Rue de la Couronne, Quebec, Canada G1K 9A9

[2]Agriculture et Agroalimentaire Canada, Quebec, QC, Canada G1V 2J3

[3]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

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RÉSUMÉ

Les formulations liquides et grains enrobés de *Sinorhizobium meliloti*, cultivée dans les eaux usées d'amidon ont été développées dans cette étude. Les additifs, tels que le saccharose, le sorbitol, le polyvinylpyrrolidone (10000) (PVP), le polyéthylène glycol (8000) (PEG), les alginates de sodium ont été étudiés.Les mélanges de PVP avec : le saccharose, le sorbitol et les alginates et de PEG avec : le saccharose, le sorbitol et les alginates, ont été aussi évalués pour leur capacité à protéger la viabilité de Sinorhizobium meliloti au cours du stockage réfrigéré. Les résultats montrent que toutes les formulations ont préservé la viabilité des cellules à des concentrations supérieures à 10^9CFU/ml durant 13 semaines de conservation. Le saccharose à 10% w/v était le meilleur additif avec une demi-vie de 83 semaines. Le suivi de la viabilité des cellules (formulations fraiches (FLF) et formulations conservées pendant 13semaines(OLF)) après application sur les semences de luzerne a montré que les formulations FLF de PEG et de PEG-Sorb ont maintenu des concentrations supérieures à 10^3CFU/grain (normes canadiennes) pour 4 mois de conservation. Parmi les formulations OLF, le PVP-sorb était le plus efficace, aboutissant ainsi au meilleur rendement en matière sèche (146 mg). La microscopie électronique à balayage a montré que les cellules de rhizobium sont réparties différemment à la surface des grains (comme agrégats ou répartie uniformément) selon les additifs utilisés.

Mots clés: *Sinorhizobium meliloti*, formulation, conservation, viabilité, rendement des plantes

ABSTRACT

Liquid and alfalfa seed coated formulations of *Sinorhizobium meliloti* (produced in starch industry waste water) were developed in this study. The additives, such as sucrose, sorbitol, polyvinylpyrrolidone (10000)(PVP), polyethylene glycol (8000)(PEG), sodium alginates, mixture of PVP (with sucrose, sorbitol and alginates) and PEG (with sucrose, sorbitol and alginates) were evaluated for their capacity to maintain viability of *Sinorhizobium meliloti* during 13 weeks of refrigerated storage. All formulations provided cell viability higher than 10^9CFU/ml. Sucrose at 10%w/v was the best additive to promote survival of cells as shown by the half life which was 83 weeks. Evaluation of liquid formulation (fresh and 13 weeks old) applied to alfalfa seeds showed that fresh formulation of PEG and PEG-sorbitol maintained cell viability of 10^3CFU/seed (Canadian standards) for 4 months of seed storage at ambient temperature. PVP-sorb formulation stored for 13 weeks was the most efficient, allowing the highest shoot yield on a dry matter basis. Micrographs showed that rhizobial cells were distributed differently on the seed surface (as aggregates or uniformly distributed) according to the additives used.

Keywords: *Sinorhizobium meliloti*, formulation, storage, viability, plant yield

1. Introduction

Application of bio-fertilizers has been increasing the last few decades, as an alternative to chemical fertilizers. For legume crops, inoculation of *rhizobia* in soils/ on seeds was demonstrated to be an efficient agricultural practice as bio-fertilizer (Stephens *et al.* 2000). The challenge of bio-fertilizer formulations is to provide a suitable microenvironment for the bacteria to survive in soil conditions when inoculated in the soil and guarantee its efficiency under field conditions. Selection of suitable additives, concentration of different additives and industrial formulation processes are considered as key factors in microbial formulations (Bashan 1998). The fast growing and alfalfa nodulating *Sinorhizobium meliloti* is usually formulated in solid carriers, while liquid inoculants are the most suitable formulations especially for use in planting machines (Stephens *et al.* 2000).

Solid inoculant carrier requires series of industrial processing steps, such as mixing, drying, sterilizing, milling, neutralization, packaging and use of large amount of additives which is costly and can affect commercial inoculant competitivity (Singleton *et al.* 2002; Tittabutr *et al.* 2007). In addition, the problem of carrier availability (peat) is an additional point to be considered. An overview of studies carried out on bio-inoculants has shown that liquid formulation followed by pre-inoculation of seeds is an efficient alternative that could overcome problems associated with solid carriers (Singleton *et al.* 2002; Tittabutr *et al.* 2007). Moreover, a high cost of industrial production of inoculum has been attributed to the culture media; therefore, development of formulation based on industrial wastewater is an interesting field of research. In previous work, it was demonstrated that starch industrial wastewater (SIW) supported growth of *Sinorhizobium meliloti* as well as standard medium (>10^9CFU/mL) and SIW is a low cost liquid waste (Rouissi *et al.* 2010). In this study, we have optimized the conditions for stable and efficient liquid and seed coated formulations of SIW grown *Sinorhizobium meliloti* by using several classes of additives (polymers and sugars) and their mixtures.

2. Material and methods

2.1 Microorganisms and inoculum preparation

Sinorhizobium meliloti A_2 (Agriculture and Agri-food Canada, Sainte-Foy, Québec, Canada) was used in the current study. Inoculum was produced in standard liquid medium, yeast mannitol broth (YMB) (Vincent 1970). This medium contained the following constituents: K_2HPO_4, 0.5 g/L; $MgSO_4.7H_2O$, 0.2 g/L; NaCl, 0.1g/L; yeast extract, 1.0 g/L; and mannitol, 10.0 g/L. Cells were grown at 30±1 °C for 24 h on a rotary shaker (Excella E25, NewBrunswick Scientific, Edison, New Jersey, US) at 180 rpm. Inoculum (3% v/v containing 10^9 CFU/mL) was added to the flasks.

Starch industry wastewater SIW (1.4% w/v total solids, ADM Ogilvie; Candiac, Québec, Canada) was used for the development of formulation for *Sinorhizobium meliloti* (John *et al.* 2010). pH was first adjusted to 7, yeast extract was added at 0.1% w/v (Rouissi *et al.* 2010). Sterilization was carried at 121 ± 1°C for 15 min. Fermentation was carried out for 72 h at 30°C under incubation conditions similar to inoculum production.

Cell count was estimated by plating on YMA plate, supplemented with Congo red (0.25% w/v) after a serial dilution using saline solution (NaCl, 0.85% w/v). Incubation of plates was carried out at 30 ± 1°C for 72 h.

2.2 Liquid formulations

Cells were grown until 4.5 x 10^9 CFU/mL. Suspension formulations were developed using the fermented SIW. Additives that were tested for formulations comprised: sorbitol at concentrations of 1, 2 and 5% w/v; sucrose at 2, 5 and 10 % w/v; sodium alginate at 0.2, 0.3 and 0.5 % w/v; polyvinyl pyrrolidine (PVP) (M_w=100000) at 1, 2 and 5 % w/v and polyethylene glycol (PEG) (M_w=8000) at 1, 2 and 5% w/v. Combination of polyvinyl pyrrolidine (PVP) at 1% w/v with sucrose at 5% w/v, sorbitol at 2% w/v and sodium alginate at 0.3%, and a combination of polyethylene glycol (8000 g/mol) at 1% w/v with sucrose at 5% w/v, sorbitol at 2% w/v and sodium alginate at 0.3% were studied. All additives were sterilized prior to addition. All formulations were kept at 4 °C and stability was checked based on the survival of bacteria every 3 weeks of storage.

2.3 Seed coated formulations

The selected chemicals additives used for seed coating in w/v were: polyvinyl pyrrolidine (PVP) at 1%, polyethylene glycol (8000) at 1%, sucrose at 5%, sorbitol at 2% and sodium alginate at 0.3%. Combination of polyvinyl pyrrolidine (PVP) at 1% either with sucrose at 5% or sorbitol at 2% or sodium alginate at 0.3% were studied. Formulations based on polyethylene glycol (8000) at 1% either with sucrose at 5% or sorbitol at 2% or sodium alginate at 0.3% were also studied. In these experiments, fresh fermented broth with viable cell count of $4.5x10^9$CFU/mL served as control.

Alfalfa seeds, *Medicago sativa* L seeds were coated with fresh (FLF) and 13 week old stored liquid formulations (OLF) and viability of the coated *S. meliloti* was determined with storage time. In order to coat the cells, 4 g of seeds were vigorously mixed for 2 min with 2 ml of each formulation (at ratio 2:1) and kept in contact for 2 h at ambient temperature. Drying was achieved by placing the samples in laminar flow for 12 h. Coated seeds (initial viable cells ≈ 10^6CFU/seed) were stored at ambient temperature (23 ±1°C) in petri-dishes until viable cells concentration decreased below 10^3CFU/seed.

In order to determine cell viability on the coated seeds, about 45 coated seeds were mixed with 4.5 ml of saline solution (NaCl, 0.85% w/v, supplemented with Tween 80 at 0.1% w/v) for 2 min; serial dilutions were used for cell count. YMA plate supplemented with Congo red (0.25% w/v) was used for cell plating and incubation of plates was carried out at 30±1 °C for 72 h.

2.4 Plant yield and nodulation index

Pre-inoculated alfalfa seeds with FLF (PEG 1% and PEG1%-Sorb 2%; 24h and 12 weeks of storage) and with OLF (sucrose 5% and PVP1%-sucrose 5%; 24h and 6 weeks of storage) were tested for their symbiotic efficiency. For each formulation, 10 alfalfa coated seeds were sown in sterilized plastic growth pouches (Mega international of Minneapolis, Mexico) fed with nutrient N-free solution (Vincent 1970). Fifteen pouches were used for each formulation. After germination, plants were kept in a growth room (at 20°C with a photoperiod of 16 h and at 15°C during the nights). Plants were irrigated regularly with deionized water. Plant yield was determined based on dry matter (plants were dried at 70°C for 48 h) after 6 weeks of growth. To calculate the nodulation index, visual estimation of nodule number (3 for many, 2 for several, and 1 for few)

was multiplied by the color (2 for pink and 1 for white) and by the size of root nodules (3 for large, 2 for medium and 1 for small); maximum nodulation index is18 (Ben Rebah *et al.* 2007).

These tests were carried after 24 h of coating, and after 12 and 6 weeks of seed storage for SLF and OLF, respectively (which corresponded to viable cells per seed of ≈ $3x10^3$CFU/seed).

2.5 Microscopic observation

Distribution of *Sinorhizobium meliloti* and additives used on seeds were examined using a scanning electron microscope (Carl Zeiss SMT, Cambridge, England). Coated seeds were first fixed to stud metal, coated with mild gold at a thickness of 0.01 mm and then exposed to an accelerate voltage of 10KV.

2.6 Statistical analyses

Statistical analyses were carried using R version 2.10.1 (Copyright (C) 2009, The R Foundation for Statistical Computing ISBN 3-900051-07-0). Analysis of variance (ANOVA) was used to evaluate significance of the difference between plant yield formulations. Difference was considered significant at $p < 0.05$ (α was fixed to 0.05). The error bars on the Figures indicate the mean standard deviations for three replicates.

3. Results and discussion

3.1 Survival of cells in liquid formulation during refrigerated storage

3.1.1 Sucrose formulations

Figure4.1a shows the cell viability during 13 weeks of storage at 4°C for sucrose at concentrations of 2, 5 and 10% w/v. These formulations maintained higher cell viability than the control (fermented broth). Viable population was similar at all concentrations studied (2, 5 and 10% w/v) until 9 weeks of storage (≈ 4.4 $\times 10^9$CFU/ml). At 13th week, formulations with 5 and 10 % w/v sucrose exhibited higher stability, which was close to the initial cell concentration (4.1 and 4.2$\times 10^9$CFU/ml respectively); viability of cells was 3.6$\times 10^9$CFU/ml in the formulation of 2% w/v of sucrose. Viability of cells in the control decreased steeply, by approximately one log cycle, after 6 weeks of storage and reached 2.8 $\times 10^8$CFU/mL (Fig 1a). At the end of storage period, viable cell count was 4.17$\times 10^5$CFU/ml (13 weeks of storage at 4°C).There was a major decrease was in the first six weeks only, after, it tended to decrease slowly. Sucrose has rarely been investigated as suspending agent; it has been commonly used as growth substrate and non-ionic osmoregulatory agent. Addition of sucrose helps to maintain the stability of proteins and cell structure during osmotic stress (Deaker *et al.* 2004). In the current study, efficiency of sucrose as a suspending and protecting agent during refrigerated storage was proved; and cell count remained higher than 4 × 10^9 CFU/mL for all concentrations studied after 13 weeks of storage.

Mechanisms involved in the stability of formulation are mainly related to the hydrogen bonding between sucrose and proteins, which may be responsible for the cell stabilizing effect (Tsevtkov *et al.* 1989; Deaker *et al.* 2004). Gouffi et al (1999) demonstrated that sucrose is an effective osmoprotectant for *S. meliloti*, *R. leguminosarum* bv. *trifolii* and *phaseoli.* Sucrose has been showed to be a superior suspending agent in comparison to glucose, xylose, tryptophane, salicin, saline and water for the protection of *Streptococcus pyogenes* C203 and *Escherichia coli* communion during desiccation (Heller, 1941; Deaker *et al.* 2004) and was able to enhance survival of *R. japonicum* USDA 138 in biopolymer gel formulations (Mugnier and Jung, 1985).

3.1.2 Sorbitol formulations

The storage profile of formulations with sorbitol at different concentrations of 1, 2 and 5 % w/v are given in Fig. 4.1b. In the first six weeks of storage (at 4°C), viability increased with the concentration of sorbitol added. For instance, at 3rd week, a cell count ≈ 3.5×10^9 CFU/mL was observed with the formulation of 1% w/v sorbitol whereas cell count of ≈4×10^9 CFU/ml for 2 and 5% w/v of sorbitol was obtained. From 6th to 13th week of storage, all sorbitol formulations were equally effective and maintained an unchanged *S. meliloti* population and the final cell count was 3.3×10^9 CFU/mL, 3.4×10^9 CFU/mL and 3.5×10^9 CFU/mL for 1, 2 and 5% w/v of sorbitol, respectively.

One of the major microbiological applications of sorbitol is cell protection during freeze drying (Carvalho *et al.* 2003; Abadias *et al.* 2001; Linders *et al.* 1997). Annear (1962) pointed out the efficiency of sorbitol-peptone mixture in preserving several vacuum dried bacteria on cellulose fibers at room temperature. The mechanisms by which sorbitol can protect living cells could be the stabilization of lipids and proteins of the cell membrane (Crowe *et al.* 1988). Sorbitol was also demonstrated to be efficient in stabilization of the structure of collagen (Usha *et al.* 2006) and in protection of ovalbumin, lysozyme, conalbumin, and α-chymotrypsinogen against heat denaturation (Back *et al.* 1979). Sorbitol has been reported to strengthen the bridge interactions between polypeptide chains by replacing the water molecules (Gekko and Koga 1983).

3.2 Polymeric formulations

3.2.1 Polyethylene glycol (PEG) formulations

Polyethylene glycol (PEG) (average molecular weight =8000 g/mol) at concentrations of 1, 2 and 5% w/v was used as an additive. Fig. 4.1c shows the viability profile during storage of these formulations. *S. meliloti* viability varied among the concentrations studied in the first nine weeks of storage. A steady phase in cell count was observed for first 6 weeks with formulation of 1 and 2 % w/v polyethylene glycol and for 3 weeks for the formulation of 5 % w/v. Later, there was a continuous decline in terms of viable cell count. The slope of decline of viable cells count with time was more pronounced in the 1% w/v formulation of than that of 2 and 5% w/v, leading to comparable values in the 9th week of storage (≈1.9×10^9CFU/mL). From the 9th week to the end of storage period, a stable phase was observed for all polyethylene glycol concentrations studied, and the final cell count ranged from 1.6 X 10^9 to 1.7 X 10^9 CFU/mL.

Polyethylene glycol is a seed coating adhesive for bacteria with an effective cryoprotectant for proteins (Pofits 1994) and microorganisms prior to freezing or drying (Hubalek 2003). Addition of polyethylene glycol 6000 to soil provided protection for fast growing strains (Bushby and Marshall 1977; Deaker *et al.* 2004). Priming soybean seeds in PEG 8000 solution improved germination and seedling establishment by stimulating physiological and biochemical activities (Khalil *et al.* 2001). PEG 3000 was studied by Tittubtr *et al.* (2007) in liquid formulation and they observed that addition of this polymer led to the development of stable formulations of *Azorhizobium caulinodans* IRBG23 and *Bradyrhizobium japonicum* USDA110 during 6 months of storage.

3.2.2 Polyvinyl pyrrolidine (PVP) formulations

Polyvinyl pyrrolidine (PVP) (average molecular weight =10000 g/mol) was also tested as an additive at 1, 2 and 5 % w/v as seen in Fig 4.1d. Among these concentrations, 2%w/v of PVP maintained the highest cell viability (final viable cell count $\approx 1.8\times10^9$ CFU/ml). For the concentrations of 1 and 5% w/v, final cell count ranged from 1.45 to 1.54 $\times$ 10^9 CFU/ml.

PVP has been described as a polymer with high water-binding capacity, largely used as seed coating adhesive for bacteria. Some researchers observed that the insoluble PVP form was able to bind seed exudates, naturally mobilized during inoculation and seed germination (Deaker *et al.* 2007). PVP improved viability of *B. japonicum* SEMIA 5019 when mixed with the culture broth and as added to peat (Denardin and Freire 2000) and when applied to soybeen seed (Singleton *et al.* 2002). Generally, under the storage conditions studied, PVP formulations maintained comparable cell viabilities compared to PEG. Tittubtr et al (2007) proposed an adsorption mechanism by which the polymer formed a thin molecular layer on the surface of the individual particles, which resulted in a stabilized suspension that prevents cell membrane damage, consequent reduction of O_2 and nutrient diffusion from media to cells, leading to a stable cell count during storage.

3.2.3 Sodium alginate formulations

Fig 4.1e shows the storage profile of sodium alginate formulations. Formulations based on sodium alginate as an additive showed a viability of cells more than $3X10^9$ CFU/ml for all concentrations studied (0.2, 0.3 and 0.5% w/v), over 13 weeks of storage at 4°C as seen in Fig 1e. Sodium alginate added at 0.3 % w/v was the most effective formulation in promoting

rhizobial survival: cell count was ≈3.7×10^9 CFU/ml, at the end of storage whereas it was 2.7×10^9 CFU/mL and 3.3×10^9 CFU/mL for the concentrations of 0.2 and 0.5% w/v, respectively.

Efficiency of sodium alginate has been already demonstrated for cell encapsulation and it is the most common biopolymer used for industrial purposes (Chen and Huang 1988; Fenice *et al.* 2000; Paul *et al.* 1993; Bashan *et al.* 2002). One of the main advantages of alginate is non-toxicity and biodegradability in the soil which is an interesting point in rhizobia formulation (Bashan 1998). Tittabutr *et al.* (2007) observed that sodium alginate added at 0.1% w/v to liquid inoculants improved the survival of *Bradyrhizobium japonicum* USDA110, and *Mesorhizobium ciceri* USDA2429 cultivated in standard media, which remained at cell concentrations of 10^7-10^8 cells/ml and *Sinorhizobium fredii* HH103 from $10^5$to 10^6 cells/ml after 6 months of storage at 28-30°C. Mugnier and Jung (1985) allowed the protective nature of alginate and biopolymer in general to its high water activity and stabilizing effect of cell membrane (Holcberg *et al.* 1981; Groboillot *et al.* 1994).

3.3 Formulation with combination of additives

Mixture formulations were developed in order to study the effect of combination properties of sorbitol, alginates, sucrose, PEG and PVP in refrigerated storage. Results with mixtures of PEG-sorbitol, PEG -sucrose, PEG-alginates, PVP sorbitol, PVP-sucrose, and PVP-alginates are shown in Figure 1f and 1g. The survival of bacteria was slightly higher in the formulations of (PEG 1% w/v + sorbitol at 2% w/v) and (PVP 1% w/v + sorbitol at 2% w/v) during storage at 4°C. After 12 weeks, the cell count was 1.53 and 1.45 $x10^9$ CFU/ml for PEG (1% w/v) + sorbitol (2% w/v) and PVP (1% w/v) + sorbitol (2% w/v), respectively.

3.4 Comparison between formulations

Comparison was carried out between formulations to select the most effective additives and concentrations that can be used to promote the *Sinorhizobium meliloti* survival in liquid formulation, during storage at 4°C. The comparison was based on calculation of half life ($t_{1/2}$) according to the following Equation:

Viable cell count = a*exp (-b*t)), t: storage period in weeks, a and b are constants

$$t_{(1/2)} = -[\log(\text{(initial viable cell count)}/2*a)]/b \qquad (1)$$

The half life results for the different formulations are shown in Figure 2. All probability values (*p*) related to the models used were < 0.05. The most efficient formulations were those containing sucrose followed by alginates, sorbitol, PEG, PVP and finally combination formulations. The best sucrose concentration was 10% w/v; with a half life of approximately 83 weeks, while half life of control was 1.6 weeks. The half lifves of sucrose at 5 and 2% w/v were 72.5 and 42 weeks, respectively. Alginates at 0.3 % w/v was the second best formulation, this concentration guaranteed a half life of 44 weeks. Half-life of *Sinorhizobium meliloti* at all sorbitol concentrations were similar (around 29 weeks). When sucrose was mixed at 5% w/v with PEG or PVP at 1% w/v, the half life decreased to 6.4 and 5.8 weeks, respectively. Sucrose formulation at 5% w/v showed half life of 72.5 weeks, half life for PEG and PVP at 1% w/v were 9.8 and 7.2 weeks, respectively. The same reduction was observed in the case of sorbitol 1% w/v when mixed with polymers, from 29.5 weeks to 7.4 and 7.9 when mixed with PEG and PVP at 1% w/v respectively.

3.5 Effect of storage and additives on *Sinorhizobium meliloti* viability on coated seeds

3.5.1 After 24h of storage

Old liquid formulation (OLF) and fresh liquid formulation (FLF) were compared for their ability to support survival of *Sinorhizobium meliloti* when applied to alfalfa seeds, immediately after 24h of storage at ambient temperature, without controlling the humidity to simulate real storage conditions. Inoculant efficiency (%) was defined as:

(%) cells viability on seeds = viable cell count per seed after 24h/ initial cell count applied per seed (2)

Initial cell count applied per seed= cell count in formulation (CFU/ml) / Number of seeds mixed with 1 ml of formulation (3)

There was a substantial decrease of *Sinorhizobium meliloti* viability on alfalfa seeds (%) after 24h in all formulations (Table1).

The most efficient seed formulations were PEG 1% -sucrose 5% and PEG 1%-alginates 0.3% for both FLF and OLF. These formulations maintained 22.3 and 19.2 % of cells added per seed

for FLF; and 15.9% and 13% for OLF, respectively. Addition of alginates at 0.3% w/v, sorbitol at 2% w/v or sucrose at 5% w/v to PEG and PVP formulation 1% w/v improved inoculant survival.

By comparison between seeds coated with FLF and OLF, cell viability decreased for all additives used when liquid formulations were stored. This was mainly observed for sucrose 5% w/v (from 21.7% for FLF to 1.5% for OLF) and for sorbitol 2% w/v (from 13.8% for FLF to 0.4% for OLF). During seed coating, the first important formulation property was adhesiveness and protection against desiccation (Tittabutr *et al.* 2007): weak adhesion resulted in low cells concentration entrapped on seed surface. This could explain the low inoculant efficiency observed in the case of alginate formulation at 0.3 % w/v, where quantity of polymer added was the lowest used in this study. It was demonstrated that survival of rhizobia on seed depends on kinetics of water loss and/or moisture sorption characteristics of the polymeric adhesives and anti-desiccant used (Deaker *et al.* 2007) within a studyi of the survival of *R. leguminosarum* bv. *trifolii* coated to glass beads as model under dry conditions, Vincent et al (1962) described two distinct phases of death in relation to loss of water, the first: between 0 and 24 h, characterized by a rapid loss in cell numbers, the second phase was characterized by a decline of the death rate and continued loss of water. Efficient formulation should protect cells after coating, especially against desiccation, the phenomenon which induces changes in cells membrane permeability during storage. When polymers are combined with sucrose and sorbitol, the cell membrane is protected against osmotic stress conditions, increasing oxygen permeability and toxic effect of seed exudates, by complementary properties of the additives used; PVP and PEG have a high water binding capacity which could maintain sufficient water around cells, reduced permeability and death and explain these efficiencies (Tittabutr *et al.* 2007). Sorbitol and sucrose stabilized cell membrane during osmotic stress conditions and improved survival of rhizobia on seeds and beads (Carvalho *et al.* 2004, Deaker *et al.* 2004). Therefore, formulation of these components will combat against desiccation and osmotic pressure.

When liquid formulations were stored for 13 weeks (OLF) prior to seed coating, a general decrease was observed in the percentage of cells viability in comparison to FLF. This could be mainly due to the altering effect of long time storage in cell membranes (Russel 2002), resulting in increase of cell sensitivity to desiccation and higher percentage of death.

3.5.2 During storage

Results of cells viability on coated seeds are shown in Figures 4.3 and 4.4 for FLF and OLF, respectively. During storage, the effect of desiccation continued following drying. To select the most efficient additives, a comparative study was carried out between seeds coated with (FLF) with those coated with (OLF). Seeds were stored at ambient temperature. The high number of live rhizobial cells is one of the most important criteria for inoculant quality. Improving survival of the cells on seeds would directly affect nodulation and subsequent yield of the plant (Deaker *et al.* 2004). According to Canadian Standards (CFIA), requirement of minimum number of *Sinorhizobium meliloti* cells delivered per seed for commercial products is 10^3 (Ben Rebah *et al.* 2007).

All the formulations tested were equally effective in maintaining the initial rhizobial population of higher than 10^4CFU/seed after 24h of coating. Best protection during storage was achieved by application of fresh formulations. Survival of cells on seeds coated with PEG at 1% w/v and combination of PEG 1% w/v with sorbitol at 2% was in conformity with Canadian standards over 130 days of storage as seen in Figure 4 b.

The protective nature of these formulations was possibly due to PEG properties. In general, polymers have the ability to limit water transfer (evaporation) by acting as a barrier, which could protect cells during long term storage (Mugnier and Jung 1985). However, in this research, when seeds were coated with stored formulations, higher cell viability were obtained with formulations of sucrose at 5% w/v, followed by combination of PVP 1% with sorbitol at 2% w/v; cell viability remained higher than $3.5x10^3$ CFU/seed over 35 days of storage (Canada standards: minimum number =3 $x10^3$ CFU/seed).

These results suggested an existing interaction between period of storage of liquid inoculant and additives before application to seeds. In fact, physiology of cells seems to be negatively affected by prolonged liquid inoculant storage due to morphological changes (Albareda *et al.* 2008). When applied to seeds, *S. meliloti* cells are directly exposed to oxygen which acts as an additional factor affecting viability, damaging proteins, membranes and nucleic acids (Deaker *et al.* 2004). Sorbitol and sucrose were demonstrated to be efficient in maintaining macromolecular structure which may improve biological integrity, and consequently survival of cells. The protective function was related to the ability of these additives to replace the water lost

during desiccation and thus preventing formation of unfavourable conformations in labile structures (*Salema et al.* 1982).

3.6 Inoculant evaluation for plant yield and nodulation index

Shoot dry matter plant yield of stored and 24h coated seeds with OLF and FLF, respectively, are shown in Figures 4.5a and 4.5b. For fresh coated seeds, best results were obtained in seeds coated with OLF of PVP 1%-sorbitol 2% w/v, followed by seeds coated with FLF of PEG 1%-sorbitol 2% w/v. Plant yields for these two combined formulations were 145 mg and 122 mg, control (non coated seeds) was 38 mg, respectively. When the sown seeds were coated with formulations of sucrose at 5% w/v and PEG at 1% w/v, dry matter content was almost comparable to each other and it was around 105 mg.

Stored coated seed formulations were sown when viable cell count was between 3 and 3.5 10^3 CFU/seed, which corresponded to a storage period of 6 and 12 weeks for OLF and FLF respectively before sowing, respectively. Plant yields were all comparable.

All formulations allowed similar nodulation indicies of alfalfa as determined by nodulation index. This similar observed nodulation (index value was at maximum, 18) could be due to the fact that all formulations were applied at very high number of *Sinorhizobium meliloti* cells (higher than 10^5CFU/seed). The same tendency was observed for stored coated seed formulations with a nodulation index of 12.

The effect of formulations on plant yield was not dependent only on the number of viable cells per seed at sowing but also with additives. For example, plant yield was 122 mg for the formulation of PEG-sorb and 147mg for the formulation PVP-sorb (containing 8 times fewer rhizobial cells than PEG-sorb).

The same trend was observed when comparison was made between formulations of sucrose at 5% (OLF) and of PEG at 1% w/v (FLF): the plant yields were almost the same, although viable cells was 10 times higher with PEG (8.5 x 10^5CFU/seed), respectively.

The higher performance of some formulations containing a lower number of rhizobia cells could be due to specific chemical and physical properties that may protect cells from dehydration damage on coated seeds. This hypothesis is supported by microscopic observations of coated seeds (Fig.4.6 and 4.7) which showed that rhizobia are distributed on seed surface in a varied manner depending on the additives. With PVP-sorbitol (OLF), cells were entrapped in a matrix

and they bound together in clusters: seed surface was rough due to the higher repartition of these aggregates with approximately 50 cells per cluster. This kind of arrangement allowed better protection of cells, binding more water which improved cellular metabolism activity, the initial step of the nodulation process and would consequently increase nitrogen fixation and plant yield.

With PEG and PEG-sorbitol formulations, individual cells were uniformly distributed in multiple layers. Sucrose was similar to PEG and PEG-sorbitol, but with fewer of layers of cells on the seed surface. Thus, these non-aggregated cells are not likely protected against the damage caused by water uptake during seed imbibition. This distribution may slow the rhizobia growth by delaying the recovery of optimal cellular activity and consequently delaying the nodulation process. In our experiment, although we did not see differences in the nodulation index after 6 weeks, appearance of nodules could be delayed with these formulations. Thus, formulation efficiency is not only related to the initial cell count applied and the period of storage, other factors concerning cell distribution on the seed surface do play a major role during nodulation.

This study showed the complexity of criteria for determining bioinoculant efficiency. Many questions remain regarding the physiological response of cells to the liquid formulation from storage phase to the final application phase of sowing. In fact, maintaining high level of viable cells in liquid or seed coated formulations is not a sufficient criterion to select best formulation. During all storage phases and subsequent final application, bioinoculant formulation should guarantee cell performance until seed germination and plant growth.

4. Conclusion

A low cost effective biofertilizer formulation can be developed by growing *Sinorhizobium meliloti* in starch industry waste (as an alternative to standard media). In this research, all liquid formulations that were developed maintained cell viability higher than 10^9 CFU/mL. Sucrose was the best additive to promote viability of *Sinorhizobium meliloti* during storage in liquid formulation: the longest half life was obtained at concentration of 10 %w/v (83 weeks). For seed coated formulations, FLF (fresh liquid formulation) supplemented with PEG and PEG-sorb guaranteed viability higher than 10^3CFU/seed during 4 months of storage. The effectiveness of formulations was tested on alfalfa and the highest shoot dry matter was obtained with combined formulation (PVP-sorb and PEG-sorb, for OLF and FLF, respectively) that also gave best results despite lower viability during liquid phase storage compared to other developed formulations. Thus, we recommend these formulations for development of *Sinorhizobium meliloti* liquid biofertlizer. It was also noted that the cell distribution on the seed surface following coating varied among the additives used. Further studies are needed to understand mechanisms which affect cell viability on seeds.

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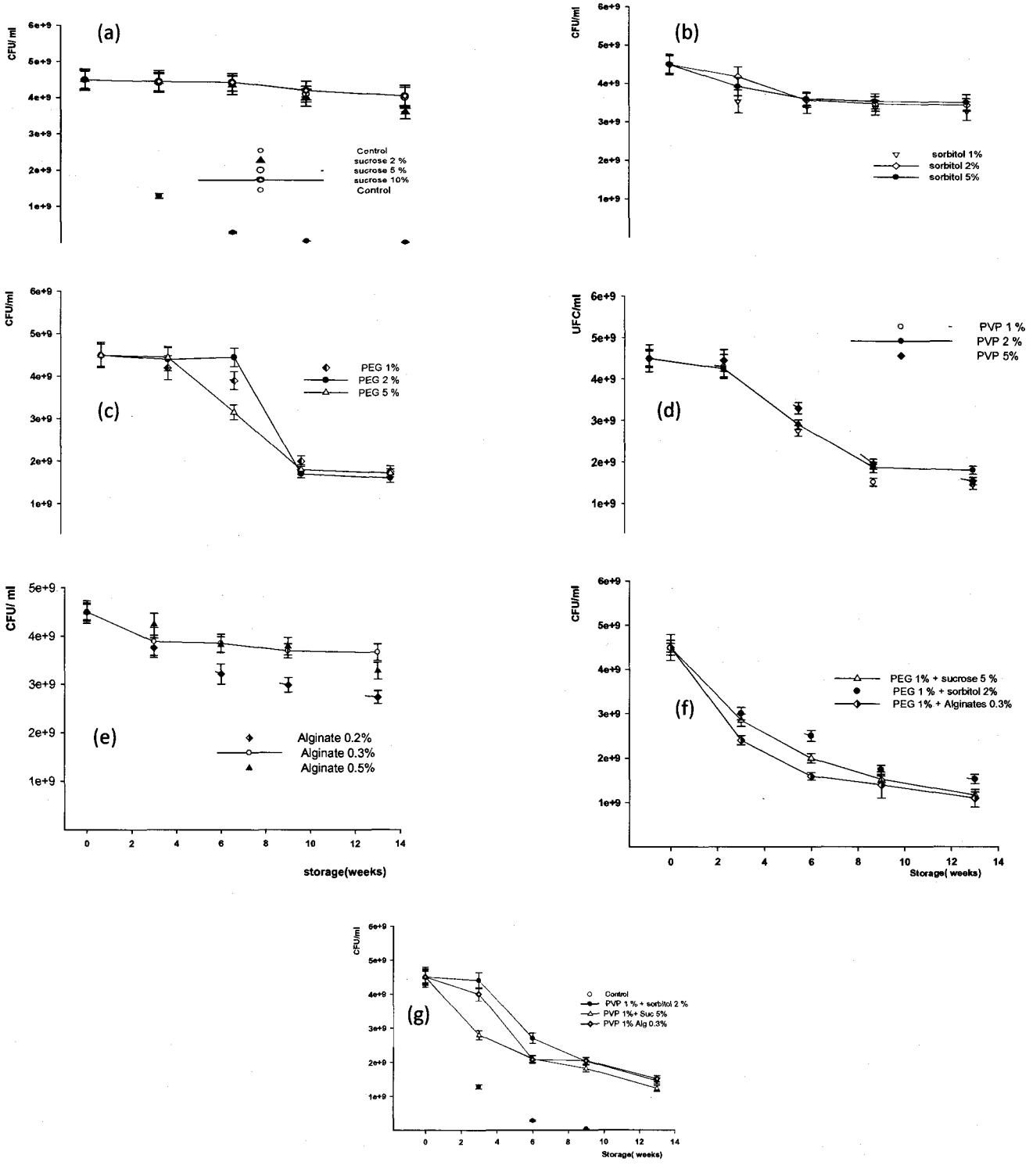


Figure 4.1. Survival of *Sinorhizobium meliloti* in liquid formulations at 4°C (a) sucrose; (b)sorbitol; (c) PEG; (d) PVP; (e) alginate; (f) PEG + sucrose, PEG+ sorbitol, PEG + alginates; (g) PVP + sucrose, PVP+ sorbitol, PVP+ alginates

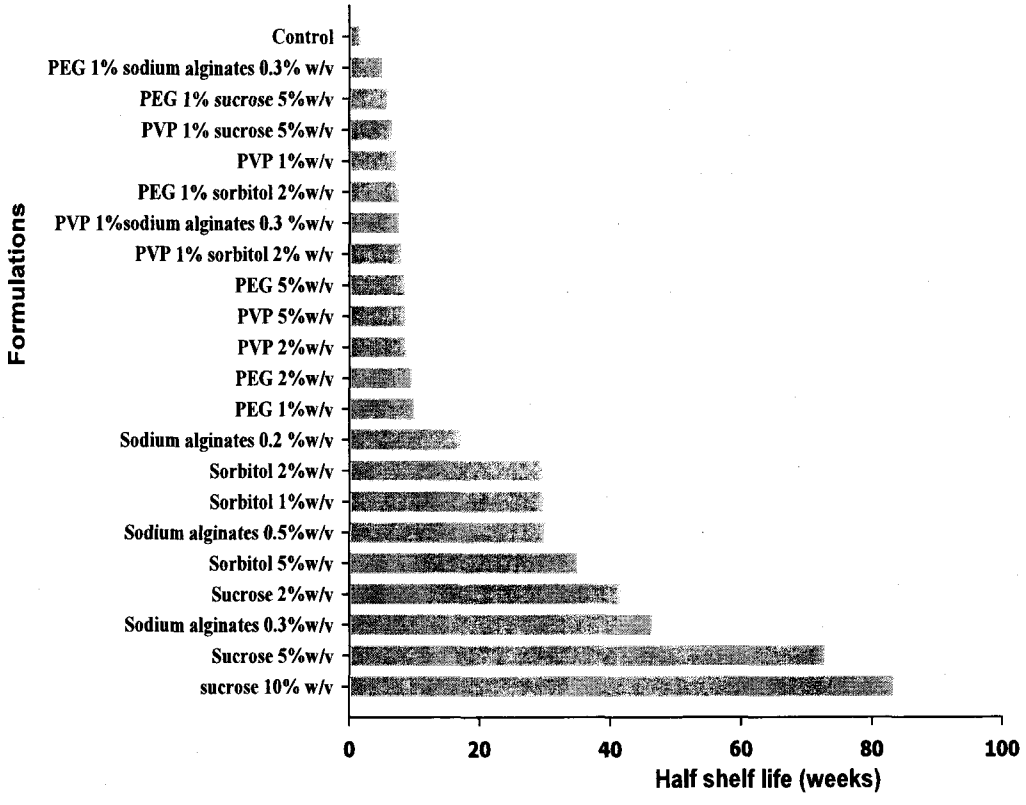


Figure 4.2. Half life of different rhizobial liquid formulations

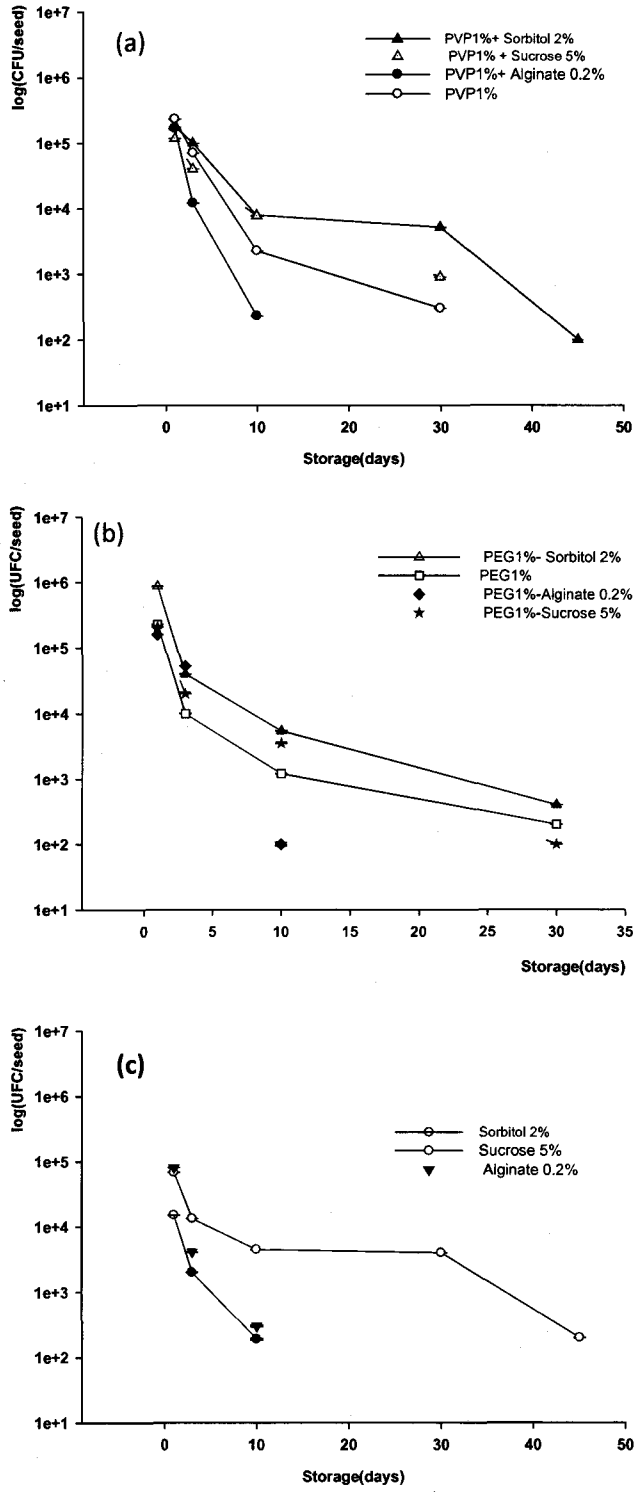


Figure 4.3 Survival of *Sinorhizobium meliloti* cells on alfalfa seeds coated with OLFsupplemented with: (a) PVP formulations ;(b) PEG formulations; (c) sucrose , sorbitol and alginates

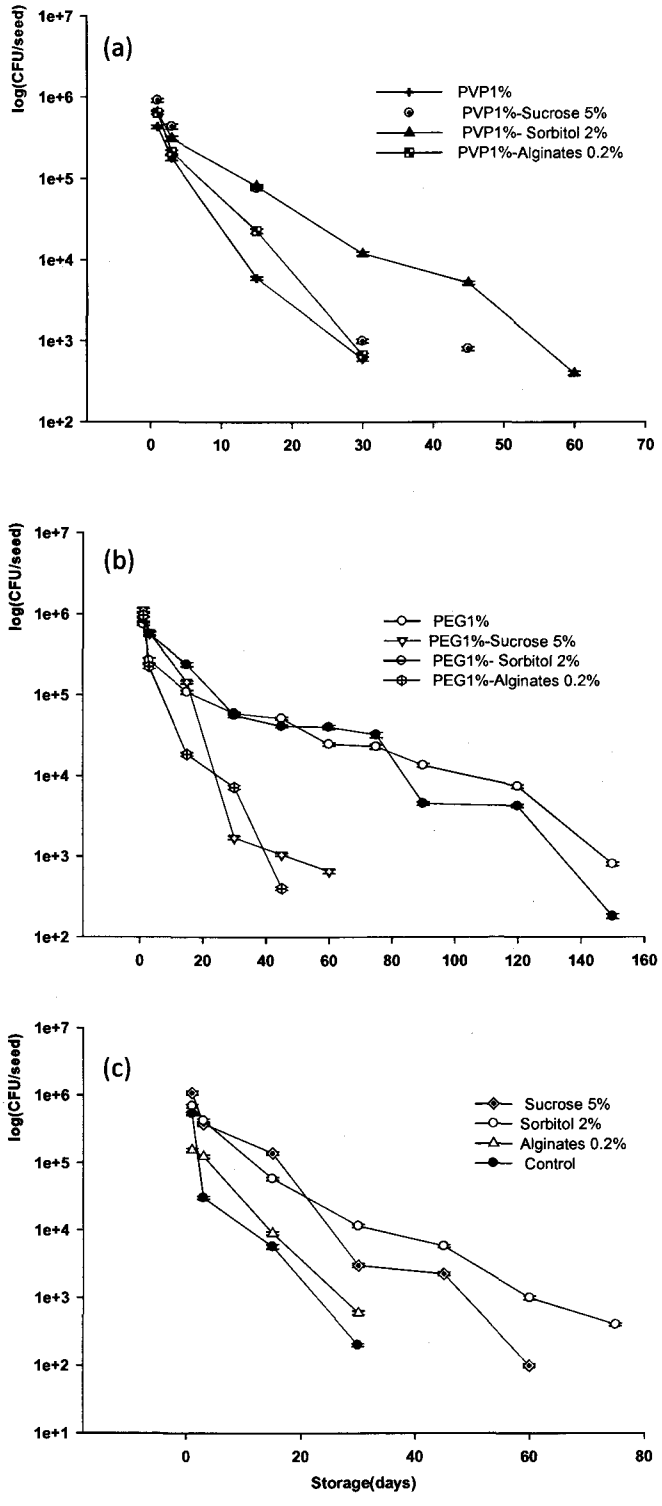
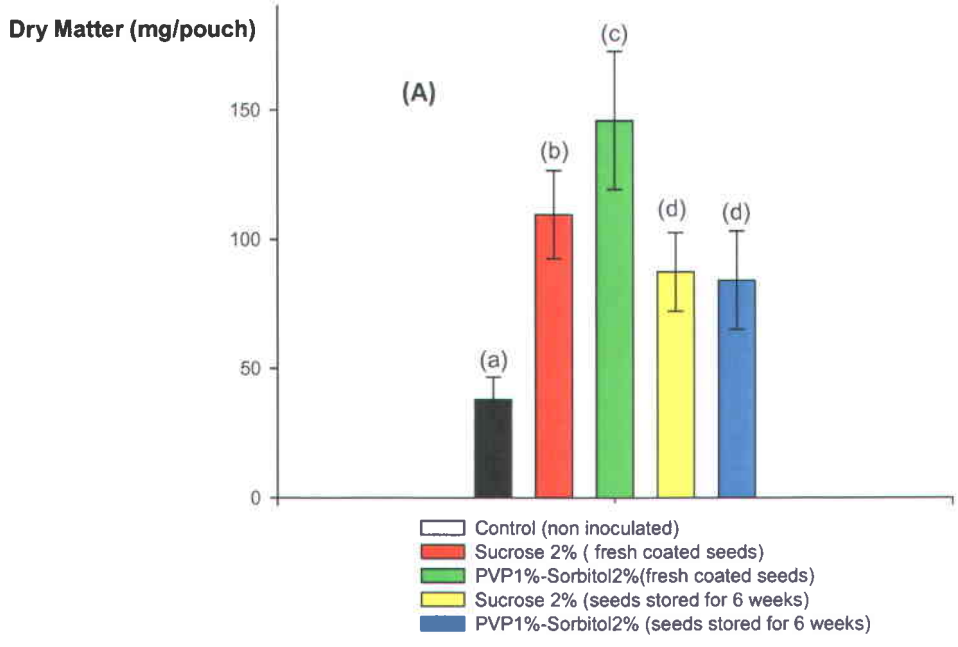


Figure 4.4 Survival of *Sinorhizobium meliloti* cells on alfa alfa seeds coated with fresh liquid formulation (FLF) supplemented with: (a) PVP formulations; (b) PEG formulations ;(c) sucrose, sorbitol and alginates, control is fermented SIW

Table 4.1: Percent cell viability on seeds after 24h of coating

Formulations	PVP1%	PVP1%-Sucrose 5%	PVP1%-Sorbitol 2%	PVP1%-Alginates 0.3%	PEG1%	PEG1%-Sucrose 5%
FLF	14.4	18.2	13.0	12.8	14.9	22.3
OLF	8.0	8.6	11.0	10.0	11.6	15.9

PEG1%-Sorbitol 2%	PEG1%-Alginates 0.3%	Sucrose 5%	Sorbitol 2%	Alginates 0.3%
17.2	19.2	21.7	13.8	3.1
ND	13.0	1.5	0.4	2.0



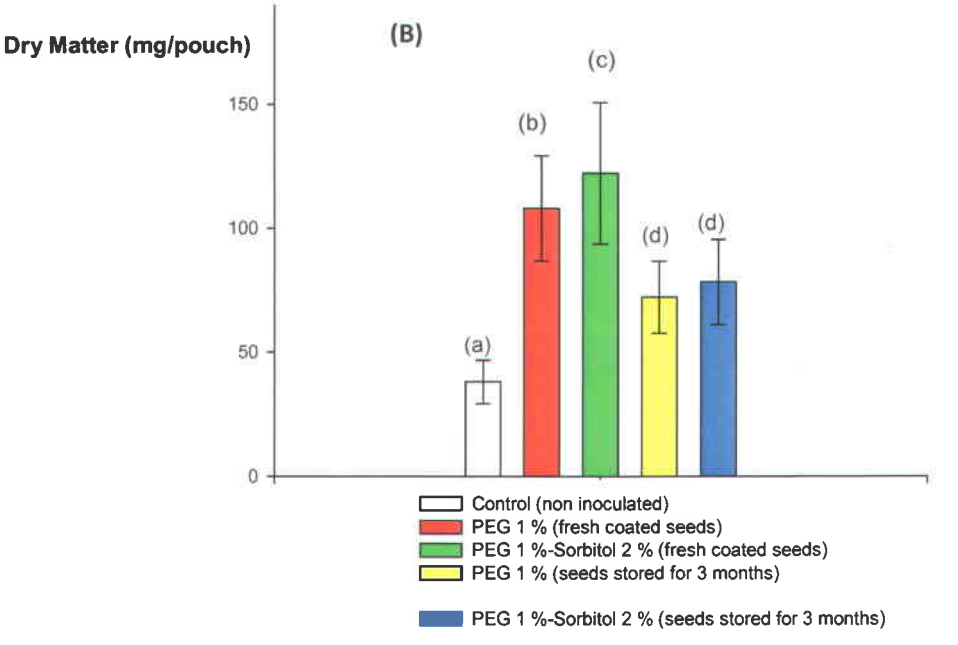


Figure 4.5: Shoot dry matter yield of alfa alfa inoculated with: (A) formulations based in 13 weeks stored liquid formulation(OLF); (B) formulations based in fresh liquid formulation(FLF), formulations followed by the same letter, in the Figure are not significantly different at P <0.05.

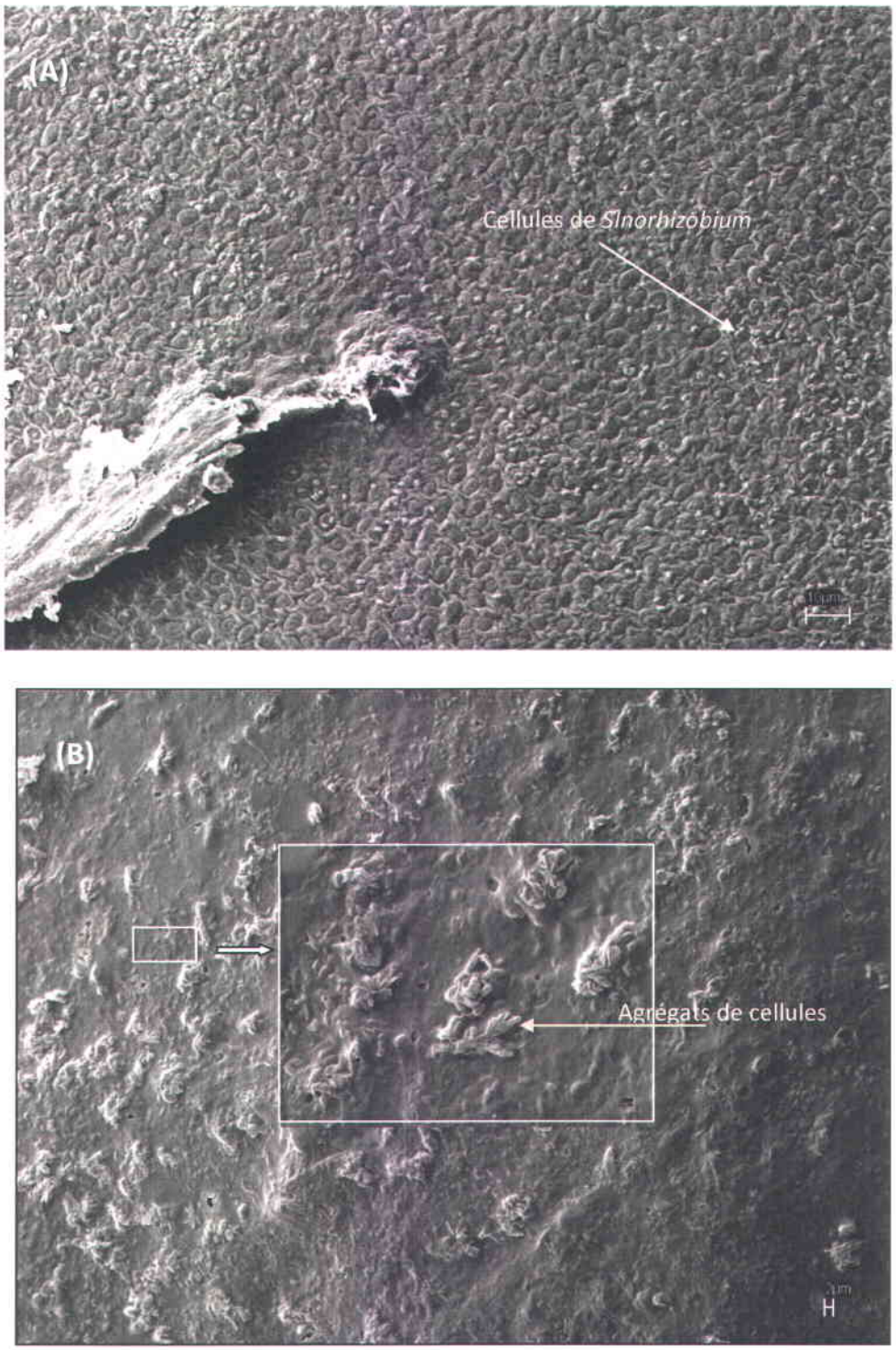


Figure 4.6: Micrographs of *Sinorhizobium meliloti* on alfa alfa seeds coated with sucrose 5%(A), and PVP1%-sorb2%(B)

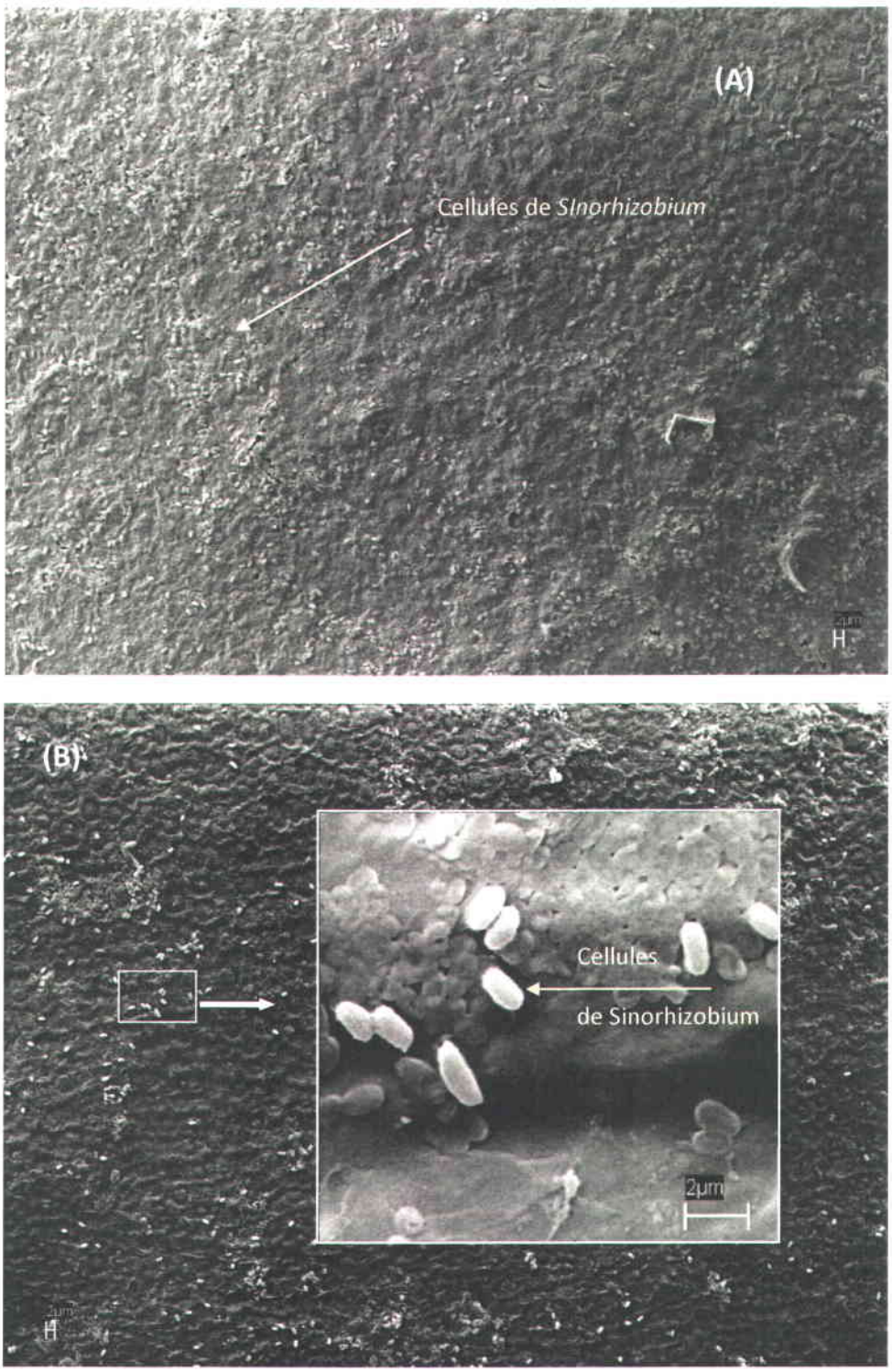


Figure 4.7. Micrographs of Sinorhizobium meliloti on alfa alfa seeds coated with PEG (A), and PEG sorbitol (B)

CHAPITRE 5

OPTIMISATION DU SÉCHAGE PAR ATOMISATION DU SINORHIZOBIUM MELILOTI PAR LA MÉTHODE DE RÉPONSE DE SURFACE ET DÉVELOPPEMENT DE FORMULATION EN POUDRE

OPTIMIZATION OF SPRAY DRYING BY RESPONSE SURFACE METHODOLOGY FOR THE PRODUCTION OF *SINORHIZOBIUM MELILOTI* POWDER FORMULATION BY USING STARCH INDUSTRY WASTE WATER

OPTIMISATION DU SÉCHAGE PAR ATOMISATION DU SINORHIZOBIUM MELILOTI PAR LA MÉTHODOLOGIE DE SURFACE DE RÉPONSE ET DÉVELOPPEMENT DE FORMULATION EN POUDRE

T. Rouissi[a], R.D. Tyagi[a], S.K. Brar[a *], R.P. John[a], D. Prévost[b] , R Y Surampalli[c]

[a] INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec (Qc) G1K 9A9, Canada

[b]Agriculture and Agroalimentaire Canada, Quebec, QC, Canada G1V 2J3

[c]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

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RÉSUMÉ

Le séchage par atomisation a été optimisé en utilisant la méthodologie de réponse de surface. Les paramètres optimisés sont : la température de séchage, la pression de pulvérisation, le débit d'air de séchage et le débit d'alimentation. Le lactosérum à 10% w / v, le sorbitol à 1% w/v et le saccharose à 1% w/v ont été ajoutés au bouillon fermenté concentré avant séchage. Les réponses étudiées étaient la viabilité des cellules, l'humidité et la température à la sortie. Dans les conditions optimales (température de séchage de 105°C, un débit d'air de séchage de 0.56m3/min, une pression d'atomisation de 0.07 MPa et un débit d'alimentation de 8 ml/min) qui ont permis d'atteindre le maximum de désirabilité (0,81), la viabilité des cellules obtenue était de $6.8x10^9$CFU /g, l'humidité était de 11,4% et la température de sortie de 42°C. La viabilité des cellules dans la poudre séchée a été maintenue supérieure à10^9 UFC / g au cours de 4 mois de conservation réfrigérée. Les formulations séchées ont donné le meilleur rendement en matière sèche (144.6mg) ainsi que le meilleur indice de nodulation (15), en comparaison par rapport aux cellules fraîches cultivées dans l'YMB (131 mg, 7.2). Les micrographes ont montré que les cellules de rhizobium étaient incorporées dans le mélange d'additifs (lactosérum-saccharose-sorbitol) qui les enrobait comme une matrice protectrice.

Mots clés: *Sinorhizobium meliloti,* formulation, optimisation, séchage par atomisation.

ABSTRACT

A Spray-drying process was optimized by response surface methodology for the development of a powdered formulation of *Sinorhizobium meliloti* (produced in starch industry waste water). The process parameters comprised temperature drying, air drying rate, spray pressure and feed sample rate. Cheese whey powder, sorbitol and sucrose were added at 10, 1 and 1 % w/v, respectively before drying. Responses studied were cell viability, moisture and outlet temperature. Under the optimal conditions (maximum desirability =0.81), the formulation showed cell viability of 6.8 10^9 CFU/g, moisture of 11.4% and temperature outlet of 42°C. During 4 months of refrigerated storage, viability was maintained higher than 10^9 CFU/g. Analysis of inoculated alfalfa plants showed that powder formulation induced similar shoot yield on dry basis (144.6mg)(p=0.23) and nodulation index (15.2) (p=0.18) compared to fresh cells cultured in standard media (shoot yield and nodulation index were 131mg and 14, respectively). Micrographs showed that cells were embedded in the mixture of additives (whey-sucrose-sorbitol) which acted as a matrix

Key words: *Sinorhizobium meliloti,* formulation, optimization, spray-drying.

1. Introduction

There have been increasing interests in biofertilizer application over the last few decades (Stephens and Rasks, 2000). Development of research in this area has demonstrated the effectiveness of rhizobia inoculation of seed legumes and the feasibility of development of practical formulations (Brockwell and Bottomley, 1995; Bashan, 1998). In field applications, peat powder formulations are the most commonly used bioinoculant form by farmers (Bashan, 1998). For large scale industrial production, multiple and successive processing is required to obtain a successful and efficient product, which involves sterilization, grinding, inoculum preparation, the curing phase and packing (Olsen *et al.*, 1995). Thus, in addition to peat availability, risk of contamination, high cost, and storage problems make formulation a challenge for inoculant industry (Olsen *et al.*, 1996; Smith, 1992). In literature, lots of researches have been focused on finding an alternative carrier than peat (Daza *et al.*, 2000; Ferreira et Castro, 2005; Albareda *et al.*, 2008). As processing (such as, carrier sterilization, inoculation and maturation phase) was the same as for peat formulation, development of new solid bioinoculants is still not interesting for large scale industrial production.

Spray drying is the predominant industrial process for high production rate of bacterial cells in dry powder, at relatively lower cost (Morgan *et al.*, 2006). In biotechnology, this process was mainly applied to lactic acid bacteria cultures (Kim *et al.*, 1990; To *et al.*, 1997; Wen *et al.*, 2002) which allowed production of instant powdered cell formulations. Despite development of this technology, it has rarely been investigated for production of rhizobial powdered bioinoculants (Ochin *et al.*, 1986; Mary *et al.*, 1993). During spray-drying, rhizobia cells are exposed to heat and dehydration damage. Thus, optimization was necessary to reduce cell death and increase efficiency of this process, which involved a simultaneous study of all working parameters and effects on cell viability (Adjalle *et al.*, 2011).

In this case, a study on spray drying feasibility of *Sinorhizobium meliloti* cells produced in starch industry waste water was carried and the process were optimized by response surface methodology in order to develop a stable, cost effective and efficient powder formulation.

2. Materials and Methods

2.1 Bacterial strain and culture medium

Sinorhizobium meliloti strain used in this study was A2 (Agriculture and Agri-Food Canada, Quebec, (Qc), Canada). Production of cells was carried in starch industry wastewater (SIW) (1.7% w/v total solids, ADM Ogilvie; Candiac, Quebec, Canada).

2.2 Inoculum preparation and cell count

Inoculum (24 h) was prepared with standard liquid medium of yeast mannitol broth (YMB; Vincent, 1970) containing following constituents K_2HPO4, 0.5 g/L; $MgSO_4.7H2O$, 0.2 g/L; NaCl, 0.1g/L; yeast extract, 1.0 g/L; and mannitol, 10.0 g/L. Cells were grown at 30 ± 1 °C for 24 h on a rotary shaker (Excella E25, NewBrunswick Scientific, Edi son, New Jersey, US) at 200 rpm. Inoculum cell count was 1.5 $x10^9$± 0.09 CFU/ml.

Cell count was conducted in triplicates by plating on yeast mannitol agar plate, supplemented with Congo red (0.25% w/v) after a serial dilution on saline solution (NaCl, 0.85% w/v). Incubation of plates was carried out at 30 ± 1°C for 72 h.

2.3 Fermentation

Sinorhizobium meliloti broth was produced in an automatic controlled bioreactor with a total capacity of 15 L (working volume: 10 L, Biogenie, Quebec, Canada), equipped with software (iFix 3.5, Intellution, USA) to set and control parameters (dissolved oxygen (DO), pH, antifoam, agitation, aeration rate and temperature). Before sterilization, the probes were calibrated: pH probe (Mettler Toledo, USA) was calibrated by using buffers of pH 4 and 7, oxygen probe (Mettler Toledo, USA) was first calibrated to zero using sodium bisulphite (2% w/v) solution and then to 100% with air saturated water. SIW medium was supplemented with 0.5% w/v of yeast extract to achieve maximum cell growth: yeast extract was demonstrated to increase cell concentrations when added to growth media (Rouissi *et al.*, 2010). Sterilization was carried out at 121±1°C for 15 min. The oxygen probe was recalibrated at 100 % by sparging air at 400 rpm and air flow rate at 8L/min. Inoculation was carried out at ratio 3% v/v with 24 h of inoculum in exponential phase. Temperature and pH of fermentation were fixed at 30±1°C and 7,

respectively. At the end of fermentation (48 h, stationary phase), the cell count was 8.8±0.7 $x10^9$ CFU/ml.

2.4 Centrifugation and preparation of formulation for spray-drying

Fermented broth was centrifuged at 8000 g for 20 min (Rouissi *et al.*, 2010), by using a fixed angle rotor, 500 mL (Sorvall GS-3, 13 679 g). The pellet was mixed with cheese whey powder (Agropur, Ste- Hyacinthe, Quebec) at 10 % w/v, sucrose at 1% w/v and sorbitol at 1% w/v. The mixture was then slowly stirred to obtain a homogenous suspension.

Supernatant was added to a viscosity of 16 ± 3 mPas and total solids of 8.2 6± 0.06 %. Final cell count was 1.1 ± 0.07x 10^{11} CFU/ml.

2.5 Spray drying and optimization

Spray drying was carried by using a spray dryer (Yamato, Model Pulvis GB 22, of Yamato Scientific America, Inc, South San Francisco, CA, USA). The nozzle used was 0.711mm which formed liquid droplets of approximately 20 µm of diameter with a surface area of 3.00 cm^2 per mL (Yamato manual). Spray drying process was carried out in the same manner as described by Adjallae *et al.* (2011). The studied parameters (independents variables) were (1) temperature inlet of drying air; (2) rate of sample feed; (3) pressure of atomization; and (4) flow rate of air drying (flow rate of aspiration).

Experiments were designed using a Central Composite Design with 6 replicates in the center. 31 analyses were done in totality. Corresponding parameters levels studied and codes are listed in Table 5.1. Optimization was carried out by applying response surface methodology (RSM). This methodology is widely used for bioprocess optimization. RSM was known to be useful in parameter interaction studies which allowed building models and selecting optimum working ranges. Dependant variables measured were: concentrations of viable cells, powder moisture content and temperature outlet. Simultaneous optimization of the studied responses was carried out by introducing a global desirability function as described by Derringer and Suisse (1980).

2.6 Moisture, out let temperature and observation by scanning electron microscope

Powder moisture was measured by using HR 83 Halogen Moisture analyzer (Mettler 120 Toledo, Ontario, Canada). The outlet temperature was automatically measured by a probe connected to the exit port of the collecting powders (Adjalle *et al.*, 2011). At the end of the experiments, the optimal powder formulation was examined using a scanning electron microscope (10 kV) (Carl Zeiss SMT, Cambridge, England) in order to examine the external appearance of the particles. Samples were first fixed to stud metal and coated with mild gold at a thickness of 0.01 mm.

2.7 Plants yield and nodulation index

Optimal powder formulation was evaluated for its effectiveness in inoculation tests with alfalfa. Seeds were inoculated with suspensions of powders to obtain 10^5 CFU/seed. About 15 alfalfa inoculated seeds were sown in sterilized plastic growth pouches with 10 replicates (Mega international of Minneapolis, Mexico). The control used was *Sinorhizobium meliloti* grown in standard media (YMB). Alfalfa plants were grown in a growth chamber (16 h days at 20°C and 15°C nights). Irrigation was performed with deionized water. Plant growth yields were calculated based on dry matter (plants were dried at 70°C for 48h) after 4 weeks of growth. Nodulation index was recorded based on: number, color and size of root nodules as described by Rebah et al (2001).

2.8 Statistical analyses

Statistical analyses and design of experiments were carried out by using STATISTICA, STAT SOFT trial version 10. ANOVA was used to test the significance of difference between plants yield and between nodulation indexes. The difference was considered significant at $p < 0.05$ (α was fixed to 0.05).

3. Results and discussion

3.1 Viable cells

Results of viable cells count in powder are shown in Table 5. 2. In the present work, RSM has been used with a central composite design to plan experiments for the purpose to model this process. Statistical analysis was done by ANOVA with the regression models given by Equation (1).

$$Y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j \qquad \text{(Eq.1)}$$

Where: *Y*, β_0, *Xi* and *Xj*, β_i, *βii, and βij* are the predicted responses of the dependent variable, second-order reaction constant, the independent variables, linear regression coefficient, the quadratic regression coefficient, and regression coefficient of interactions between two independent variables, respectively.

The effects of the independent variables (temperature inlet of drying air; rate of sample feed; pressure of atomization(P); and drying air (flow rate of aspiration) on the response (cells viability) were analyzed according to the polynomial model given in Equation 1. The effect estimates are given in Figure 1. The statistical significance of the studied variables and their interaction was evaluated by using probability (*p*) values and the Student's t-test as a tool.

Cells viability after spray-drying ranged between 2.72 x 10^9 and 6.97 x 10^9 CFU/g (Table 5.2). Survival was higher in experiment 1, while the lower survival was observed in experiment 18. The statistical analysis showed that the temperature inlet, atomization pressure and air drying rate have significant influence on cell viability. The linear effect of the temperature inlet and the quadratic effect of air drying rate have statistically highly significant negative effects (p=0.000163 and 0.000259, respectively). This indicates the fact that they can act as limiting factors for the cell viability even at low range of values. The quadratic effect of the temperature inlet was significant and negative (p=0.010123). Estimates effect of interaction between air drying rate and atomization pressure was significant and positive (p= 0.048304). Solution feed rate did not significantly affect the model: corresponding linear and quadratic effect estimate coefficients showed *p* values superior to limit of probability acceptance (0.05). This is probably due to The

relatively high air drying rate used and pressure applied. Thus, in this case, increasing feed solution rate will mainly lead to increased volume and moisture of the collected powder.

The statistical analysis of the regression coefficients showed an acceptable R-squared (R-sqr = 0.79607) which means that approximately 79 % of the variability can be explained by the model. Thus, the general model presented in Equation 1 can be simplified considering only the significant regression coefficients and interaction ($p < 0.05$).

$$\textbf{Cells viability } *10^9 = -215.256 + 602.279 \text{ Air drying} - 625.914 \text{ Air drying flow rate}^2 - 0.009T^2_{inlet} + 1150.503 \text{ Air drying flow rate} * P \quad \text{(Eq.2)}$$

To test if the model can adequately estimate the cell viability values, an analysis of the variance was carried out (by introducing the pure error) to determine the ``lack of fit". This analysis allowed measuring the failure of the model to represent data in the experimental region in which points were not included in the regression (Varnalis *et al.*, 2004). Results showed a p value of 0.23> 0.05 which means that the model presented can be used to adequately estimate cell viability.

Based on literature, only a few studies have been carried out on the development of rhizobia powder formulations using spray drying (Mary *et al.*, 1993; Ochin *et al.*, 1986). In fact, a challenge of optimizing this process mainly lies in the high sensibility of cells to heat and the need to formulate protective spraying suspension. Moreover, many factors were reported to affect the thermal sensibility of microorganisms, such as stage of cell growth and composition of the media (Teixeira *et al.*, 1994) which demands additional study and selection of optimal cell production conditions.

The 2D response surface plots described by the regression model given in Equation .2 are presented in Figures 2a and 2b: Figure 2a illustrates a fitted response profile based on the pressure of atomization against the variation of air drying rate. The temperature of air drying and feed sample rate were kept constant at 111.5°C and 8 mlL/min, respectively. Increasing pressure of atomization reduced cell viability to less than $1x10^9$CFU/g, which could be due to the decrease of the pulverized droplet size. Heat energy transferred to cells inside droplet will be accelerated in this case due to the reduction of barrier thickness formed by protective formulation (mixture whey-sucrose-sorbitol) surrounding rhizobia cells. However, when the air drying rate increased, there is an optimal interval for each range of value of pressure applied.

As observed, the maximum estimated cell viability was obtained by spray drying at an air drying rate of 0.56 m^3/min and pressure of atomization of 0.08 MPa. *Figure 2b* was obtained by varying the temperature of drying and air drying, fixing the pressure of atomization at 0.08 MPa and feed sample rate at 8 ml/min. This figure revealed that continuous increase of air drying flow rate or temperature of drying could lead to higher cells damage and mortality. The optimal values were 111 °C (temperature of drying air) and 0.56 m^3/min (air drying flow rate).

3.2 Powder moisture and temperature out let

Moisture content is one of the most important parameters affecting powder formulation stability during storage. As observed in Table 5.2, a concomitant decrease in cells viability and in water content was found, especially when considering runs 8 and 20: corresponding values of moisture content were 2.56 % and 12.72% whereas those of cell viabilities were 4.17 and 6.22 10^9CFU/g for, respectively. In fact, decreasing moisture content was demonstrated to lower water activity and consequently lower enzymes and microbial activity in powder formulation (Mugnier and Jung, 1984; Deaker *et al.*, 2007). When analysing the effect estimates, the quadratic effect of air drying flow rate was statistically significant (p<0.05). All interaction effects were statistically significant (p<0.05) except between feed sample rate and pressure of atomization. The R^2 value obtained was 0.77 which indicated that 77% of the moisture data variability can be explained by the model presented in Eq.3.

Moisture (%) =-745.29 Air drying flow rate ^{2}+ 12.82 Sample feed rate* Air drying flow rate + 7.01T drying *Air drying flow rate + 3231.25 P* Air drying flow rate -0.07Sample feed rate* T drying -15.13 P* T drying

(Eq.3)

In addition to moisture analysis, outlet temperature was also recorded (Table 5.2). Many researchers have shown that increasing outlet temperature reduces the survival of microorganisms after spray-drying (Kim and Bhowmik, 1990; To *et al.*, 1997). Wen *et al.* (2004) demonstrated that reducing the outlet temperature improved the viability of bifidobacteria in the collected powder. In the current study, these observations were particularly noted when considering runs 18 (T_{outlet} and cell viability of 77°C and 2.72x 10^9 CFU/g, respectively) and 22 (T_{outlet} and cell viability of 65°C and 2.89x 10^9CFU/g, respectively) in comparison to runs 20 (T_{outlet} and cell viability of 41°C and 6.22x 10^9CFU/g, respectively) and 21(T_{outlet} and cells viability of 42°C and 6.29x 10^9 CFU/g, respectively).

Analysis of effect estimates of outlet temperature results showed that pressure of atomization and all interactions were not statistically significant. The linear and quadratic effects of air drying flow rate, sample feed, and drying temperature were significant ($p<0.05$). The increase in inlet air temperature and air drying flow rate led to increasing the supply of heat energy and consequently the outlet temperature. When sample feed rate increased (Fig 5.3) outlet temperature decreased: heat energy supplied to the spray dryer contributed mainly in this case to water evaporation in the drying chamber. The general model presented in Equation 1 can be simplified to:

$$\mathbf{T_{out\ let} = 3608.20 - 8909.21 Air\ drying\ flow\ rate + 8106.81\ Air\ drying\ flow\ rate^2 + 0.89 Sample\ feed\ rate - -17.58 T\ drying^2 + 0.08 T_{in\ let}}$$

(Eq.4)

The minimum outlet temperature value obtained according to the general model given in Eq.1 was 39.79 °C with a confidence interval of [36.63; 42.94]. This optimal condition could be achieved by using an air drying rate of 0.56 m^3/min, a feed sample rate of 9.4 mL/min, an inlet temperature of 113.5 °C and pressure of pulverization of 0.09 MPa. Experimentation at optimal values, which allowed highest cell viability, showed an outlet temperature of 48°C. Lowest moisture that can be reached according to Equation 3 was 10.25 %, with a confidence interval of [8.44; 12.06]. Measured moisture when spray drying was carried out under optimal cell viability operating conditions was 12.4±0.3 % (mean of three runs).

There was difficulty in finding a compromise between the antagonist responses (cell viability and powder moisture content) during optimization and the need to proceed to a simultaneous optimization.

3.3 Simultaneous optimization by desirability functions of moisture content, cells viability and outlet temperature during spray drying

The study of simultaneous optimization of the responses was conducted by defining a global desirability function as described by Derringer and Suisse (1980). Results are illustrated in Figure 4. This method consisted of transformation of the predicted response, such as cells viability, moisture and outlet temperature to a dimensionless partial desirability function, d_i.

Depending on the objective of the studied response, two-sided functions were used (maximizing function for cell viability and minimizing function for moisture and outlet temperature)

$$d_{cells\ viability} = [(\text{Cell viability-A})/(\text{A-B})]$$

$$d_{cells\ viability} = 1 \text{ if Cell viability} > A$$

$$= 0 \text{ if Cell viability} < B$$

(Eq.4)

$$d_{moisture} = [(\text{moisture-A})/(\text{A-B})]$$

$$d_{moisture} = 1 \text{ if moisture} < B$$

$$= 0 \text{ if moisture} > A$$

(Eq.5)

$$d_{T\ outlet} = [(\text{T outlet-A})/(\text{B-A})]$$

$$d_{T\ outlet} = 1 \text{ if T outlet} < B$$

$$= 0 \text{ if T outlet} > A$$

(Eq.6)

where: A and B are the lowest and the highest values obtained for each corresponding response, so that any combination which resulted in viability lower than 2.7 10^9 CFU/g, outlet temperature higher than 77°C or moisture higher than 12.7% will be rejected. The global desirability function D was obtained after combining $d_{cells\ viability}$, $d_{moisture}$ and $d_{Toutlet}$ as described in Equation 7.

$$D = (d_{cells\ viability} . d_{moisture} . d_{T\ outlet})^{1/3}$$

(Eq.7)

When D reached a value higher than zero, this implied that all responses are in a desirable range. A continuous increase of D close to 1 means that combination of the different independent variables was globally optimum (Sivakumar *et al.*, 2007).

Profiles for predicted values and desirability showed (Figure 4) that best compromise (maximum of desirability ≈0.81) could be achieved at drying temperature of 105°C, air drying rate of 0.56m^3/min, pressure of atomization 0.07 MPa and feed flow rate of 8 mL/min. In order to validate respective theoretical working conditions optimum, three independent runs were carried out. The corresponding predicted responses, confidence interval for each individual response optimal and experimental values are shown in Table 4. When considering individual optimization, the highest predicted viable cells was ≈ 6.18 E+09, with a confidence interval values of [4.66E+09, 7.70E+09 CFU/g]. At maximum global desirability, experimentations showed a viable cells count of 6.8 ±0.4 x 10^9 CFU/g, which is covered by the confidence interval and confirms the effectiveness of considering the desirability methodology. The same comparison was carried out between individual optimal values of moisture and outlet temperature and showed that experimental values were in the confidence interval of the respective responses (Table 5.3). Thus, to obtain respectable cell viability during storage, spray drying was conducted at the optimal condition of maximum desirability function.

3.4 Storage of powder formulation, nodulation index and plant yield

Viability of spray dried *Sinorhizobium meliloti* cells (at optimal conditions) was followed for 4 months of storage at 4°C; the results are presented in Figure 5. Cell count remained higher than 10^9 CFU/g during all period of refrigerated storage. Analysis after the 4^{th} month showed a satisfactory survival of 3.19 x10^9 CFU/g (initial cell count was 6.62 x10^9 CFU/g) which is in the concentration range required to meet the 10^5 CFU/seed for bioinoculant commercialization (Lupwayi *et al.*, 2000). The addition of sucrose 1% w/v, sorbitol 1 % w/v and whey powder at 10% w/v performed good protection of cells during spray drying and further storage. The main protective effect could be due to initial protein and carbohydrate (from whey) abundance in the sprayed formulation (Wen *et al.*, 2004). Mary *et al.* (1993) demonstrated that a combination of skimmed milk (10% w/v) and sucrose (2% w/v) allowed good protection during storage of *Bradyrhizobium japonicum* cells after spray-drying at 130°C. Milk protein in cheese whey was demonstrated to improve *Rhizobium meliloti* viability during refrigerated storage (Estrella *et al.*, 2004). Examination of spray dried powders by scanning electron microscope (in Figure 6) showed spherical microparticles, varying in size with an average diameter between 4 and 20 µm. *Sinorhizobium meliloti* cells seemed to be embedded by the mixture of whey-sucrose-sorbitol acting as a matrix. This matrix could act as a barrier which limits heat transfer during spray drying and consequently improved cell viability. During storage, this type of formulation

(*Sinorhizobium meliloti* surrounded by a matrix) could reduce the rate of water evaporation from cells, which prevents the cytoplasmic membrane from desiccation damage.

In order to evaluate the effectiveness of the spray dried *Sinorhizobium meliloti* cells were applied to alfalfa seeds and a comparison was made with fresh cells cultivated in the standard media (YMB). Results of plant yield and nodulation index are presented in Table 4. Inoculation of seeds with 10^5 *Sinorhizobium meliloti* /seed from spray dried formulation gave similar results of plants yield (144.6mg) and nodulation index (15.2) with the control (plants yield and nodulation index were 131 mg, and 14, respectively). At this inoculation rate of *Sinorhizobium meliloti*, it seemed that the additives used (sorbitol, sucrose and cheese whey powder) have a protective and stimulating effect on the cells root colonization which could explain current results. The powder formulation when suspended in water was highly soluble which allowed easy coating seed operation. Moreover, the average particle diameter varied between 4 and 20µm which could improve the powder repartition on the seed surface.

It was demonstrated in this study that application of spray drying process can be a successful alternative to traditional peat formulation for the development of instant bioinoculant powder, which decreased the time of processing. Production of *Sinorhizobium meliloti* in starch industrial waste water and use of cheese whey powder as additive reduced the cost and improved competitiveness of the final product. The developed spray dried formulation was observed adaptable to the common method applied to alfalfa seeds due to its high solubility in water, which could be practical for large scale seed coating processes. This study also showed the feasibility of the optimization and production of bioinoculant powder formulation of cells cultivated in agro-industrial wastes (starch industrial waste water and use of low cost additives (cheese whey powder and sucrose).

4. Conclusion

In this study, it was possible to optimize a spray drying process and find a compromise between the parameters which affected the product quality (cell viability, moisture and temperature outlet). Under optimal conditions, cell viability ≈ 6.8 $x10^9$ CFU/g, which was maintained at higher than 1x 10^9 CFU/g after 4 months of storage. Nodulation index and plant yield test demonstrated the efficiency of the additives used which protected cells against heat and guaranteed their activity during nodulation. Powder particle morphology showed that cells were embedded in a matrix (formed by cheese whey powder-sucrose-sorbitol) which could act as protecting barrier against heat damage during drying.

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Table 5.1 Independent variables used in the optimization

	Codes and values of independent variables of experimental designs for screening using response surface methodology				
Levels	-2	-1	0	+1	+2
T drying (°C)	105	111.25	117.5	123.75	130
Air drying flow rate (m^3/min.)	0.50	0.53	0.56	0.59	0.62
Pressure of atomization (MPa)	0.08	0.09	0.10	0.11	0.12
Feed sample rate(ml/min)	4	6	8	10	12

Table 5. 2 Design showing the set of each run and studied response.

Experiment Code	Air Drying flow rate (m^3/min)	Feed sample rate ml/min)	T drying(°C)	Pressure of atomization (MPa)	Cells count (10^9CFU/g)	T_{outlet} (°C)	Moisture (%)
1	0.53	6	111.25	0.08	6.97	59	10.00
2	0.53	6	111.25	0.1	5.26	59	10.70
3	0.53	6	123.75	0.08	4.50	65	11.24
4	0.53	6	123.75	0.1	4.21	61	10.28
5	0.53	10	111.25	0.08	5.96	51	10.14
6	0.53	10	111.25	0.1	4.69	49	11.11
7	0.53	10	123.75	0.08	5.83	58	9.29
8	0.53	10	123.75	0.1	4.17	58	2.56
9	0.59	6	111.25	0.08	5.89	61	3.29
10	0.59	6	111.25	0.1	6.32	61	8.94
11	0.59	6	123.75	0.08	4.42	68	9.89
12	0.59	6	123.75	0.1	5.14	68	10.74
13	0.59	10	111.25	0.08	6.32	54	7.01
14	0.59	10	111.25	0.1	5.86	54	8.99
15	0.59	10	123.75	0.08	5.20	62	9.52
16	0.59	10	123.75	0.1	5.11	55	10.53
17	0.5	8	117.5	0.09	4.76	63	9.59
18	0.62	8	117.5	0.09	2.72	77	7.71
19	0.56	4	117.5	0.09	5.65	69	9.33
20	0.56	12	117.5	0.09	6.22	41	12.72
21	0.56	8	105	0.09	6.29	42	11.20
22	0.56	8	130	0.09	2.89	65	11.11
23	0.56	8	117.5	0.07	5.82	43	11.18
24	0.56	8	117.5	0.11	6.36	47	9.94

Table 5. 2 Design showing the set of each run and studied response (continued).

25(C)	0.56	8	117.5	0.09	5.54	48	9.27
26(C)	0.56	8	117.5	0.09	5.54	42	10.64
27(C)	0.56	8	117.5	0.09	6.08	42	11.42
28(C)	0.56	8	117.5	0.09	6.58	42	11.06
29(C)	0.56	8	117.5	0.09	6.33	42	11.24
30(C)	0.56	8	117.5	0.09	6.24	42	9.95
31(C)	0.56	8	117.5	0.09	6.96	42	10.50

Table 5.3 Validation of maximum desirability conditions

	Theoretical[1] optimal value	Confidence interval of the theoretical optimal value	Experimental value[2]
Cells viability(10^9 CFU/g)	6.18	[4.66; 7.70]	6.8 ±0.4
Temperature out let(°C)	39.79	[36.63; 42.94]	42±1
Moisture (%)	10.25	[8.44; 12.06]	11.6 ±0.3

[1]Theoretical optimal value by individual optimization

[2] mean of 3 independent runs at maximum of global desirability conditions.

Table 5.4 Effectiveness of spray dried *Sinorhizobium meliloti* on nodulation index and plant yield/pouch

	Nodulation index			Plants yield (dry matter in mg)		
Rate of *S. meliloti* application per seed	Control cells cultivated in standard media YMB	Spray dried formulation	*p* value	control	Spray dried formulation	*p value*
10^5 CFU/seed	14^a	15.2^a	0.18	131^b	144.6^b	0.23

*Values followed by different letter are statistically different (*p* value=0.05)

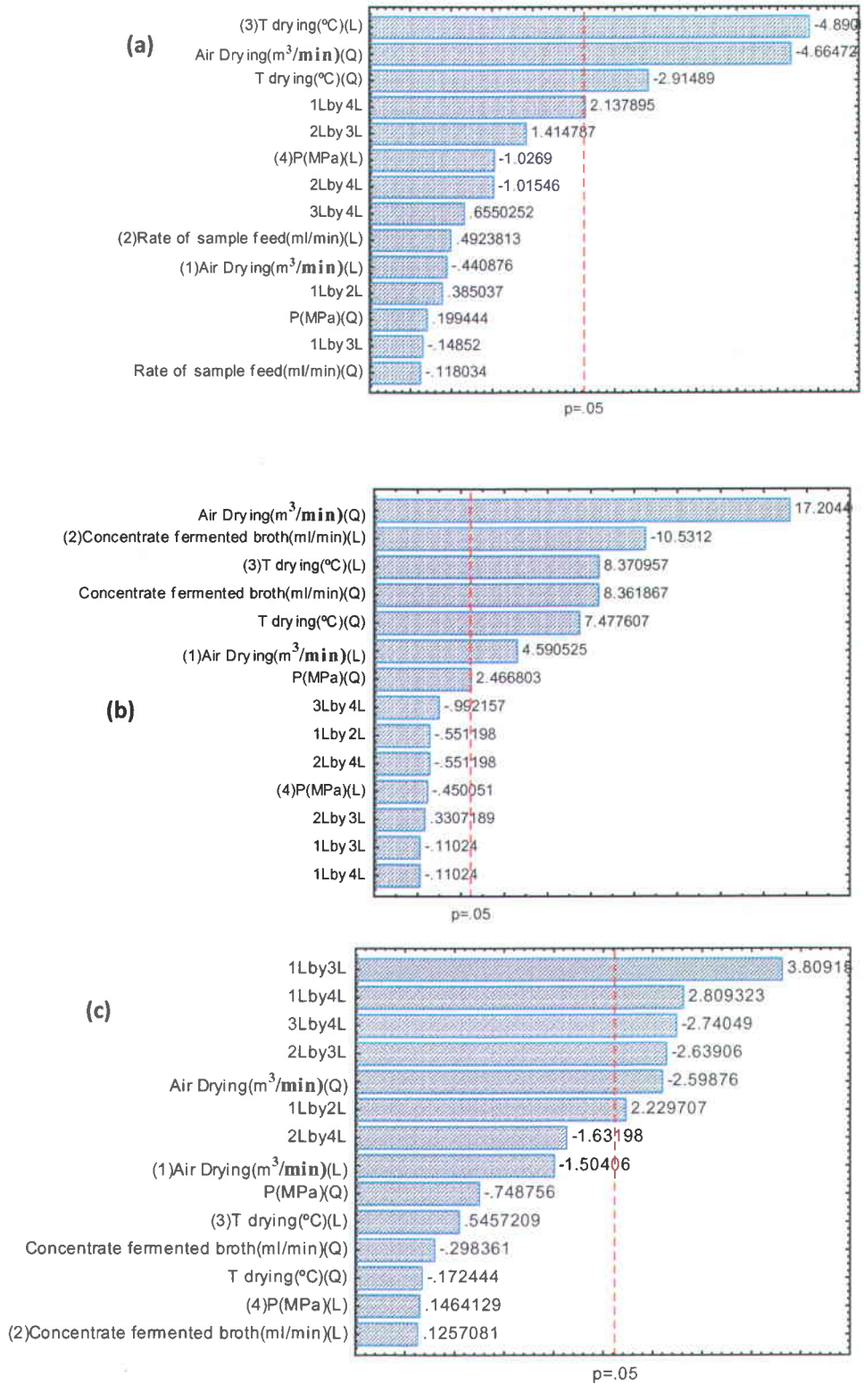

Figure 5.1 Pareto chart of standardized effects: (a) cell count (CFU/g); (b) T_{outlet} (°C), (c) moisture (%).

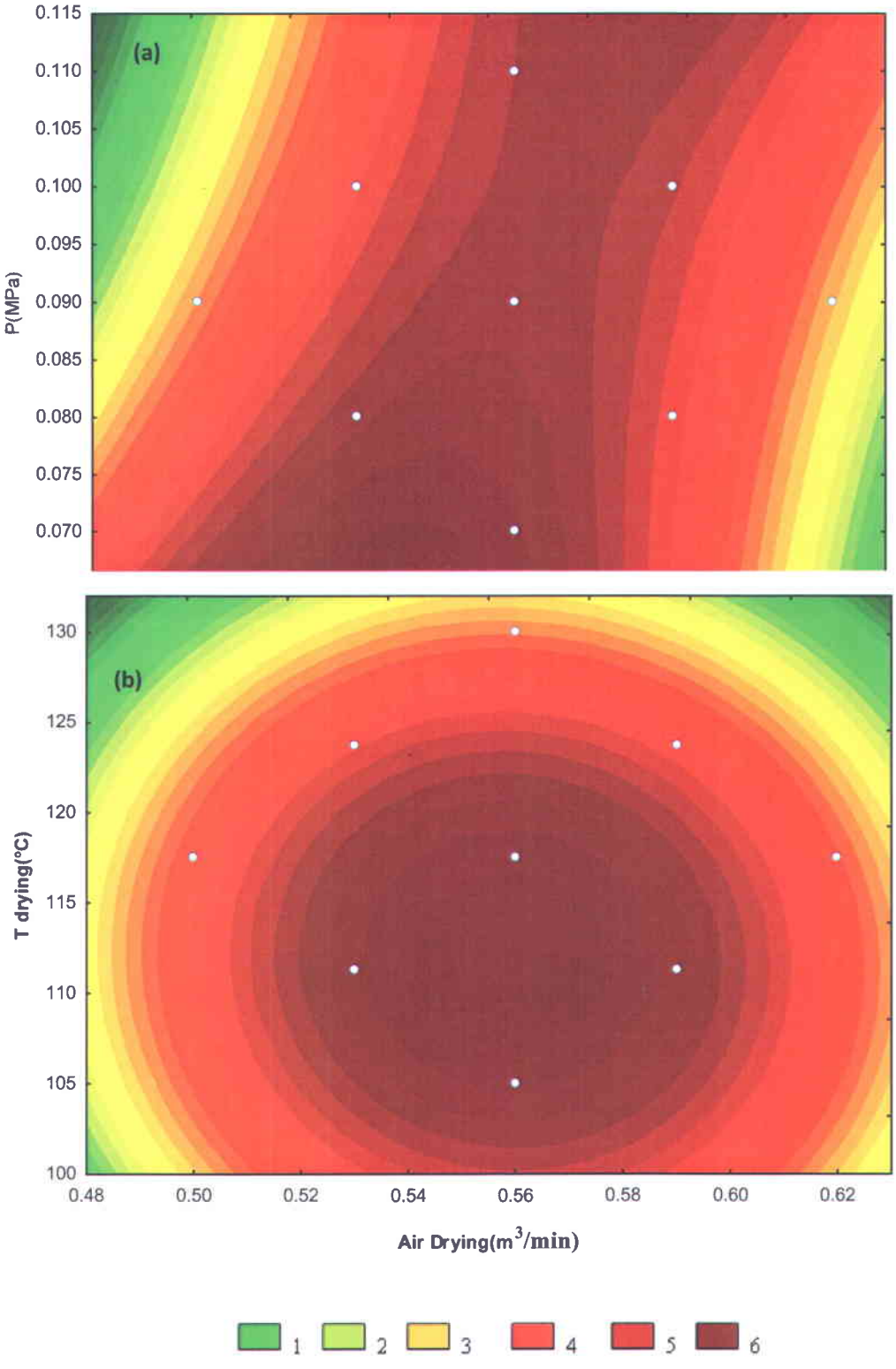

Figure 5.2 (a) Effect of pressure of atomization and air drying rate, (b) Effect of temperature and air drying flow rate, on the viability of *S. meliloti* (x 10^9 CFU/g)

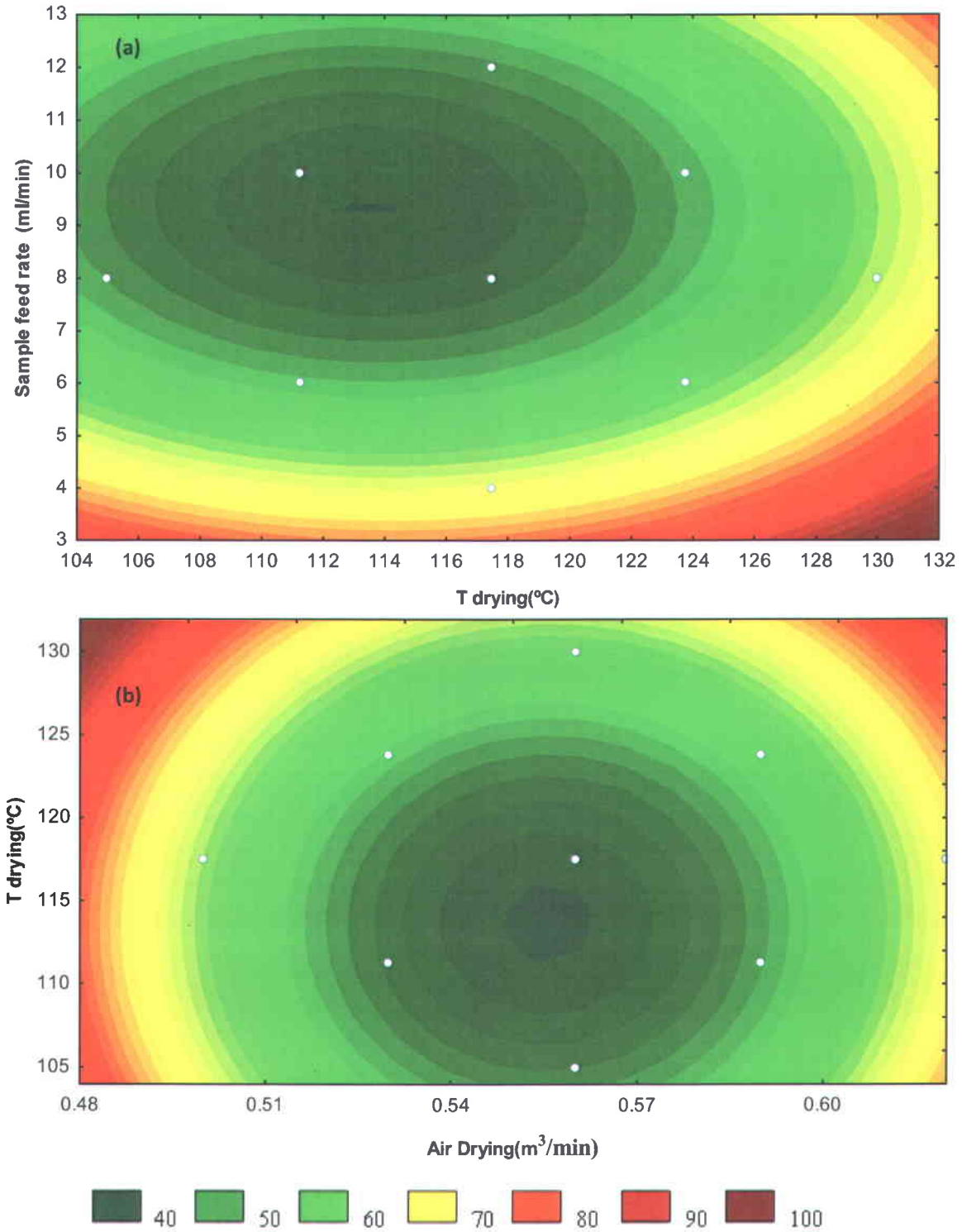

Figure 5.3: (a) Effect of rate of sample feed (ml/min) and Temperature of drying(°C), (b) Effect of pressure of atomization (MPa) and air drying flow rate (m^3/min), on the temperature out let (°C).

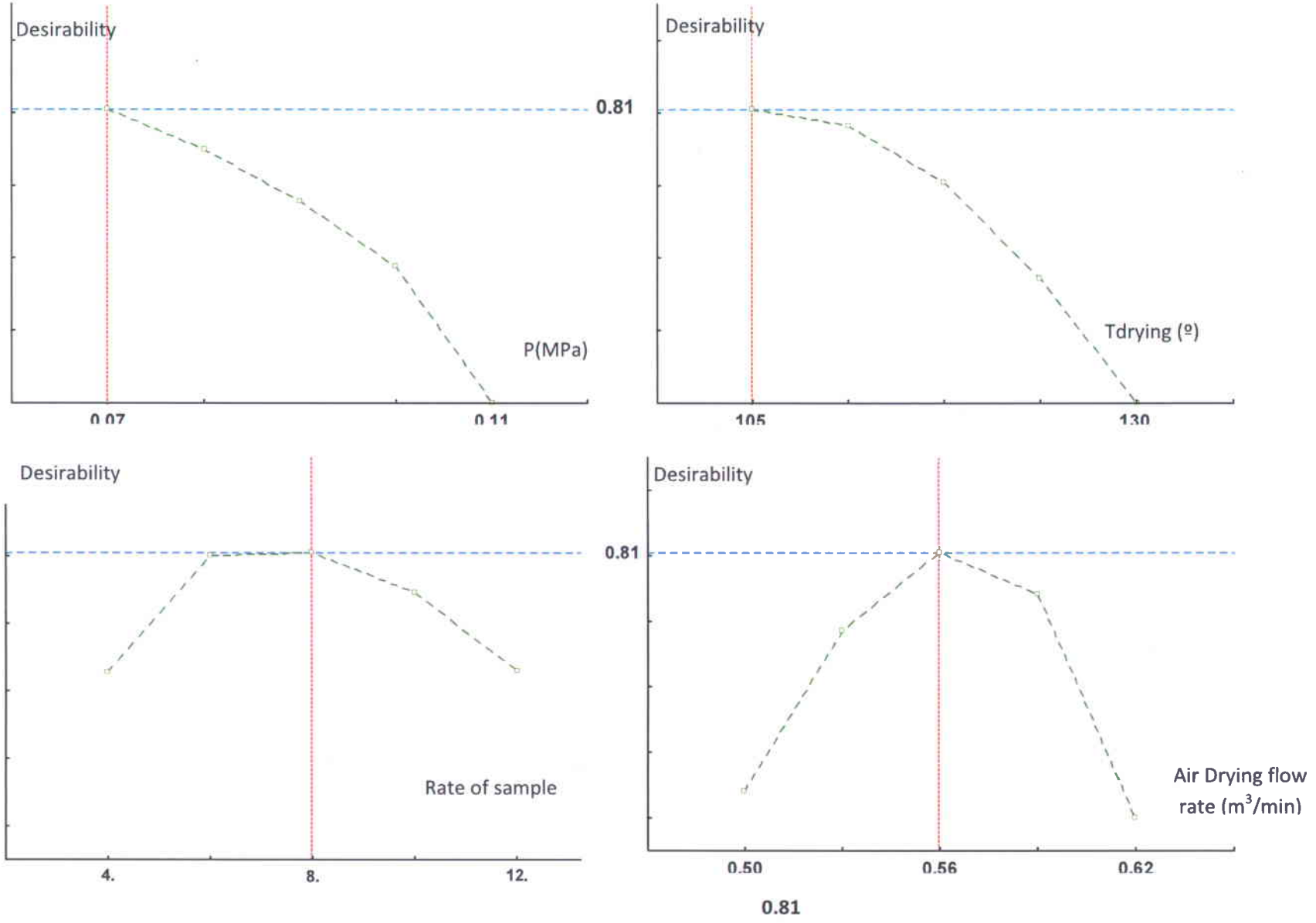

Figure 5.4 Profiles for desirability

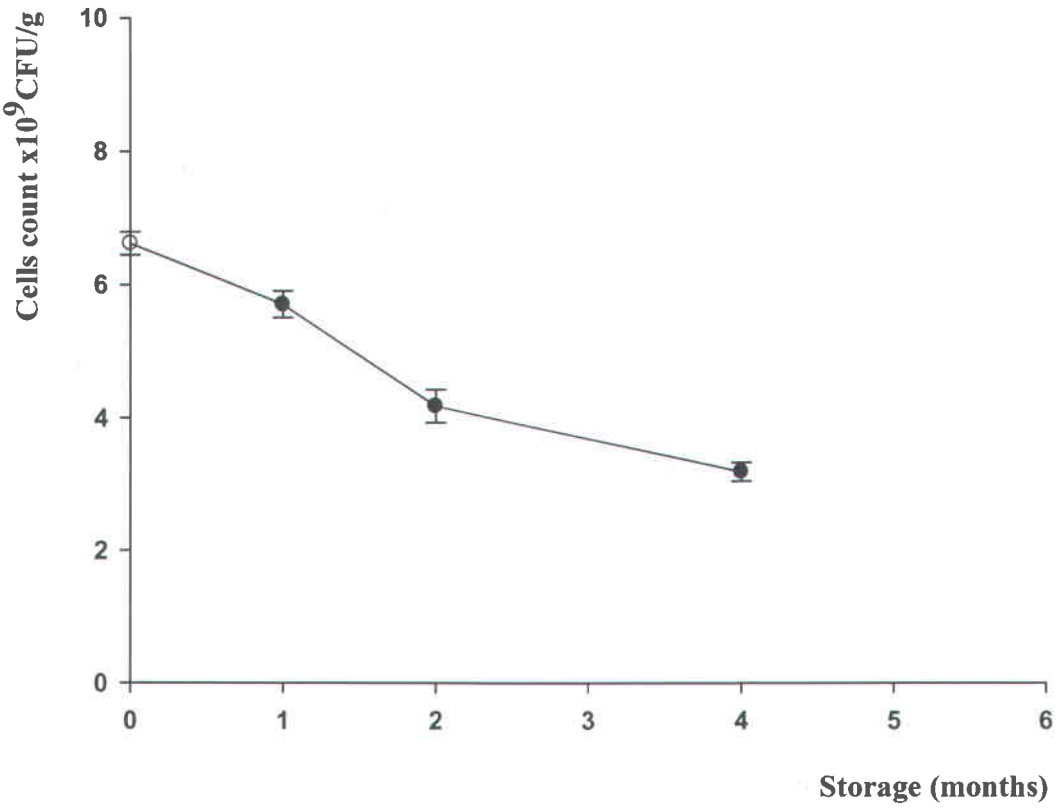

Figure 5.5 Viability of *Sinorhizobium meliloti* spray dried at optimal conditions during storage at 4°C

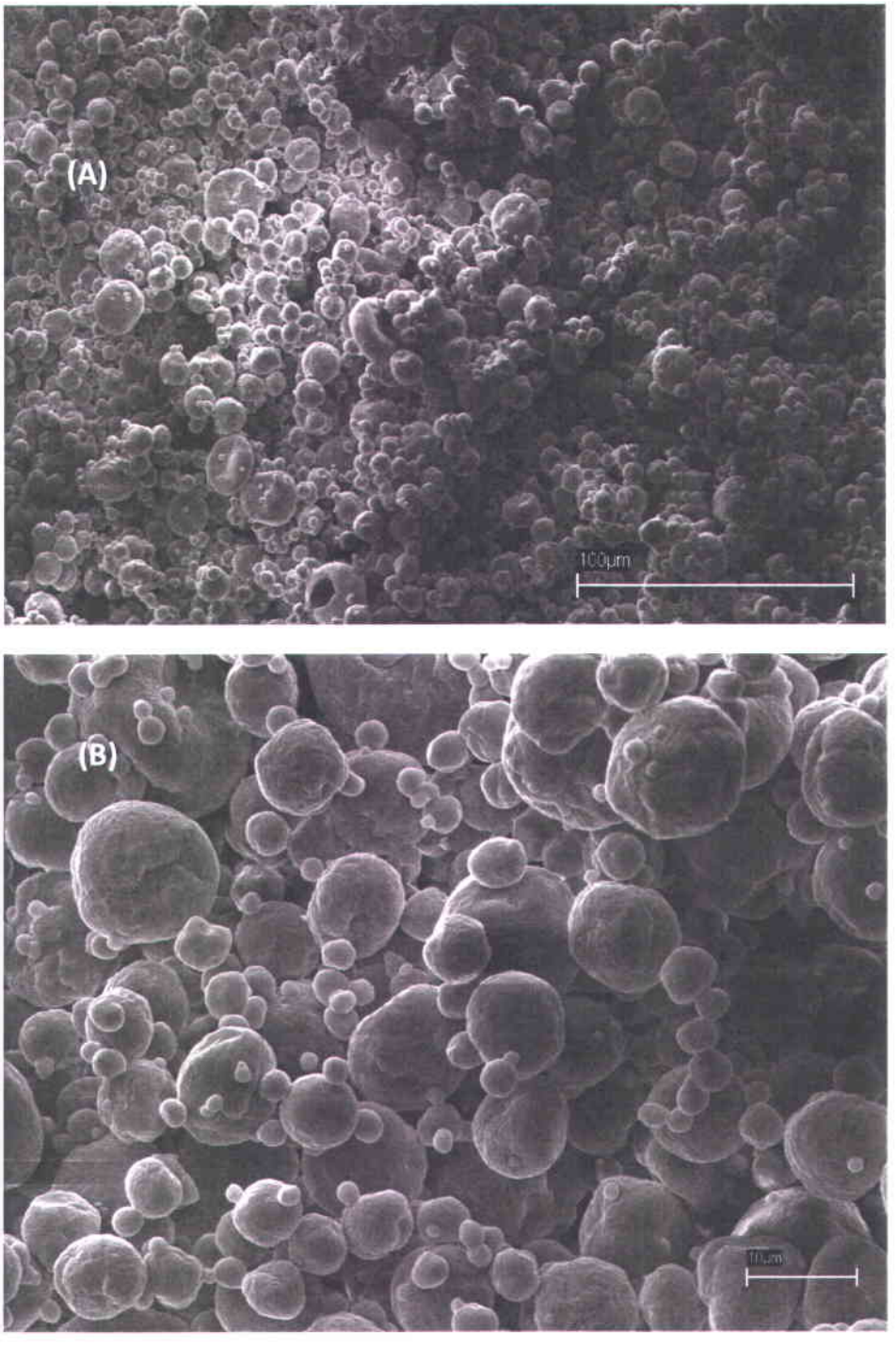

Figure 5.6 A and B: SEM micrographs of spray-dried formulation of *Sinorhizobium meliloti*

CHAPITRE 6

AMÉLIORATION DE LA QUALITÉ DES FORMULATIONS DE SINORHIZOBIUM MELILOTI PAR CO INOCULATION AVEC AZOSPIRILLUM BRASILIENSE : OPTIMISATION DES CONDITIONS DE PRODUCTION DANS LES EAUX USÉES D'AMIDON ET ÉTUDES DES DOSES APPLIQUÉES

AZOSPIRILLUM BRASILENSE PRODUCTION IN STARCH INDUSTRY WASTE WATER AND CO-INOCULATION EFFICACY OF *SINORHIZOBIUM MELILOTI –AZOSPIRILLUM ON* ALFALFA PLANTS

AMÉLIORATION DE LA QUALITÉ DES FORMULATIONS DE SINORHIZOBIUM MELILOTI PAR CO INOCULATION AVEC AZOSPIRILLUM BRASILIENSE : OPTIMISATION DES CONDITIONS DE PRODUCTION DANS LES EAUX USÉES D'AMIDON ET ÉTUDES DES DOSES APPLIQUÉES

T. Roussi[a], A.Mahmoudi [a], R.D. Tyagi[a]*, D. Prévost[b], S.K. Brar[a],

R.Y. Surampalli[c]

[a] Institut National de la Recherche Scientifique- Eau, Terre & Environnement (INRS-ETE), Université du Québec, 490, Rue de la Couronne, Québec G1K 9A9, Canada

[b] Agriculture et Agroalimentaire Canada, Sainte-Foy, Quebec G1V 2J3, Canada

[c] US Environmental Protection Agency, Kansas City, USA

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RÉSUMÉ

Dans le but d'améliorer la qualité des formulations de *Sinorhizobium meliloti*, la co-inoculation des suppléments de croissance à savoir le gluconate à 0.5%w/v, extrait de levure à 0.5% et (extrait de levure 0.5%w/v +gluconate0.5%w/v) ont été étudiés dans le but d'augmenter la production d'*Azospirillum brasiliense* dans les eaux usées d'amidon, la comparaison a été réalisée avec le milieu Bashan et le SIW. Les résultats ont montré que l'ajout de l'extrait de levure ou de gluconate améliore significativement le taux de croissance de $0.266h^{-1}$ (pour SIW) à respectivement $0.315h^{-1}$ et $0.316h^{-1}$. Ces taux étaient statistiquement comparables au milieu Bashan ($0.324h^{-1}$). La méthodologie de réponse de surface a été appliquée afin d'optimiser la quantité d'extrait de levure ajoutée et la température de croissance d'*Azospirillum brasiliense* dans le milieu SIW.

Le modèle obtenu (R^2 = 0,92) a montré que la production maximale de cellules était à 34 ° C et avec 0.28% w/v d'extrait de levure. Un test de confirmation « check point » a été effectué : *Azospirillum brasiliense* a été produit dans le fermenteur 7.5L. La concentration atteinte était de ≈ $0.90x10^9$CFU/ml, valeur située dans l'intervalle de confiance de la variable prédite. La comparaison entre les rendements en matière sèche des plantes de luzerne co-inoculés avec : *Azospirillum Brasiliense* (à10^4,10^5et 10^6CFU/plante)et *Sinorhizobium meliloti* (à 10^5CFU/plante) a montré que la combinaison d'*Azospirillum* à 10^5 ou 10^6CFU /plante avec *Sinorhizobium meliloti* à 10^5CFU/plante a permis d'atteindre le plus haut rendement en matière sèche (augmentation respectives de 20 et 17%,) comparativement aux plants de luzerne inoculés avec *Sinorhizobium meliloti* seul à10^5CFU/plant.

Mots clés: *Azospirillum brasilense*, *Sinorhizobium meliloti*, co-inoculation, optimisation.

ABSTRACT

Supplement growth selection was carried out in order to increase the production of *Azospirillum brasilense* ATCC29710 cells in starch industry waste water (SIW). For this purpose, gluconate, yeast extract and their combination (yeast extract+gluconate) were added to SIW; comparison was made with standard Bashan medium and SIW. Results showed that addition of gluconate or yeast extract improved the growth rate form $0.266h^{-1}$ (for SIW) to $0.315h^{-1}$ and $0.316h^{-1}$, respectively; values not statistically different to standard Bashan medium ($0.324h^{-1}$). Response surface methodology was applied in order to optimize the amount of yeast extract added and growth temperature of *Azospirillum brasilense* in SIW. The model obtained (R^2=0.92) showed that optimal temperature and yeast extract amount for cell production was 34°C and 0.28% w/v, respectively. A checkpoint analysis carried out by culturing cells in a 7.5L fermenter showed a concentration of $0.90x10^9$ CFU/ml, which was included in the confidence interval of the predicted variable.

Comparison of co-inoculated alfalfa plants with *Azospirillum brasilense* (at 10^4, 10^5 and 10^6 CFU/plant) and *Sinorhizobium meliloti* (at 10^5 CFU/plant) showed that a combination of 10^5 or 10^6 CFU of *Azospirillum* /plant with 10^5CFU of *Sinorhizobium meliloti*/plant allowed the highest plant dry matter yield (increase of 20 and 17%, respectively) compared to alfalfa plants inoculated with *Sinorhizobium meliloti* at 10^5CFU/plant.

Keywords: *Azospirillum brasilense*, *Sinorhizobium meliloti*, co-inoculation, optimization.

1. Introduction

Various plant growth promoting rhizobacteria (PGPR) have been shown to be interesting for the development and commercialization of industrial biofertilizers. Among the PGPR, *Azospirillum spp* has been demonstrated to improve the development and yield of various crops such as cereals and grasses [1]. Bashan *et al.* [2] considered *Azospirillum spp* as general plant-growth-promoting bacteria for over 100 plant species.

For leguminous plants, co-inoculation with *Azospirillum spp* and rhizobia enhanced plants' yield, by increasing mineral and water uptake and NPK availability [3-5]. Co-inoculation of *Sinorhizobium meliloti* with *Azospirillum spp* increased the number of nodules and N_2 fixation of alfalfa. This increase could be due to simulating hormones which increase the susceptibility of legumes to rhizobium colonization [6]. Other studies showed that *Azospirillum spp* induced the formation of epidermal cells that differentiated into root hairs and consequently improved primary rhizobium infections [7]. Co-inoculation of rhizobia with *Azospirillum spp* is also interesting since it can be used as biological control [8].

Efficient bio-inoculant or co-inoculant formulations required culture media with high yield production capacity [9]. In industry, most media used for large scale production consisted of synthetic media which are costly and their composition is patented. The challenge of biofertilizer commercialization is the production of low cost and efficient formulations. In previous works, it was demonstrated that starch industry waste water (SIW) supported growth of *Sinorhizobium meliloti* at a level higher than $4x10^9$ CFU/mL [9, 10]. In this study, we assessed the optimization of growth conditions of *Azospirillum brasilense* in SIW, and we studied the effect on inoculation of *Azospirillum brasilense* with *Sinorhizobium meliloti* on alfalfa plants.

2. Materials and methods

2.1 Bacterial strains and inoculum preparation

Strains used in this study were *Sinorhizobium meliloti* strain A2 and *Azospirillum brasilense* ATCC29710 (Agriculture and Agri-Food Canada, Quebec, Quebec, Canada). *Sinorhizobium meliloti* inoculum was produced in standard liquid medium (yeast mannitol broth, YMB) [11]. The constituents of the media are: K_2HPO_4, 0.5 g/L; $MgSO_4.7H_2O$, 0.2 g/L; NaCl, 0.1g/L; yeast extract, 1.0 g/L; and mannitol, 10.0 g/L. Cells were grown at 30 ±1 °C for 24 h on a rotary shaker (Excella E25, NewBrunswick Scientific, Edi son, New Jersey, US) at 180 rpm (exponential phase, $\approx 10^9$ CFU/mL).

Azospirillum brasilense inoculum was prepared in Bashan media [12] with the following constituents: yeast extract, 5 g/L, Na-gluconate, 5 g/L; NaCl, 1.2 g/L; MgSO4·7H2O, 0.25 g/L; K2HPO4 0.13 g/L; CaCl2, 0.22 g/L; K2SO4, 0.17 g/L; Na2SO4, 2.4 g/L; NaHCO3, 0.5 g/L; Na2CO3, 0.09 g/L; Fe(III) EDTA, 0.07 g/L; the pH was adjusted to 7.0 after sterilization. Fermentation was carried at 35±1°C for 24 h at 150 rpm in 250 ml Erlenmeyer flasks [12].

Cell count was estimated by plating *Sinorhizobium meliloti* and *Azospirillum brasilense* after a serial dilution using saline solution (NaCl, 0.85% w/v) on their corresponding agar medium (YMB and Bashan media, respectively). Plates were incubated at 30 ± 1°C for 48-72 h for *Sinorhizobium meliloti* and at 35°C for 36 h for *Azospirillum brasilense.*

2.2 Azospirillum production optimization

In the first part of this study, we evaluated the efficacy of supplementing starch industry wastewater (SIW) (1.4% w/v total solids, ADM Ogilvie; Candiac, Québec, Canada) with yeast extract at 0.5% w/v and with Na-gluconate (at 0.5% w/v) to improve the growth of cells. Controls were Bashan media [12] and SIW. Fermentations were carried at 35°C at 150 rpm in 250 ml flasks for 33 h, pH was adjusted to 7 after sterilization. Inoculum (containing ≈ 4 x 10^8 CFU/ml) was added the ratio of 4% v/v. Each experiment was carried out in triplicates.

In the second part, we studied the effect of yeast extract added to SIW and the effect of growth temperature on cell growth. Response surface methodology was applied for this purpose. Experiments were organized with a central composite plan. Studied factors included growth

temperatures which varied from 31.5°C to 38.5°C and yeast extract was added at concentrations varying from 0.002 to 0.058%w/v). Factors levels and experimental plan are shown in Table 6. 1. *Azospirillum* fermentation was carried out for 24 h on a rotary shaker at 150 rpm. Equation 1 was used for calculation of specific growth rate (μ).

$$\mu = \ln(Nt_0) - \ln(Nt_1) / (t_0 - t_1) \qquad \text{(Eq.1)}$$

where Nt_0 and Nt_1 are the number of cells in the beginning and at the end of the exponential phase, respectively. Generation time (tg) was calculated using the Equation 2.

$$t_g = \ln 2 / \mu \qquad \text{(Eq.2)}$$

2.3 Cell production in a 7.5 l fermenter

Cells were produced in 7.5L fermenter as a check for optimized growth conditions and for plant inoculation tests. *Sinorhizobium meliloti* and *Azospirillum brasilense* were cultured in SIW at 30°C, during 48h, and at 34°C during 24 h, respectively. For *Azospirillum brasilense* production, SIW was supplemented with 0.28%w/v of yeast extract. Fermentation was carried out in 7.5l automatic controlled bioreactor (working volume: 4 l, Labfors), equipped with software (iris NT 5.0) which allows one to set and control parameters (dissolved oxygen (DO), pH, antifoam, agitation, aeration rate and temperature). The dissolved oxygen probe (Mettler Toledo, USA) was calibrated to zero using sodium bisulphite (2% w/v) solution and then to 100% with air saturated water before sterilization and recalibrated at 100 % by sparging air at 500 rpm and air flow rate at 4l/min. pH probe (Mettler Toledo, USA) was calibrated by using buffers of pH 4 and 7. SIW medium was sterilized at 121±1°C for 15 min. pH was automatically maintained at 7. Final cell count was ≈ 6.4×10^9 CFU/mL and ≈0.9×10^9 CFU/mL for *Sinorhizobium meliloti* and *Azospirillum brasilense*, respectively.

2.4 Plant yield and nodulation index

Inoculated alfalfa plants with *Azospirillum brasilense* (at 10^4, 10^5 or 10^6 CFU/plant), *Sinorhizobium meliloti* (at 10^5CFU/plant), and combination of *Sinorhizobium meliloti* (at 10^5CFU/plant) + *Azospirillum brasilense* (at 10^4, 10^5 or 10^6CFU/plant) were tested for their symbiotic efficiency. About 13 alfalfa seeds were sown for each treatment in sterilized plastic growth pouches (Mega international of Minneapolis, Mexico). Ten pouches (replicates) were

used for each treatment. After germination, plants were kept in an environmental chamber (at 20°C with a photoperiod of 16 h and at 15°C during night). Plants were irrigated regularly with demonized water. After 5 weeks of growth, plant yield was determined based on dry matter (plants were dried at 70°C for 48h). Nodulation index was calculated as was described by Ben Rebah *et al.* [13]: a visual estimation of nodule number (3 for several, 2 for many, and 1 for few) was multiplied by the color (2 for pink and 1 for white) and by the size of root nodules (3 for large, 2 for medium and 1 for small).

2.5 Statistical analyses

Statistical analysis was carried out using R version 2.10.1 (Copyright (C) 2009, The R Foundation for Statistical Computing ISBN 3-900051-07-0). Analysis of variance (ANOVA) and contrast analysis were used to evaluate significance of the difference between plant yield treatments. Differences were considered to be significant at $p < 0.05$ (α was fixed to 0.05). Tukey's post hoc analysis was applied for comparison among the growth supplements studied.

3. Results and discussion

3.1 Growth supplement selection

Figure 6.1 shows results of supplement selection for the cultivation of *Azospirillum brasilense* in SIW. Yeast extract added at 0.5% w/v, Na-gluconate added at 0.5% w/v, or combined yeast extract at 0.5% +Na-gluconate 0.5% improved cell growth, when compared to the control (SIW without additives). After 24 h of fermentation, cell concentration increased more than 2 times when SIW was supplemented with yeast extract (≈8.8 $x10^8$ CFU/mL), and with Na-gluconate ≈8.96$x10^8$CFU/ml, compared to SIW (≈3.9$x10^8$ CFU/ml). When yeast extract and Na-gluconate were added simultaneously, cell number of 24 h increased to 6.3$x10^8$ CFU/ml. The highest cell concentration was observed with Bashan (1.11$x10^9$ CFU/mL).

Table 6.2 showed growth results of supplements studied based on generation time and specific growth rate: addition of yeast extract and/or Na-gluconate significantly improved SIW capacity to support *Azospirillum* growth (p= 0.0205 and 0.0212, respectively). Growth rate increased significantly from 0.266h^{-1} for SIW to 0.315 h^{-1} and 0.316 h^{-1} when yeast extract and Na-gluconate were added, respectively. Consequently, generation time decreased significantly from 2.610 h for SIW to 2.201 h and 2.195 h for SIW+yeast extract and SIW+Na-gluconate, respectively. Tuckey's post hoc tests based on generation time showed that the efficiency of SIW+ yeast extract and SIW + Na-gluconate were not different from standard Bashan medium. Thus, by adding yeast extract or N-gluconate at 0.5% w/v, SIW can support growth of *Azospirillum* cells similarly to standard Bashan medium. Specific growth rate was improved when yeast extract and Na-gluconate were added simultaneously and it increased from 0.266 to 0.290 h^{-1}, but was lower than SIW supplemented with only one of these additives. SIW has been demonstrated to support growth of many bacteria, such as *Sinorhizobium meliloti, Bacillus mucilaginosus, Bacillus thuringiensis, Trichoderma viride* [9,14-16]. Gluconate and yeast extract served as an additional carbon source which could explain the increase in specific growth rate and the reduction in generation time when compared to the control (SIW). Composition of SIW medium [17] showed that the C/N ratio increased from 8.9 to 15 by through addition of gluconate (at 5g/L) which resulted in significant increases in specific growth rate and consequently cell concentrations. To the best of our knowledge, there is no study so far which described gluconate enzymatic pathways in *Azospirillum*. However, Westby *et al.* [18] demonstrated that *Azospirillum brasilense* sp7 could grow well with gluconate as the sole carbon source and demonstrated high

activities for key enzymes in the Entner-Doudoroff pathway. Döbereiner and Day [19] have found that the preferred carbon source for *Azospirillum brasilense* were based on organic acids, such as malic, succinic, a-ketoglutaric, gluconic, or lactic [20,21]. Leyv and Bashan [22] observed that addition of gluconate increased gluconokinase activity and cell growth. Supplementing NH_4^+-medium with gluconate at 0.5% w/v gave comparative cell production as with malate or fructose when added at same ratio [20]. The same trend was observed by Bashan *et al.* [13] who observed that gluconate enhanced the growth of *Azospirillum brasilense*.

Results obtained with yeast extract showed that *Azospirillum brasilense* possessed the same ability to degrade yeast extract as gluconate when added at the same ratio (0.5% w/v). Yeast extract is considered as a suitable source of nitrogen and growth factors which contained more than 50 mg/g of thiamine [23]. In literature, it was reported that addition of yeast extract improved the growth of many bacterial species such as *Sulfolobus solfataricus*[24], *Sinorhizobium meliloti* [25], *Torulopsis glabrata* [26] and *Lactobacillus helveticus* [27]. Okon *et al.* [28] found that addition of yeast extract to Dobereiner-Day medium reduced the lag phase and aided vigorous growth of *Azospirillum brasilense*. Bashan *et al.* [29] suggested that nitrogen supplementation due to addition of yeast extract enhanced the growth of *Azospirillum brasilense.*

Simultaneous addition of gluconate and yeast extract to SIW improved the specific growth rate when compared to the control, however the best result was observed when each of these additives were added separately, which meant that SIW medium should be supplemented by gluconate or yeast extract in order to obtain a high growth rate.

3.2 Optimization of *Azospirillum brasilense* condition production

Results of cells count (2^2 central composite design) after 24 h of fermentation are shown in Table 6.3. The presented data were analyzed by ANOVA with the regression models given by Equation 3.

$$Y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j \qquad \textbf{(Eq.3)}$$

Y, β_0, *Xi* and *Xj*, β_i, *βii, and βij* are consecutively: the predicted responses of the dependent variables, second-order reaction constant, the independent variables, linear regression coefficient, the quadratic regression coefficient, and regression coefficient of interactions between the independent variables).

The effects of growth temperature and yeast extract concentration (independent variables) were analyzed according to the model given in Equation 3. Student's t-test was used in order to evaluate the statistical significance of the studied variables and their interaction. The effect estimates and regression coefficients are presented in Table 6.4.

After 24 h of fermentation, the cell count ranged between 0.12 $x10^9$ CFU/g and 0.9 $x10^9$ CFU/g (Table 3). Higher cell concentration (0.9±0.055 x 10^9 CFU/mL) was obtained in experiment 12 when fermentation was carried out at 35°C and 0.3%w/v of yeast extract added. The lowest value was observed in experiment 4 (0.12±0.007x 10^9 CFU/mL),

where temperature of fermentation was fixed at 37.5°C and yeast extract added was 0.5% w/v. Analysis of effect estimates showed that the quadratic effects of the temperature of fermentation and yeast extract added were highly significant ($p < 0.001$), the linear effect of temperature was significant ($p < 0.05$). There was no linear effect of added yeast extract or interactive effect (between temperature of fermentation and yeast extract added).

The polynomial proposed model given in Equation 3 with the entire regression coefficient was:

Cells viability *10^9 CFU/ml=-49.063012401461+2.8716578643768*T-0.041740000000008*T^2+3.1465196609398*Yeast-6.5218750000003*$Yeast^2$+015000000000031*T*Yeast (Eq.4)

The correlation coefficient (R^2) was 0.92, which meant that 92% of the variability can be explained by the proposed model. To test if the presented model could be expanded in the experimental regions in which points were not included in the regression, pure error was introduced in the analysis of variance to test the lack of fit. Results showed that the model presented could be used to adequately estimate the cell concentration (*p* value= 0.37> 0.05). Thus, the proposed model presented in Equation 4 could be simplified considering only the significant regression coefficients and interactions ($p < 0.05$) (Table 4).

Cells viability *10^9 CFU/ml=-49.063012401461 + 2.8716578643768*T-0.041740000000008*T^2 -6.5218750000003* $Yeast^2$ (Eq.5)

The 2D response surface plot described by the polynomial model (Equation 2) was drawn. The analysis of Figure 2 reveals that maximum of cell concentration can be obtained at a growth temperature of 34°C and yeast extract of 0.28% w/v.

To the best of our knowledge, no previous studies were carried out in order to optimize the growth temperature and amount of yeast extract added. In the literature, *Azospirillum* strains were produced at a range of temperatures of fermentation: Fages [30] cultured *Azospirillum lipoferum* in 20 *l* fermenter at 35°C for 24 h. Askary *et al.* [31] produced *Azospirillum brasilense* at 30°C for 24h. Dardanelli *et al.* [32] grew *Azospirillum brasilense* at 28°C. Bashan *et al.* [13] proposed two new media for mass cultivation of *Azospirillum* and produced cells at 36°C. In parallel, as demonstrated in the previous paragraph, yeast extract has a beneficial effect when added to SIW. However, in the literature, the recommended amount to be added was not clearly specified and varied from 0.1g/L [28, 29] to 5 g/lL[13].

A checkpoint analysis was performed to confirm the theoretical optimum parameters for the production of *Azospirillum brasilense* in SIW predicted by the model (growth temperature of 34°C and yeast extract of 0.28% w/v). Three independent fermentations were runs in shake flasks followed by two independent fermentations in a 7.5L fermenter. Results of cell count (shake flasks: 0.74 ± 0.26 x 10^9 CFU/mL; fermenter: 0.90 ± 0.21 x 10^9 CFU/mL) within the confidence interval of the studied response (cell count) [0.72 x 10^9 CFU/mL; 0.95 x 10^9 CFU/mL] which confirmed the applicability of the optimized conditions for the production of *Azospirillum brasilense*.

3.3 Effect of co-inoculation with *Sinorhizobium meliloti*

Azospirillum brasilense and *Sinorhizobium meliloti,* cultured in SIW, were inoculated on alfalfa plants. Plant dry matter production data after 5 weeks of growth are presented in Table 6.5. Simultaneous co-inoculation significantly improved alfalfa plant growth when compared to the control (inoculated with *Sinorhizobium meliloti* at 10^5 CFU/plant) (p = 0.038). Contrast analysis carried between the treatments showed that inoculation with (10^5 *Sinorhizobium meliloti* /plant +10^6*Azospilrillum brasilense*) or (10^5 *Sinorhizobium meliloti* + 10^5 *Azospirillum brasilense*) gave similar results and enhanced plant growth in comparison to control (increase of plant dry matter

between 17 and 20%).

By calculation of nodulation index, it was not possible to observe any difference among the treatments applied (data not shown) which could be due to the application of the same high rate of *Sinorhizobium meliloti* per plant for all the treatments studied and/or the method of calculation (based on visual observation). The same trend was observed by Groppa *et al.* [33] who noted a significant increase of soybean plant yield when co-inoculated with *Bradyrhizobium japonicum* and *Azospirillum brasilense* although no effect on nodulation.

When concentration of *Azospirillum brasilense* applied decreased to 10^4 cells, plant dry matter decreased. Analysis of variance showed that observed values were not different from the control: co-inoculation of *Sinorhizobium meliloti* (at 10^5CFU/plant) with 10^4*Azospirillum brasilense*/plant seemed to be not interesting when considering plant dry matter yield. Thus, the recommended co-inoculation treatments according to our results were 10^5 *Sinorhizobium meliloti* /plant +10^6 *Azospilrillum brasilense* or 10^5 *Sinorhizobium meliloti* + 10^5 *Azospirillum brasilense*.

Those results are in conformity with the studies of Hassouna *et al.* [34] and Itzigsohn *et al.* [35] who observed an increase in alfalfa plant dry and fresh weight plant when plants were co-inoculated with *Azospirillum brasilense* + *Sinorhizobium meliloti*. Parmar and Dadarwal [36] showed a significant increase in root and shoot biomass, and total plant nitrogen of chickpea when co-inoculated with effective rhizobium strains + *Azospirillum brasilense*. The improved growth due to co-inoculation effect was mainly attributed to the production of plant growth promoting substances and to the cumulative effects of an enhanced supply of nutrients (mainly nitrogen and phosphorus) [37]. *Azospirillum brasilense* is known to produce large amounts of vitamin such as thiamine, niacin and pantothenic acid which could enhance rhizobia root colonization and plant growth [38, 39].

When considering the rate of cells applied in co-inoculation, many studies focused on understanding its effect on alfalfa plant growth. Burdman etal. [40] noted that application of relatively high concentration of *Azospirillum* (10^8 *Azospirillum* cells/plant) combined with 10^5 *Rhizobial* cells */plant* inhibited the plant growth and recommended combined treatment: 10^5 *Rhizobia /plant* + 5 x10^6*Azospilrillum brasilense*/plant. Groppa *et al.* [33] observed that co-inoculation of soybean plants with (5 x 10^6 *Bradyhrizobium japonicum*/plant + 2 x 10^7 *Azospilrillum brasilense*/plant) was efficient and observed an increase in nitrogen content by 23 % over *Bradyrhizobium japonicum*-single inoculated plants. Co-inoculation of faba bean with 5 x 10^6 *Rhizobium /plant* +10^6 *Azospilrillum brasilense*/plant improved yield by 36.5% when

compared to the control (inoculated with $5x10^6$ *Rhizobium /plant*) [20]. Yahalom *et al.* [7] observed that application of *Azospirillum brasilense* and *Sinorhizobium meliloti* at the same rate ($5x10^5$CFU/plant) improved nodulation and plant growth.

Overall, the research was in concordance with results of the present study, which suggested that the rate of *Azospirillum brasilense* applied as a co-inoculant should be the same or one log higher than the rate of *Sinorhizobium meliloti* (10^5 CFU/plant). Moreover, starch industry wastewater was demonstrated to support the growth of *Azospirillum brasilense* and *Sinorhizobium meliloti*, which is promotional for the development of low cost and effective co-inoculant formulation.

4. Conclusion

This study showed that addition of yeast extract or gluconate to SIW at 0.5%w/v significantly improved the growth of *Azospirillum brasilense* (p = 0.0205 and 0.0212, respectively) which is particularly of interest for both the development of inoculant formulation and recycling industrial wastes. In addition, simultaneous optimization of the concentration of added yeast extract and growth temperature demonstrated the possibility to reduce the cost of cell production: optimal cells production (≈0.9 x 10^9 CFU/mL) was obtained when fermentation was carried out at 34°C, with the addition of yeast extract at 0.28%w/v. Our results indicated that co-inoculation of alfalfa plants with *Azospirillum brasilense* and *Sinorhizobium meliloti*, cultured in SIW have the potential to promote plant growth: based on dry matter, best results were observed when plants were inoculated with *Azospirillum brasilense* at 10^5 or 10^6 CFU/plant + *Sinorhizobium meliloti* at 10^5 CFU/plant.

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Table 6.1 Independent variables used in the optimization

	Codes and values of independent variables of experimental designs for screening using response surface methodology				
Levels	-1.414	-1	0	+1	+1.414
Temperature (°C)	31.46	32.5	35	37.5	38.5
Yeast extract (%w/v)	0.02	0.10	0.30	0.50	0.58

Table 6.2 Growth rates, generation time and cells concentration (after 24h) of *Azospirillum brasilense*

	SIW	SIW+ yeast extract (0.5%w/v)	SIW+ Na-gluconate (0.5%w/v)	Bashan medium	SIW+ Yeast extract (0.5%w/v)+ Na-gluconate (0.5%w/v)
Growth rate(h^{-1})	0.266^{a}	0.315^{b}	0.316^{b}	0.324^{b}	0.29^{c}
Generation time(h)	2.610^{a}	2.201^{b}	2.195^{b}	2.141^{b}	2.380^{c}
Cell concentration (CFU/ml)	$3.9x10^{8}$	$8.8\ x10^{8}$	$8.96\ x10^{8}$	$1.11\ x10^{9}$	$6.35x10^{8}$

Values denoted with different lower case letter differ significantly at $P<0.05$ by one-way ANOVA and Tukey's post hoc analyses. Each value is the mean of three independent replicates

Table 6.3 Design showing the set of each run and studied response

	Temperature (°C)	Yeast extract added(%w/v)	Cells count (10^9CFU/ml)
1	32.50	0.10	0.60± 0.035
2	32.50	0.50	0.42± 0.026
3	37.50	0.10	0.27± 0.013
4	37.50	0.50	0.12± 0.007
5	31.46	0.30	0.36±0.021
6	38.54	0.30	0.16±0.03
7	35.00	0.02	0.28±0.04
8	35.00	0.58	0.24±0.012
9	35.00	0.30	0.90±0.049
10	35.00	0.30	0.87±0.051
11	35.00	0.30	0.81±0.048
12	35.00	0.30	0.90±0.055
13	35.00	0.30	0.66±0.032

Table 6.4 Effect of estimation and regression coefficient of the components of the fitted model

	Effect Estimates		Regression coefficients	
	Effect	p	Coefficients	p
Mean/Interc.	0.82800	0.000001*	-49.0630	0.000526*
(1)Temperature (°C)(L)	-0.22821	0.020567*	2.8717	0.000436*
Temperature (°C)(Q)	-0.52175	0.000385*	-0.0417	0.000385*
(2)Yeast extract(%)(L)	-0.09664	0.247638	3.1465	0.440393
Yeast extract(%)(Q)	-0.52175	0.000385*	-6.5219	0.000385*
1L by 2L	0.01500	0.893807	0.0150	0.893807

Table 6.5 Effect of co-inoculation of *Azospirillum Brasilense* and *Sinorhizobium meliloti* on alfalfa plant dry weight

inoculated cells per plant	Average plant dry weight (mg)
(T1) : 10^5 *S. meliloti* + 10^6 *A. Brasilense*	$31.58^{a} \pm 2.85$
(T2) : 10^5 *S. meliloti* + 10^5 *A. Brasilense*	$32.50^{a} \pm 2.10$
(T3) : 10^5 *S. meliloti* + 10^4 *A. Brasilense*	$28.68^{ab} \pm 2.94$
(T4) : 10^5 *S. meliloti*	$27.12^{b} \pm 3.03$
P value	0.037912*
Contrast Analysis	P value
(T1) vs (T2)	0.548400
(T1) vs (T3)	0.118697
(T1) vs (T4)	0.024309*
(T2) vs (T3)	0.058094
(T2) vs (T4)	0.012156*
(T3) vs (T4)	0.478356

Values denoted with different lower case letters differ significantly at $P<0.05$ by contrast analysis.

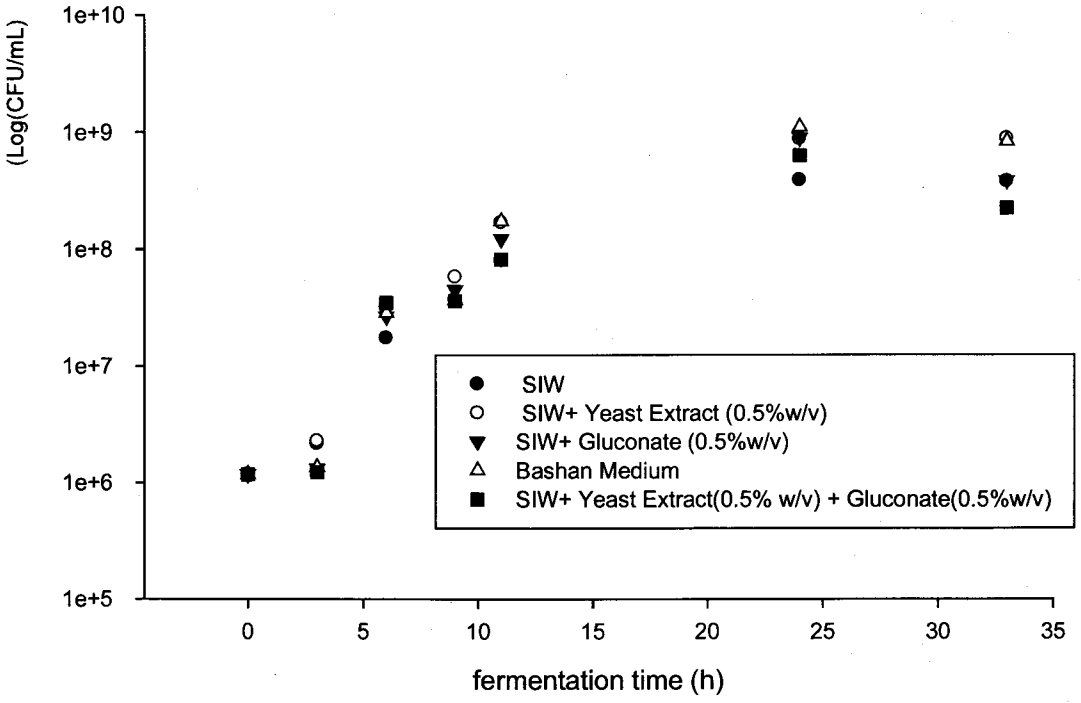

Figure 6.1 Growth of *Azospirillum brasilense in SIW, in SIW withs supplements and in standard Bashan medium* (SIW: starch industrial waste water)

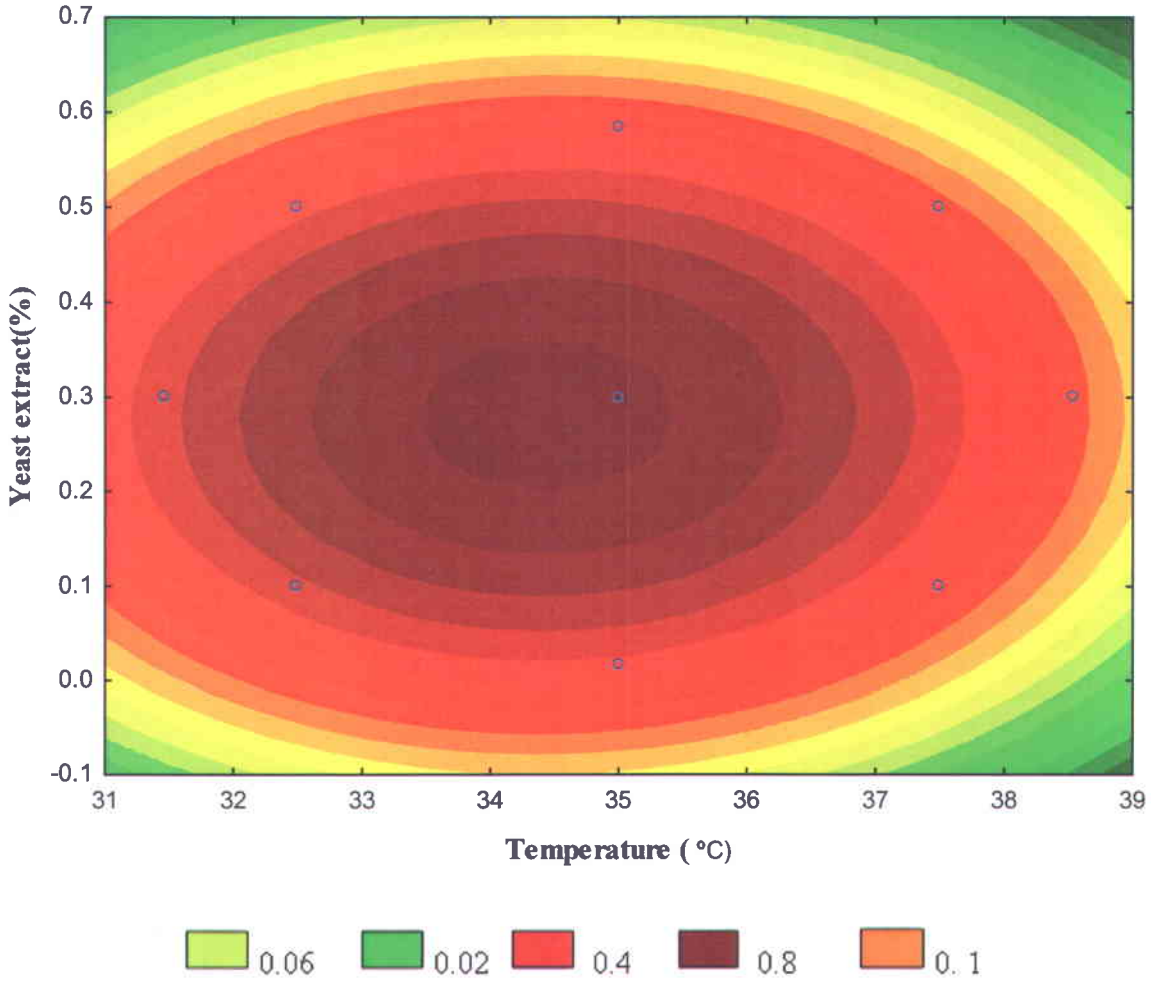

Figure 6.2: Effect of pressure of yeast extract added and growth temperature of fermentation on the production of *Azospirillum brasilense* in starch industry waste water (x 10^9 CFU/ml)

CHAPITRE 7

CONCLUSION ET RECOMMANDATIONS

1. Conclusion

Cette étude a démontré la faisabilité de développement de formulation (liquides, en poudre et de grains enrobés) de *Sinorhizobium meliloti*, cultivée dans les eaux usées d'industries d'amidon. Elle a démontré également que ces formulations sont stables au cours de la conservation et répondent aux normes canadiennes d'inoculants. Les procédés optimisés dans cette thèse peuvent être appliquées pour des productions à l'échelle industrielle.

1. La récolte des *S.meliloti* est une étape indispensable pour le développement des formulations concentrées en cellules, efficaces et stables. La bonne optimisation de cette étape permet de réduire les coûts de transports des produits (en diminuant les volumes) et augmente les marges de conservation (par augmentation de la concentration des cellules récoltées). La centrifugation en utilisant le rotor à gadgets mobiles permet une récupération maximale des cellules lorsqu'elle est effectuée entre 2000 to 4000 *g* pour des durées de 20 à 25 minutes, ou entre 8000 et 10000 *g* pour des durées de 5 à 10 minutes.
2. Dans la plupart des cas, les formulations de bioinoculants sont diluées dans l'eau avant d'être directement appliquées sur les semences. Le passage ainsi par la phase « formulation en suspension » est parfois indispensable. Le bon choix des additifs, et des concentrations permet de garantir l'efficacité de la formulation durant la conservation à l'état liquide et après application sur les semences.
    - ⇨ Les formulations liquides développées dans cette étude ont permis de maintenir la viabilité des cellules à une concentration supérieure à 10^9 CFU/mL durant la conservation à 4°C. Il s'est avéré que le sucrose était le meilleur additif qui permet la protection des *Sinorhizobium meliloti* au cours de la conservation: la plus longue durée de demi-vie a été obtenue avec la concentration de 10 %w/v (83 semaines).
    - ⇨ Concernant les formulations de grains enrobés, le FLF supplémenté avec du PEG et PEG-sorb ont permis de maintenir la viabilité des cellules à une concentration supérieure à 10^3CFU/grain durant 4 mois de conservation.
    - ⇨ L'efficacité des formulations a été testée en évaluant l'indice nodulaire et le rendement en matière sèche. Les meilleurs résultats ont été obtenus avec les formulations combinées (PVP-sorb et PEG-sorb, pour respectivement OLF et FLF).

- ⇨ La distribution des cellules à la surface des grains varie dépendamment des additifs utilisés au cours de la formulation ce qui affecte la viabilité au cours de la conservation.

3. En plus de la viabilité des cellules, la stabilité physique des formulations liquides de *Sinorhizobium meliloti* et des bioinoculants (liquides) en toute généralité est un critère important pour l'évaluation de la qualité des produits. Cette stabilité a été démontrée dépendante des interactions entre les particules en suspensions.
   - ⇨ La détermination de la viscosité ou du potential zeta peut être utilisée comme une nouvelle alternative pour évaluer la stabilité physique des formulations en suspensions.
   - ⇨ La suspendibilité est reliée à la viscosité et au potentiel zêta have à travers un modèle de régression sigmoïdale.
   - ⇨ Il a été démontré que le sorbitol est un agent de suspension efficace, en particulier pour le cas des bioinoculants.
4. La formulation en poudres de *Sinorhizobium meliloti* présente plusieurs avantages que ce soit pour l'industrie ou pour l'agriculteur. En effet la poudre est facilement transportable, adapté aux équipements utilisés par les agriculteurs et peut être conservée à la température ambiante.
   - ⇨ il a été possible d'optimiser simultanément les trois principaux paramètres du séchage (viabilité des cellules, l'humidité, et la température à la sortie de l'atomiseur) par la méthode de réponse de surface.
   - ⇨ il a été possible aussi de trouver un compromis entre ces paramètres et de faire une optimisation multiple en introduisant la fonction de désirabilité.
   - ⇨ Sous les conditions optimales de séchage, la viabilité des cellules obtenue était de 6.8 10^9 CFU/g, cette viabilité a été maintenue supérieure à 1x 10^9CFU/g au cours de 4 mois de conservation : les additifs utilisés (sorbitol, sucrose et lactosérum) ont permis la protection des cellules contre les effets de la température élevée et contre la dessiccation au cours de la conservation.
   - ⇨ L'évaluation de l'indice nodulaire et du rendement des plantes en matières sèches a démontré que les additives utilisés exercent un effet stimulant pour les cellules de *Sinorhizobium meliloti* au cours de la nodulation, ce qui permet une bonne croissance des plantes.
   - ⇨ L'observation de la morphologie de la poudre avec la microscopie électronique à balayage a permis de décrire la structure de particules obtenues après

séchage : les cellules sont enrobées par une matrice formée par le mélange (lactosérum, sorbitol, sucrose) ce qui pourrait jouer le rôle de barrière ou couche protectrice pour le *Sinorhizobium meliloti* au cours du séchage et de la conservation et expliquer les résultats obtenus.

5. La co-inoculation de *Sinorhizobium meliloti* avec *Azospirillum brasiliense* permet d'améliorer le rendement des plantes. Dans la littérature, plusieurs travaux ont démontré que cette association favorisent la nodulation et le développement racinaire de la luzerne (Hassouna *et al.* 1994; Itzigsohn *et al.* 1990). L'ajout ainsi des cultures d'*Azospirillum brasiliense* aux formulations de *Sinorhizobium meliloti* améliore la compétitivité des produits formulés sur le marché.
   - ⇨ Cette étude a démontré que l'addition de l'extrait de levure ou de gluconate à 0.5% aux eaux usées des industries d'amidon améliore la croissance d'*Azospirillum brasiliense* ce qui est particulièrement intéressant pour le développement des formulations à faible coût et la valorisation des rejets.
   - ⇨ L'optimisation simultanée de la concentration d'extrait de levure et de la température de croissance a démontré la possibilité de réduire le coût de production : la concentration maximale en cellules ($\approx 0.9 \times 10^9$CFU/ml) a été obtenue lorsque la fermentation a été faite à 34°C, avec l'addition de 0.28%w/v d'extrait de levure.
   - ⇨ la co-inoculation des grains de luzerne avec *Azospirillum brasiliense* et *Sinorhizobium meliloti*, cultivés dans les eaux uses d'amidon favorise la croissance des plantes: les meilleurs rendements en poids secs ont été observés lorsque les plantes ont été co-inoculées avec *Azospirillum brasiliense* à 10^5 ou 10^6CFU/plant + *Sinorhizobium meliloti* à 10^5CFU/plant.

2. Recommandations

1. Évaluer le potentiel de croissance des *Azospirillum brasiliense* et *Sinorhizobium meliloti* lorsque ces deux bactéries sont co-inoculées ensemble dans la tourbe. Étudier l'effet de l'humidité et du temps d'incubation sur la concentration finale des cellules dans ces formulations en poudres humides. l'incorporation de quelques fractions de rejets solides avec la tourbe peut être une alternative intéressante pour la réduction du coût de produit.

2. Étudier la stabilité des formulations en suspensions et les formulations de grains enrobés, développées à partir des poudres de cellules séchées par atomisation.

3. Faire des essais aux serres et sur le terrain, en effet les résultats de l'efficacité symbiotique ont été obtenus avec les tests en sachets de croissance, il serait alors recommandable de passer au test à grand échelle.

4. L'ultrafiltration peut être appliquée pour une récupération maximale des cellules, à partir du surnagent (après l'étape de centrifugation).

5. Incorporer l'*Azospirillum brasiliense* dans les formulations de *Sinorhizobium meliloti* développées au cours de cette thèse et étudier la stabilité du co-inoculant.

ANNEXES

ANNEXE 1

Tableau A.1. Caractérisation des eaux usées d'amidon

Composés	Concentration (mg /kg TS)
Carbone total	600345±6980
Nt (azote total)	35089±1473
Pt (phosphores total)	330176±2801
N-NH_3 azote ammoniacal (réduit)	104,8±58.9
N-NO_2^-. N-NO_3^- nitrites et nitrates (azote oxydé)	4,2±1.1
P-PO_4^{3-} (phosphates)	11480±2801
Al (aluminium)	56987±3798
Ca (calcium)	11567±402
Cd (cadmium)	0,51±0.1
Cr (chrome)	1,3±0.04
Cu (cuivre)	338±170
Fe (fer)	7986,4±802
K (potassium)	23056±2853
Pb (plomb)	27,2±3.4
S (soufre)	2301,4±71
Zn (zinc)	250±67
Na (sodium)	2189,4±321

Tableau A.2. Caractérisation des eaux usées d'amidon (suite)

Paramètres	Valeurs
Ph	3,35±0.1
TS(g/l)	14±1.4
TVS(g/l)	10±1.1
SS(g/l)	2±0.8
VSS(g/l)	2±0.6

Tableau A.3. Composition du milieu standard YMB

Constituants	Quantité (en g/l)
Mannitol	10
Extrait de levure	1
K_2HPO_4	0.5
$MgSO_4$	0.2
NaCl	0.1

ANNEXE 2

Table A.4 Profiles of studied parameters: (a) zeta potential, and suspendibility = f (concentration); (b) viscosity, pH and suspendibility = f(concentration)

Sorbitol concentration (g/l)	Zeta potential (Mv)	Suspendibility	Mobility(µm/s/V/cm)	Viscosity
0	-38.824±0.27355	42±4%	-2.79±0.02	18.2±0.2
1	-39.87±0.29558	52±11%	-2.88±0.01	19.46±0.11
2	-40.563±0.26784	59±8%	-2.92±0.01	20.00±0.52
3	-40.702±0.29558	79±12%	-2.94±0.02	21.26±0.31
5	-40.723±0.43285	80±12%	-2.94±0.06	21.87±0.42
7	-41.45±0.21506	85±6%	-3.06±0.01	22.60±0.23
8	-41.968±0.51456	86±5%	-3.00±0.04	23.4±0.10
10	-43.638±0.48844	88±6%	-3.16±0.04359	24.29±0.70

ANNEXE 3

Table A.5 Survival of *Sinorhizobium meliloti* in liquid formulations at 4°C

Storage (weeks)	Alginates 0.2%	Alginates 0.3%	Alginates 0.5%	sucrose 2 %
0	4.5E+09±1.9E+08	4.5E+09±1.6E+08	4.5E+09±2.3E+08	4.5E+09±3E+08
3	3.8E+09±2E+08	3.9E+09±2.9E+08	4.2E+09±2.3E+08	4.4E+09±2.6E+08
6	3.2E+09±2.1E+08	3.9E+09±1.9E+08	3.8E+09±1.6E+08	4.4E+09±2.7E+08
9	3E+09±1.5E+08	3.7E+09±1.5E+08	3.8E+09±1.8E+08	4E+09±2.4E+08
13	2.7E+09±1.3E+08	3.7E+09±1.7E+08	3.3E+09±1.8E+08	3.6E+09±1.8E+08

Storage (weeks)	PVP 2 %	PVP 5%	PEG 5 %	PVP 1 %
0	4.5E+09±3.3E+08	4.5E+09±1.9E+08	4.5E+09±2.6E+08	4.5E+09±2.1E+08
3	4.3E+09±2E+08	4.5E+09±2.6E+08	4.5E+09±2.5E+08	4.3E+09±2.9E+08
6	2.9E+09±1.2E+08	3.3E+09±1.4E+08	3.2E+09±1.7E+08	2.8E+09±1.3E+08
9	1.9E+09±1.3E+08	2E+09±1.2E+08	1.8E+09±1.1E+08	1.5E+09±9.5E+07
13	1.8E+09±9.6E+07	1.5E+09±8.9E+07	1.7E+09±9.2E+07	1.5E+09±1.1E+08

Storage (weeks)	Control	sorbitol 1%	sorbitol 2%	sorbitol 5%
0	4.5E+09±2.4E+08	4.5E+09±2.3E+08	4.5E+09±2.4E+08	4.5E+09±2.7E+08
3	1.3E+09±6.6E+07	3.5E+09±3E+08	4.2E+09±2.6E+08	3.9E+09±2.4E+08
6	2.8E+08±2.1E+07	3.4E+09±1.8E+08	3.6E+09±1.9E+08	3.6E+09±1.8E+08
9	4.2E+07±2E+06	3.4E+09±1.9E+08	3.5E+09±1.9E+08	3.5E+09±2E+08
13	4178571±2.4E+05	3.3E+09±2.4E+08	3.4E+09±1.6E+08	3.5E+09±2.1E+08

Table A.5 Survival of *Sinorhizobium meliloti* in liquid formulations at 4°C(continued)

Storage (weeks)	sucrose 5 %	sucrose 10%	PEG 1%	PEG 2 %
0	4.5E+09±2.6E+08	4.5E+09±3E+08	4.5E+09±3E+08	4.5E+09±2.7E+08
3	4.4E+09±2.6E+08	4.5E+09±3.1E+08	4.2E+09±2.9E+08	4.4E+09±2 E+08
6	4.4E+09±2.1E+08	4.4E+09±2.4E+08	3.9E+09±2.1E+08	4.5E+09±2.2E+08
9	4.1E+09±2.2E+08	4.2E+09±2.6E+08	2E+09±1.2E+08	1.7E+09±8.9E+07
13	4.1E+09±2.9E+08	4.1E+09±3E+08	1.8E+09±1.3E+08	1.6E+09±1E+08

Table A.6 Half life of different rhizobial liquid formulations

Formulations	Half life (weeks)
sucrose 10%	83
sucrose 5%	73
alginates 0.3%	46
sucrose 2%	41
sorbitol 5%	35
alginates 0.5%	30
sorbitol 1%	30
sorbitol 2%	29
alginates 0.2 %	17
PEG 1%	10
PEG 2%	9
PVP 2%	9
PVP 5%	9
PEG 5%	8
PVP 1% sorbitol 2%	8
PVP 1% alginates 0.3 %	7
PEG 1% sorbitol 2%	7
PVP 1%	7
PVP 1% sucrose 5%	6
PEG 1% sucrose 5%	6
PEG 1% alginates 0.3%	5
Control	2

Table A.7 Survival of *Sinorhizobium meliloti* cells on alfalfa seeds coated with 13 weeks stored liquid formulation

Storage in days	PVP1%- Alginates 0.2%	Sorbitol 2%	Alginates 0.2%	Sucrose 5%
1	1.7E+05±8.0E+03	1.5E+04±8.7E+04	8.1E+04±3.3E+03	6.8E+04±3.5E+03
3	1.2E+04±6.7E+02	2.0E+03±1.2E+04	4.1E+03±2.5E+02	1.3E+04±7.1E+02
10	2.3E+02±9.6E+00	1.9E+02±1.1E+03	3.0E+02±2.2E+01	4.5E+03±1.9E+02
30				4.0E+03±1.9E+02
45				2.0E+02±8.3E+00
90				

storage in days	Control	PVP1%	PVP1%-Sucrose 5%	PVP1%- Sorbitol 2%
1	5.4E+05±2.8E+04	2.3E+05±1.2E+04	1.2E+05±6.2E+03	1.8E+05±9.8E+03
3	3.0E+04±1.5E+03	7.0E+04±5.0E+03	4.0E+04±2.9E+03	1.0E+05±5.9E+03
10	5.7E+03±4.3E+02	2.3E+03±1.2E+02	7.7E+03±4.3E+02	7.9E+03±4.9E+02
30	2.0E+02±9.7E+00	3.0E+02±1.7E+01	9.0E+02±5.3E+01	5.2E+03±2.5E+02
45				1.0E+02±5.5E+00
90				

storage in days	PEG1%	PEG1%-Sucrose 5%	PEG1%- Sorbitol 2%	PEG1%-Alginates 0.2%
1	2.3E+05±1.2E+04	2.1E+05±7.4E+03	8.7E+05±4.1E+04	1.6E+05±7.8E+03
3	1.0E+04±6.5E+02	2.0E+04±1.5E+03	4.0E+04±2.6E+03	5.3E+04±3.1E+03
10	1.2E+03±6.0E+01	3.5E+03±1.7E+02	5.5E+03±2.7E+02	1.0E+02±6.7E+02
30	2.0E+02±7.4E+00	1.0E+02±3.9E+00	4.0E+02±2.1E+01	
45				
90				

Table A.8: Survival of *Sinorhizobium meliloti* cells on alfa alfa seeds coated with fresh liquid formulation (FLF)

Storage in days	PVP1%- Alginates 0.2%	Sorbitol 2%	Alginates 0.2%	Sucrose 5%
1	6.4E+05±2.7E+04	6.9E+05±4.1E+04	1.6E+05±9.0E+03	1.1E+06±7.2E+04
3	2.1E+05±1.1E+04	4.2E+05±2.6E+04	1.3E+05±7.1E+03	3.8E+05±2.6E+04
15	2.3E+04±1.5E+03	5.8E+04±2.8E+03	9.0E+03±5.0E+02	1.4E+05±7.6E+03
30	6.8E+02±3.4E+01	1.2E+04±6.4E+02	6.0E+02±3.8E+01	3.0E+03±1.9E+02
45		5.8E+03±2.8E+02		2.3E+03±1.2E+02
60		1.0E+03±6.8E+01		1.0E+02±6.3E+00
75		4.0E+02±1.9E+01		
90				
120				
150				

Storage in days	PEG1%	PEG1%-Sucrose 5%	PEG1%- Sorbitol 2%	PEG1%-Alginates 0.2%
1	7.5E+05±2.7E+04	1.1E+06±5.8E+04	8.6E+05±5.8E+04	9.6E+05±5.5E+04
3	2.7E+05±2.0E+04	5.8E+05±3.1E+04	5.6E+05±3.2E+04	2.2E+05±1.3E+04
15	1.1E+05±5.2E+03	1.4E+05±6.1E+03	2.4E+05±1.5E+04	1.9E+04±8.7E+02
30	5.9E+04±2.3E+03	1.7E+03±8.2E+01	5.6E+04±3.3E+03	7.2E+03±3.8E+02
45	5.1E+04±1.9E+03	1.1E+03±4.4E+01	4.1E+04±1.7E+03	4.0E+02±1.8E+01
60	2.5E+04±1.2E+03	6.5E+02±3.8E+01	4.0E+04±2 E+03	
75	2.3E+04±1.5E+03		3.2E+04±2.3E+03	
90	1.4E+04±6.6E+02		4.5E+03±2E+02	
120	7.4E+03±3.8E+02		4.2E+03±1.7E+02	
150	8.0E+02±3.9E+01		1.8E+02±1.2E+01	

Table A.8 Survival of *Sinorhizobium meliloti* cells on alfa alfa seeds coated with fresh liquid formulation (FLF) (continued)

Storage in days	Control	PVP1%	PVP1%-Sucrose 5%	PVP1%- Sorbitol 2%
1	5.4E+05±2.8E+04	4.3E+05±2.2E+04	9.1E+05±4.9E+04	6.5E+05±3.9E+04
3	3.0E+04±1.5E+03	1.8E+05±1.3E+04	4.3E+05±3.2E+04	3.1E+05±1.9E+04
15	5.7E+03±4.3E+02	6E+03±3.2E+02	7.7E+04±4.3E+03	8.2E+04±4.0E+03
30	2.0E+02±9.7E+00	6.0E+02±3.4E+01	1.0E+03±5.9E+01	1.2E+04±6.7E+02
45			8.0E+02±4.4E+01	5.2E+03±2.5E+02
60				4.0E+02±2.2E+01
75				
90				
120				
150				

ANNEXE 4

Table A.9 Viability of Sinorhizobium meliloti spray dried at optimal conditions during storage at 4°C

Storage (months)	Cell count x10^9CFU/g
0	6.62±0.17
1	5.71±0.20
2	4.18±0.25
4	3.19±0.14

ANNEXE 5

Table A.10 Supplement growth selection for *Azospirillum brasiliense*

SIW	SIW+ Yeast Extract	SIW+ Glyconate	Bashan Medium	SIW+ Yeast Extract + Glyconate
1.2E+06±7.2E+04	1.2E+06±5.9E+04	1.2E+06±6.1E+04	1.2E+06±6.8E+04	1.2E+06±8.6E+04
2.2E+06±1.1E+05	2.3E+06±1.1E+05	1.3E+06±7.0E+04	1.4E+06±7.7E+04	1.2E+06±5.8E+04
1.8E+07±9.7E+05	3.2E+07±1.1E+06	2.7E+07±1.1E+06	2.9E+07±1.6E+06	3.5E+07±1.4E+06
3.7E+07±2.2E+06	5.8E+07±4.4E+06	4.5E+07±2.2E+06	3.7E+07±2.3E+06	3.6E+07±2.5E+06
8.0E+07±3.3E+06	1.7E+08±8.2E+06	1.2E+08±6.6E+06	1.8E+08±9.4E+06	8.2E+07±4.4E+06
3.9E+08±2.1E+07	8.8E+08±3.5E+07	9.0E+08±5.1E+07	1.1E+09±5.3E+07	6.4E+08±2.6E+07
3.8E+08±2.5E+07	8.8E+08±4.2E+07	3.9E+08±2.3E+07	8.4E+08±5.7E+07	2.3E+08±1.3E+07