Université du Québec
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Développement d'un nouveau bioprocédé utilisant les biocoagulants/biofloculants produits par les microorganismes pour la séparation solide/liquide des boues d'épuration

Par

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PUBLICATIONS DANS CETTE THÈSE

1. **Bala Subramanian S.**, S. Yan, R. D. Tyagi and R. Y. Surampalli. (2008). A New Pellet Forming Fungal Strain: Its Isolation, Molecular Identification, and Performance for Simultaneous Sludge Solids Reduction, Flocculation, and Dewatering. *Water Environmental Research*, 80 (9): 840-852.
2. **Bala Subramanian S.**, Song Yan, R. D. Tyagi and R.Y. Surampalli. (2009)**.** Chapter-7 Bioflocculants. Sustainable Sludge Management: Production of Value Added Products. Published by *American Society of Civil Engineers*. Pages: 146-167.
3. **Bala Subramanian S.**, S. Yan, K. C. K. Lai, R. D. Tyagi and R. Y. Surampalli. (2006). Screening of Extracellular Polymeric Substances (EPS) producing Microorganisms for Sludge Settling. *IWA Specialized Conference - Sustainable Sludge Management: state of the art, challenges and perspectives*, May 29-31, 2006, Crocus Expo Exhibition Center, Moscow, Russia. Pages: 188 -195.
4. **Bala Subramanian S**, S. Yan, R. D. Tyagi and R. Y. Surampalli. 2009. Simultaneous Solids and Pathogens Reduction, Settling and Dewatering (SSPRSD) Using a Filamentous Fungal Strain Penicillium expansum BS30 Isolated from Wastewater Sludge. *Journal of Environmental Engineering*, (Accepted-In-press). http://dx.doi.org/10.1061/(ASCE)EE.1943-7870.0000217
5. **Bala Subramanian S**., Yan, S., Tyagi, R. D., and Surampalli R. Y. (2009). Extracellular Polymeric Substances (EPS) Producing Bacterial Strains Of Municipal Wastewater Sludge: Isolation, Molecular Identification, EPS Characterization And Performance For Sludge Settling And Dewatering. *Water Research.* (Accepted-In-press). http://dx.doi.org/10.1016/j.watres.2009.12.046
6. **Bala Subramanian S**., Yan, S., Tyagi, R. D., and Surampalli R. Y. (2009). EPS producing Microbial community analysis from CUQ wastewater treatment plant. (*To be communicated to Nucleic Acid Research*).

AWARDS

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BREVET HORS THÈSE

National Patent (India)

1. Gaonkar, S.N., B.S. Swami, M. Udhayakumar, C.E. Deshmukh, C.B. Jagtap, S.T. Chongdar, R. Mohanram, S. Titus, P. Kumar, P.C. Deb and **Bala Subramanian S.** 2006. "A Process For The Production Of Bioemulsifier Using A Consortium Of Marine Oil Degrading Bacteria". Patent Filed Date: 27/05/2004. Patent Application No. 964/DEL/2004. Patent Publication Date: 23/06/2006.

PUBLICATIONS HORS THÈSE

Journals

1. **Bala Subramanian S.,** S. Yan, R. D. Tyagi, R. Y. Surampalli and BN Lohani. (2008). Isolation and Molecular Identification of Extracellular Polymeric Substances (EPS) Producing Bacterial Strains for Sludge Settling and Dewatering. *Journal of Environmental Science and Health, Part A*, 43(13): 1495-1503.
2. **Bala Subramanian S.,** A. S. Kamat and K. K. Ussuf and R. D. Tyagi. (2006). Virulent gene based DNA probes for the detection of pathogenic strains of Bacillus cereus BIS59 from foods, *Process Biochemistry*. 41: 783–788.
3. Mohammedi, S., **Bala Subramanian S.,** Yan S, Tyagi, R. D and Valéro, J. R. (2006). Molecular Screening of *Bacillus thuringiensis* strains from Wastewater Sludge for Biopesticide Production, *Process Biochemistry*. 41: 829–835.
4. Sapna Pavitran, C. B. Jagtap, **Bala Subramanian S.,** Susan Titus, Pradeep Kumar and P. C. Deb. (2006). Microbial Bioremediation of Fuel Oil hydrocarbons in Marine Environment. *Defence Science Journal.* 56 (2): 209-224.
5. Yan, S., **Bala Subramanian S.,** S. Narsaiah, R. Y. Surampalli and R. D. Tyagi. (2007). Isolation, Characterization and Identification of Bacteria from Activated Sludge and Soluble Microbial Products in Wastewater Treatment Systems. *Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management*. 11 (4): 240-258.
6. Yan, S., **Bala Subramanian S.,** Mohammedi, S., R. D. Tyagi, R. Y. Surampalli and B.N. Lohani. (2007). Wastewater sludge as a raw Material For Biopesticides Production- Impact of seasonal Variations. *Journal of Residuals Science & Technology,* 4 (4): 179-184.

7. Yan S., **Bala Subramanian S.,** R.D. Tyagi and R.Y. Surampalli (2008). Polymer production by bacterial strains isolated from activated sludge treating municipal wastewater. *Water Science and Technology*. 57 (4): 533–539.
8. Yan S., **Bala Subramanian S.,** R.D. Tyagi and R.Y. Surampalli (2008). Bioplastics from waste activated sludge-Batch process. *Practice periodical of Hazardous, Toxic, and Radioactive Waste Management,* 12 (4): 239-248.
9. Yan S., **Bala Subramanian S.,** R.D. Tyagi, R.Y. Surampalli and T. C. Zhang (2010). Emerging Contaminants of Environmental Concern: Source, Transport, Fate and Treatment. *Practice periodical of Hazardous, Toxic, and Radioactive Waste Management*: 14(1): 2-20.
10. Yan S., **Bala Subramanian S.,** R.D. Tyagi and R.Y. Surampalli (2007) PHA production by activated sludge and/or waste materials – a review (Submitted to *Water Research*).
11. Yan S., **Bala Subramanian S.,** R.D. Tyagi and R.Y. Surampalli (2007) Impact of feeding pattern on polyhydroxyalkanoates (PHA) production using activated sludge from a pulp-paper industry wastewater treatment plant (Submitted to *American Society of Civil Engineers*).
12. Yan S., **Bala Subramanian S.,** R.D. Tyagi and R.Y. Surampalli (2007) Bioplastics production using activated sludge from pulp-paper wastewater treatment plant with continuous feed of carbon source. *Practice Periodical of Toxic, Hazardous and Radioactive Waste Management. American Society of Civil Engineers* (Submitted).
13. Yan S., **Bala Subramanian S.,** R.D. Tyagi and R.Y. Surampalli (2007) Isolation of polyhydroxyalkanoate (PHA) accumulating bacterial strains from pulp and paper industry wastewater activated sludge (Submitted to *Water Research*).
14. Yan S., **Bala Subramanian S.,** R.D. Tyagi and R.Y. Surampalli (2007) Molecular screening of microbial strains from pulp-paper wastewater sludge for polyhydroxyalkanoate (PHA) production (to be Submitted).
15. Yan S., **Bala Subramanian S.,** R.D. Tyagi and R.Y. Surampalli (2007) Molecular identification of polyhydroxyalkanoate (PHA) accumulating bacteria isolated from municipal wastewater sludge (to be submitted).

Book Chapters

1. Kshipra Misra, **Bala Subramanian S.**, S. K. Brar, R. D. Tyagi and R.Y. Surampalli. 2007. Chapter – 7: Biological Degradation., Book entitled "Remediation Technologies for Soils and Groundwater". Published by *American Society of Civil Engineers*. Pages 199-222.
2. Kshipra Misra, **Bala Subramanian S.**, S. K. Brar, R. D. Tyagi and R.Y. Surampalli. 2007**.** Chapter-8: Bioremediation with bacteria and enzymes. Book entitled "Remediation Technologies for Soils and Ground water". Published by *American Society of Civil Engineers*. Pages 223-258.
3. **Bala Subramanian S.**, Song Yan, R. D. Tyagi and R.Y. Surampalli. 2008**.** chapter-4: biological assimilation and degradation. Book entitled "Natural Processes and Systems for Hazardous Waste Treatment". Published by *American Society of Civil Engineers*. Pages 94-119.

4. Shankha K. Banerji, Rao Y. Surampalli, Pascale Champagne, R.D. Tyagi, **Bala Subramanian, S** and S. Yan. 2008. Chapter 6: Phytoprocesses. Book entitled "Natural Processes and systems for Hazardous Waste Treatment". Published by *American Society of Civil Engineers*. Pages 161-188.

5. **Bala Subramanian S.,** Song Yan, R. D. Tyagi and R.Y. Surampalli. 2009. Molecular Biology Techniques for CoEEC Degrading Organisms. Chapter-12. Contaminants of Emerging Environmental Concern. Sponsored by: Emerging Contaminants of Concern Task Committee, Environmental Council; Environmental and Water Resources Institute (EWRI) of the American Society of Civil Engineers. Published by The American Society of Civil Engineers. Pages 446-480.

6. Yan. S., **Bala Subramanian S.,** Barnabe, S., R. D. Tyagi and R. Y. Surampalli. (2009). Surfactants. Chapter-8. Contaminants of Emerging Environmental Concern. Sponsored by: Emerging Contaminants of Concern Task Committee, Environmental Council; Environmental and Water Resources Institute (EWRI) of the American Society of Civil Engineers. Published by The American Society of Civil Engineers. Pages 279-314.

7. Song Yan, **Bala Subramanian S.**, R. D. Tyagi, R.Y. Surampalli, and Tian C. Zhang. 2009**.** Chapter-1. Introduction. Sustainable Sludge Management: Production of Value Added Products. Published by *American Society of Civil Engineers.* Pages 1-5.

8. Song Yan, **Bala Subramanian S.**, R. D. Tyagi and R.Y. Surampalli. 2009**.** Chapter-2. Wastewater sludge characteristics. Sustainable Sludge Management: Production of Value Added Products. Published by *American Society of Civil Engineers.* Pages 6-36.

9. **Bala Subramanian S.**, Song Yan, R. D. Tyagi, R.Y. Surampalli and Tian C. Zhang. 2009**.** Chapter-5. Biosurfactants. Sustainable Sludge Management: Production of Value Added Products American Society of Civil Engineers. Published by *American Society of Civil Engineers.* Pages 101-122.

10. Song Yan, **Bala Subramanian S.**, R. D. Tyagi and R.Y. Surampalli. 2009**.** Chapter-6. Bioplastics from activated sludge. Sustainable Sludge Management: Production of Value Added Products American Society of Civil Engineers. Published by *American Society of Civil Engineers.* Pages 123-145.

11. **Bala Subramanian S.**, Song Yan, R. D. Tyagi and R.Y. Surampalli. 2009**.** Chapter-7. Bioflocculants. Sustainable Sludge Management: Production of Value Added Products American Society of Civil Engineers. Published by *American Society of Civil Engineers*. Pages 146-167.

12. **Bala Subramanian S.**, Song Yan, R. D. Tyagi, R.Y. Surampalli, and Tian C. Zhang . 2009**.** Chapter-9. Biofertilizers/bioinoculants. Sustainable Sludge Management: Production of Value Added Products American Society of Civil Engineers. Published by *American Society of Civil Engineers.* Pages 203-230.

13. **Bala Subramanian S.**, Song Yan, R. D. Tyagi and R.Y. Surampalli. 2009**.** Chapter-10. Enzymes: Production and Extraction. Sustainable Sludge Management: Production of Value Added Products American Society of Civil Engineers. Published by *American Society of Civil Engineers* Pages 231-261.

CONGRÈS ET CONFÉRENCES

1. **Bala Subramanian S.,** S. Yan, R. D. Tyagi, and R. Y. Surampalli. 2009. Simultaneous Sludge Solids and Pathogens Reduction, Settling and Dewatering (SSPRSD) using filamentous fungal strain isolated from wastewater sludge. 44th CENTRAL Canadian Symposium on Water Quality Research. *February 23-24, 2009*. Canada Centre for Inland Waters, Burlington, ON, Canada.
2. Yan, S., **Bala Subramanian, S.,** R. D. Tyagi, And R. Y. Surampalli. 2009. Greenhouse Gas Emissions Analysis of Biosolids Management Options. 44th CENTRAL Canadian Symposium on Water Quality Research. *February 23-24, 2009*. Canada Centre for Inland Waters, Burlington, ON, Canada.
3. Tyagi, R. D. and **Bala Subramanian S**. 2008. Bioconversion of wastewater sludge into value added products: Biocontrol agents and bioplastics. Technical seminars from the Edmonton Waste Management Centre of Excellence. October 30, 2008. Wastewater Research & training centre, Gold Bar wastewater treatment Plant, 10977-50 street, Edmonton, AB, Canada.
4. **Bala Subramanian S**, Yan, S., R. D. Tyagi and R. Y. Surampalli. 2007. Characterization of Extracellular Polymeric Substances (EPS) Extracted from both Sludge and Pure Bacterial Strains Isolated from Wastewater Sludge for Sludge Dewatering. IWA conference on "*Moving Forward,* Wastewater Biosolids Sustainability: Technical, Managerial, and Public Synergy" June 24-27, 2007. Moncton, NB, Canada.
5. Yan, S., **Bala Subramanian S**, R. D. Tyagi and R. Y. Surampalli. 2007. Polymer production by bacterial strains isolated from activated sludge treating municipal wastewater. IWA conference on "*Moving Forward,* Wastewater Biosolids Sustainability: Technical, Managerial, and Public Synergy" June 24-27, 2007. Moncton, NB, Canada.
6. **Bala Subramanian S,** Yan, S., R. D. Tyagi, R. Y. Surampalli and B.N. Lohani. 2007. Isolation and Molecular Identification of Extracellular Polymeric Substances (EPS) Producing Bacterial Strains for Sludge Dewatering. *IWA Facing Sludge Diversities: Challenges, Risks and Opportunities 28 - 30 March 2007, Pine Beach Hotel, Belek - Antalya, TURKEY.*
7. Yan, S., **Bala Subramanian S,** Mohammedi, S., R. D. Tyagi, R. Y. Surampalli and B.N. Lohani. 2007. Wastewater sludge as a raw Material For Biopesticides Production- Impact of seasonal Variations. *IWA Facing Sludge Diversities: Challenges, Risks and Opportunities 28 - 30 March 2007, Pine Beach Hotel, Belek - Antalya, TURKEY.*
8. **Bala Subramanian S**, Vu, D. K., Yan, S., R. D. Tyagi and R. Y. Surampalli. 2006. Evaluation of bioflocculants produced by sludge microorganisms for sludge settling and dewatering. "22nd Eastern Canadian Symposium on Water Quality Research" on November 3, 2006, at Concordia University, Montreal, QC, Canada.
9. Yan, S., **Bala Subramanian S**, R. D. Tyagi and R. Y. Surampalli. 2006. Isolation of bioplastics accumulating bacterial strains from municipal wastewater sludge. "22nd Eastern Canadian Symposium on Water Quality Research" on November 3, 2006, at Concordia University, Montreal, QC, Canada.
10. D. K. Vu, **Bala Subramanian S**, R. D. Tyagi, J. R. Valero and R. Y. Surampalli. 2006. Induction Of Chitinases Production For Enhancement The Entomotoxicity of *Bacillus*

thuringiensis Based Biopesticides Using Starch Industry Wastewater As Raw Material. "22nd Eastern Canadian Symposium on Water Quality Research" on November 3, 2006, at Concordia University, Montreal, QC, Canada.

11. **Bala Subramanian S**, S. Yan, R. D. Tyagi and R. Y. Surampalli. 2006. Isolation of Extra Cellular Biopolymer Producing Microorganisms from Wastewater Sludge for Sludge Settling and Dewatering. Water Environment Federation (*WEFTEC-06), 79th Annual Technical Exhibition and Conference,* October 21-25, 2006, Dallas Convention Center, Dallas, TX, USA.

12. Yan, S., **Bala Subramanian S**, R. D. Tyagi and R. Y. Surampalli. 2006. Polyhydroxyalkanoates (PHA) production using activated sludge from a pulp-paper industry wastewater treatment plant. *IWA World Water Congress and Exhibition.* September 10-14, 2006 Beijing, China.

13. **Bala Subramanian S**, S. Yan, K. C. K. Lai, R. D. Tyagi and R. Y. Surampalli. 2006. Screening of Extracellular Polymeric Substances (EPS) producing Microorganisms for Sludge Settling. *IWA Specialized Conference - Sustainable Sludge Management: state of the art, challenges and perspectives,* May 29-31, 2006, Crocus Expo Exhibition Center, Moscow, Russia.

14. Yan, S., **Bala Subramanian S**, R. D. Tyagi and R. Y. Surampalli. 2006. Bioplastics production using activated sludge from pulp-paper wastewater treatment plant with continuous feed of carbon source. *IWA Specialized Conference - Sustainable Sludge Management: state of the art, challenges and perspectives,* May 29-31, 2006, Crocus Expo Exhibition Center, Moscow, Russia.

15. Yan, S, **Bala Subramanian S**, R. D. Tyagi and R. Y. Surampalli. 2006. Screening and identification of PHA (Bioplastics) accumulators among the diverse microbial community of wastewater sludge. *8th CSChE Ontario-Quebec Biotechnology Meeting,* May 25-26, 2006, McGill University, Montreal (QC), Canada.

16. **Bala Subramanian S,** Yan, S and R. D. Tyagi. 2006. Zetaphoremeter Characterization of Microbial Extracellular Polymeric Substances (EPS) for bioflocculation of Wastewater sludge. *8th CSChE Ontario-Quebec Biotechnology Meeting*, May 25-26, 2006, McGill University, Montreal (QC), Canada.

17. **Bala Subramanian S**., Dang-Khanh Vu, Yan S, R. D. Tyagi and R.Y. Surampalli. 2005. Isolation and Characterization of Bioflocculants/Biocoagulants Producing Microorganisms and Their Use for Wastewater Sludge Dewatering. 21st Congress of the East Canada Research on the Water Quality, CAWQ, ACQE, Nov 4, 2005, INRS-ETE, Québec, (QC), Canada.

18. Dang-Khanh Vu, **Bala Subramanian Sellamuthu**, Satinder Kaur Brar, Somsak Sivichai, Rajeshwar Dayal Tyagi. 2005. Collection, Isolation and Identification of Insect-pathogenic fungi from forests for biopesticides production. 21st Congress of the East Canada Research on the Water Quality, CAWQ, ACQE, Nov 4, 2005, INRS-ETE, Québec, (QC), Canada.

SCIENTIFIC REPORTS

1. Amine, M., **Bala Subramanian S.,** and R. D. Tyagi. 2009. Scientific report- HET. Project work was carried out for Canadian HET company, Confidential scientific report was submitted to INRS library, entitled "Analyses microbiologiques et physicochimiques du procédé de fermentation de lactosérum de la compagnie HET." **Numéro de rapport de recherche R-1090**. Dated March 11, 2009, INRS-Eau, Terre et Environnement, University of Quebec, Canada, p1-29.
2. **Bala Subramanian S.,** Adjalle, K., and R. D. Tyagi. 2009. Scientific report- HET. Project work was carried out for Canadian HET Company, Confidential scientific report was submitted to INRS library, entitled "**Production of Animal Feed (Base-L) from Whey**." **Numéro de rapport de recherche R-1089**. Dated March 11, 2009, INRS-Eau, Terre et Environnement, University of Quebec, Canada, p1-21.
3. **Bala Subramanian S.,** Chabot, F., and R. D. Tyagi. 2009. Scientific report- HET. Project work was carried out for Canadian HET Company, Confidential scientific report was submitted to INRS library, entitled "Inoculum Production for HET Pilot Plant Operation." **Numéro de rapport de recherche R-1047**. INRS-Eau, Terre et Environnement, University of Quebec, Canada, p1-29.
4. Vu, D. K., **Bala Subramanian S.,** Adjalle, D. K. and R. D. Tyagi. 2009. Scientific report- HET. Project work was carried out for Canadian HET company, Confidential scientific report was submitted to INRS library, entitled "Molecular Biology, Microbiological and Biochemical Identification of Microbial Strains Isolated from HET Process and their Final Products" **Numéro de rapport de recherche R-1046**. INRS-Eau, Terre et Environnement, University of Quebec, Canada, p1-30.
5. Antoine Coyac and **Bala Subramanian S**. 2007. Project report entitled, "Réalisation de la biofloculation des boues d'épuration par les biopolymères produits à partir des microorganismes", submitted to "Université de Perpignan", Institut Universitaire de Technologie, Département Genie Biologique option Genie de l'environnement, France, p1-52.
6. Vu, D. K., **Bala Subramanian S.,** Adjalle, D. K. and R. D. Tyagi. 2008. Scientific report- HET. Project work was carried out for Canadian HET company, Confidential scientific report was submitted to HET, entitled "Production of Animal Feed from Whey: Isolation and Identification of Microbial Strains" **Numéro de rapport de recherche R-1017**. INRS-Eau, Terre et Environnement, University of Quebec, Canada, p1-52.
7. **Bala Subramanian S**., Song Yan, R. D. Tyagi and J. R. Valero. 2007. Mini-project work was carried out for Canadian BioTEPP company, Confidential scientific report was submitted to INRS library, entitled "Effet des Conditions Opératoires de Lyophilisation sur le Potentiel Infectieux et Létal des Granulovirus Utilisés pour le Contrôle de la Carpocaspe de la Pomme" **Numéro de rapport de recherche 944**. INRS-Eau, Terre et Environnement, University of Quebec, Canada, p1-55.

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RÉSUMÉ

Au cours du traitement des eaux usées, une importante quantité de boues (contenant ~ 2-5% de solides) est produite. Afin de réduire leur volume, les boues sont décantées et déshydratées. Les méthodes physiques exigent beaucoup de main-d'œuvre, d'électricité, et d'entretien. Le recours aux méthodes chimiques utilisant des polymères cationiques de synthèse (CSP) pour neutraliser les particules de boues chargées négativement s'avère être coûteux, polluant et très toxique. L'alternative pour réduire au minimum l'utilisation des CSP est le recours à une approche biologique utilisant les biocoagulants ou biofloculants.

Depuis cinq décennies, les chercheurs étudient le rôle des substances polymériques extracellulaires microbiennes [SPE] produites au cours du processus de boues activées. Les SPE jouent un rôle majeur dans la formation des bioflocs, leur structuration et leur charge à la surface et dans les procédés de décantation et de déshydratation des boues. Les bioflocs sont formés avec des agrégats microbiens, des souches bactériennes filamenteuses, des particules organiques et inorganiques, qui sont maintenus par les SPE. Ces dernières sont principalement constituées de polysaccharides, de protéines, d'acides nucléiques et d'autres composants cellulaires. Il a été constaté que les SPE sont soit sécrétées à l'extérieur de la cellule (dépôt gluant) ou liées à la paroi cellulaire (EPS capsulaire). Les dépôts gluants sont généralement détachés de la cellule au cours de la centrifugation alors que les SPE capsulaires sont stables et restes fixées à la paroi cellulaire des micro-organismes pendant le processus de séparation.

Jusqu'à ce jour le développement de procédés de biofloculation des boues se heurte à plusieurs problèmes : (i) les interactions entre les SPE et les boues solides ne sont pas bien connues, (ii) le rôle d'autres composants actifs des SPE (protéines, lipides et glucides) dans la formation des bioflocs est indéterminé et (iii) la production de SPE dans les boues activées est un

procesus non contrôlé. Sur la base des expériences passées, il est difficile de contrôler une communauté microbienne au cours du traitement des eaux usées pour produire des SPE spécifiques et/ou de favoriser la croissance d'une souche microbienne aux dépends des autres.

Pour remédier à ces problèmes, nous avons entrepris d'isoler vingt-cinq souches bactériennes productrices de SPE, à partir des boues d'épuration municipales. Les micro-organismes ont été choisis en se basant sur leur production de SPE sur le milieu de croissance. Les souches microbiennes productrices de SPE ont été identifiées par séquençage de leur ADNr 16S. Ces souches ont été cultivées individuellement sur un milieu synthétique et ont produit une quantité importante de SPE (jusqu'à 36 g/L). Toutefois, lorsqu'elles sont cultivées dans un consortium de flores microbiennes (conditions naturelles de croissance dans les boues activées), elles produisent une quantité plus faible de SPE. Trois types de SPE [dépôt gluant, SPE capsulaires et le bouillon bactérien (combinaison des deux SPE)] ont été récoltés et leurs caractéristiques ont été étudiées. La concentration des biofloculants (poids sec), la viscosité et leur charge à la surface (potentiel zêta variant entre -18 à -80mv) ont également été mesurées. L'aptitude des SPE à floculer a été évaluée par la mesure de l'activité de floculation du kaolin (FA). Cette activité a varié entre 77 et 90% dans l'ordre dépôt gluant > SPE capsulaire > SPE dans le bouillon bactérien.

Malgré leur charge négative, les dépôts gluants ont révélé la meilleure activité de biofloculation par rapport à celle du bouillon bactérien et des SPE capsulaires. Les dépôts gluants sont sous forme de longues chaînes polymériques que et comportent de ce fait plus de sites de fixation des particules de boue, des micro-organismes et des agents de liaison (cations). Les SPE capsulaires forment des bioflocs en formant des co-agrégats avec d'autres microbes; la taille du floc est donc moins importante. Les mauvais résultats obtenus avec le bouillon bactérien sont le plus probablement dus à l'obstruction des sites actifs du dépôt gluant par les masses bactériennes. Cette observation explique le fait que les SPE produites naturellement dans les procédés de boues

activées (ASP) ou de digestion aérobi ne sont pas très efficaces pour floculer les boues. En fait, une concentration trop élevée de SPE conduit à une mauvaise floculation. Ainsi les dépôts gluants ont montré une meilleure biofloculation que les SPE capsulaires et le bouillon bactérien. En se basant sur la mesure de l'activité de floculation du kaolin, nous avons sélectionné six souches bactériennes (BS2, BS8, BS9, BS11, BS15 et BS25). Les dépôts gluants produits par ces 6 souches ont été étudiés pour leur capacité à décanter et à déshydrater les boues [par la mesure de l'indice de volume des boues (SVI)] et le temps de succion capillaire (CST), respectivement]. Il a été constaté que la capacité de biofloculation de ces extraits était similaire à celles des polymères cationiques synthétiques (CSP).

Des études sur la caractérisation chimique des dépôts gluants ont également été réalisées. Nous avons constaté que le taux d'hydrates de carbone (TC) dans les SPE est plus élevé que celui des protéines totales (TP) dans tous les cas, sauf dans le cas du consortium bactérien. Après avoir calculé le pourcentage de TC et de TP, il a été constaté que les SPE produites par la culture de souches pures étaient principalement composées de lipides, d'ADN et d'ARN. Les SPE des boues sont, quant à eux, majoritairement constituées de lipides, d'une phase minérale, d'acides organiques, d'acides nucléiques et d'autres (non identifiés). Aussi, le ratio TC/TP des SPE des souches pures est plus élevé que ceux produites par le consortium bactérien. Mais dans cette étude, nous avons constaté que les hydrates de carbone ainsi que les autres composants des SPE (lipides, ADN, ARN) jouent un rôle majeur dans la décantation des boues. Cela montre que les souches bactériennes isolées à partir de boues d'eaux usées municipales produisent une plus grande quantité d'hydrates de carbone et d'autres composants. C'est pour cette raison que le rôle de ces composés dans la formation de floc et la décantation des boues doit être étudié.

Dans le même contexte, une souche de champignon filamenteux a été isolée à partir des boues d'épuration. Elle a été utilisée pour évaluer sa capacité à réduire les solides et les agents

pathogènes, à décanter et à déshydrater les boues. Le champignon a révélé une bonne réduction des matières solides et une déshydratabilité améliorée. L'étude de reproductibilité, menée dans un bioréacteur de 10L, a confirmé les résultats obtenus à savoir une réduction des MES d'environ 54%, une réduction de 2 à 4 unités log d'agents microbiens pathogènes et une meilleure déshydratabilité des boues (CST <20s).

ABSTRACT

During wastewater treatment, a substantial amount of sludge is produced and to reduce its volume (which contains ~2-5% solids) the sludge is settled and subsequently dewatered. Physical methods requires high manpower, electricity, high operational and maintenance cost. Chemical methods employ expensive and toxic cationic synthetic polymers (CSP) to neutralize negatively charged sludge particles. To minimize the use of CSP an alternative and suitable, novel way is to use biocoagulants/bioflocculants.

Since the past five decades researchers have been studying the role of microbial extracellular polymeric substances (EPS) produced during activated sludge process. EPS are known to play a major role in bioflocs formation, bioflocs structure and surface charge and in sludge settling and dewatering. Bioflocs are formed together with microbial aggregates, filamentous bacterial strains, and organic and inorganic particles, which are held together by EPS. They mainly consist of polysaccharides, proteins, nucleic acids and other cellular components. It was found that these types of EPS are either secreted outside the cell (slime EPS) or cellular bound (capsular EPS). Slime types of EPS are generally washed out from cells during centrifugation/harvesting whereas the capsular EPS are stable and attached on the cell wall of microorganisms during the separation process.

To date the problems in successful development of sludge bioflocculation process are mainly due to (i) the interactions between EPS and sludge solids is not known; (ii) the role of other active components of EPS (proteins, lipids and carbohydrates) in biofloc formation also have not been identified; and (iii) uncontrolled production of EPS in activated sludge process. Based on the past experience, it is difficult to control a specific microbial community during wastewater treatment to generate preferred EPS and/or foster the growth of favorable microorganisms to produce EPS.

To address these problems, twenty-five EPS producing bacterial strains were isolated from municipal wastewater sludge. Microorganisms were selected based on EPS production property on the growth medium. EPS producing microbial strains were identified based on the 16S rDNA gene sequencing. These strains when grown individually in synthetic medium produced substantial quantity of EPS, up to 36 g/L. However, when grown in consortium (as they grow naturally in activated sludge or aerobic sludge digestion process), they produced lower quantity of EPS. Three types of EPS [slime, capsular and bacterial broth (combination of both slime and capsular)] were harvested and their characteristics were studied. Bioflocculant concentration (dry weight), viscosity and their charge (using Zetaphoremeter, zeta potential varied between -18 to -80 mv) were also measured. Bioflocculation ability of obtained EPS was evaluated by measuring the kaolin clay flocculation activity (FA). Kaolin (FA) varied 77-90% in the order of slime EPS >capsular EPS >EPS in bacterial broth.

Slime EPS revealed the best bioflocculation efficiency compared to those in bacterial pellet and bacterial broth in spite of their negative charge. Slime EPS possessing longer polymeric chain than the others (capsular and broth) carries more active sites to bind with the sludge particles, microorganisms and bridging agents (cations). Whereas, capsular EPS forms biofloc by forming co-aggregates with other microbes; therefore, the floc size was less. The poor performance of bacterial broth on sludge settling was most likely due to the blockage of the slime EPS active sites by bacterial pellet. This fact also explained that EPS produced naturally in activated sludge process (ASP) or aerobic sludge digestion process was not very effective in sludge flocculation; in fact, high EPS concentration led to very poor flocculation. Thus slime EPS was better in bioflocculation than EPS in capsular and bacterial broth. Six bacterial strains (BS2, BS8, BS9, BS11, BS15 and BS25) were selected based on the kaolin clay flocculation. Slime EPS from six bacterial strains were studied in terms of sludge settling [sludge volume index (SVI)] and dewatering [capillary

suction time (CST)]. The bioflocculation capacity was compared with CSP, which was found to be similar.

Studies on chemical characterization of extracted slime EPS were also conducted. The total carbohydrates (TC) concentration in EPS was higher than total protein (TP) concentration in all cases except the bacterial consortium. After calculating the percentage of TC and TP, it was found that EPS produced by pure culture strains mainly consisted of other components such as lipids, DNA and RNA. In sludge EPS, the major portion could be lipids, mineral phase, organic acids, DNA, RNA and other undefined compounds. Calculated TC/TP ratio showed pure bacterial strains possess higher TC/TP ratio than the consortium and sludge EPS. But in this study we found that carbohydrates and other (lipids, DNA, RNA) components play a major role in sludge settling. This showed that bacterial strains isolated from municipal wastewater sludge produced more quantity of carbohydrates and other components. Further, role of these compounds in floc formation and sludge settling needs to be studied.

Similarly, a floc forming filamentous fungal (FF) strain was isolated from wastewater sludge. FF strain was used to evaluate their potential in solids reduction, pathogen reduction, sludge settling and dewatering. FF strain revealed better solids reduction and enhanced dewaterability. The reproducibility study, conducted in a 10 L bioreactor, revealed enhanced suspended solids reduction (54%), 2-4 log cycle pathogen reductions and better sludge dewaterability (CST <20 s).

Abbreviations:

ASP – Activated sludge process

ATCC – American type culture collection

BOD – Biological Oxygen demand

C/N ratio – Carbon/nitrogen ratio

CFU – Colony forming units

CSP – Cationic synthetic polymer

CST – Capillary suction time

CTAB – Cetyltrimethylammonium bromide

CUQ – Communauté Urbaine du Québec

DLVO – Derjaguin, Landau, Verwey, and Overbeek theory

DNA – Deoxy ribonucleic acid

DO – Dissolved oxygen

EA – Endo agar

EPS – Exocellular or Extra cellular polymeric substances

FA – L'activité de floculation

FF – Filamentous fungal strain

IVB – Indice de volume de boues

K_d – First-order degradation rate constant

LSTA – Lactose selenite and tetrathionate agar

MES – Matières en suspension

MVS – Matières Volatiles en Suspension

NCBI – National center for biotechnology information

NST sludge – Non-sterilized sludge

NSTB – Nonsterile sludge inoculated with beads

NSTNT – Nonsterile sludges not treated with fungal biomass

NSTS – Nonsterile sludge inoculated with spores

PCA – Plate count agar

PCR – Polymerase chain reaction

PDA – Potato dextrose agar

PS – Poids sec

RNA – Ribonucleic acid

rRNA – ribosomal RNA

SBR – Sequencing batch reactor

SDA – Sabouraud's dextrose agar

SDS – Sodium dodecyl sulphate

Sludge – Biosolids

SP – Synthetic polymers

SPE – Substances polymériques extracellulaires

SRF – Specific resistance to filtration

SRT – Sludge retention time

SS – Suspended solids

SSPRSD – Simultaneous solids and pathogens reduction, settling, and dewatering

SSRSD – Simultaneous sludge–solids reduction, settling, and dewatering

ST sludge – Sterilized sludge

STB – Sterile sludge inoculated with beads

STNT – Sterile sludge not treated with fungal biomass

STS – Sterile sludge inoculated with spores

SVI – Sludge volume index

$SVI_{0.1}$ – Sludge volume index (measured at 100 mL volume)

SVI_1 – Sludge volume index (measured at 1000 mL volume)

TC – Total carbohydrate

TCS – 3,3′,4′,5-tetrachlorosalicylanilide

TP – Total protein

TSB – Tryptic soy broth

UASB – Upflow anaerobic sludge blanket

UTEU – Usines de traitement des eaux usées

VSS – Volatile suspended solids

WWS – Waste water sludge

WWTP – Wastewater treatment plant

ZP – Zetapotential

ζ – Zetapotential

CHAPITRE 1.

SYNTHÈSE

BREF HISTORIQUE DES PROCÉDÉS DE TRAITEMENT DES EAUX USÉES

Les procédés de traitement des boues activées ont été développés pour la première fois au début des années 1900 en Angleterre (Ardern et Lockett, 1914) par un célèbre ingénieur anglais nommé William Lindley. Avec ses fils, il a conçu des systèmes d'assainissement de l'eau pour plus de 30 villes européennes. Au début, ils ont développé des procédés de traitement en « batch » puis les ont modifiés en systèmes fonctionnant en flux continu. Malgré le fait qu'ils présentaient beaucoup de difficultés dans leur installation et leur mise en œuvre, les systèmes fonctionnant en flux continu se sont propagés dans le monde entier. Donaldson (1932) a suggéré que le bassin d'aération devait être cloisonné afin d'optimiser la décantation des boues. En 1959, Pasveer a étudié l'utilisation des systèmes de type « fill-and-draw », devenus populaires en Europe pendant quelques années. Cependant, presque tous ces systèmes ont été rapidement transformés en bassins d'oxydation fonctionnant en flux continu par l'ajout d'un deuxième décanteur avec recyclage des matières solides. Au cours des années 1960, Pasveer a démontré que les bassins d'oxydation alimentés séquentiellement permettaient de produire des boues avec une meilleure décantabilité que celles produites par des systèmes alimentés en continu. À la fin des années 1960 et durant la décennie suivante, Irvine et ses collègues ont attribué aux procédés séquentiels discontinus le nom de SBR (sequential batch reactor) et ont été à l'origine de la diffusion de cette technologie dans le monde (Irvine et Busch, 1979; Ketchum et Irvine, 1989; Irvine *et al.* 1997; Wilderer *et al.* 2001). Dans les années 1970, Chudoba *et al.* ont mis au point des réacteurs sélecteurs qui sont devenus la technologie la plus répandue dans le domaine du traitement des boues.

Bien que l'utilisation de ce type de réacteurs ait été un succès et ait permis de réduire les problèmes de gonflement des boues activées dans de nombreux systèmes, il y a régulièrement eu des rapports scientifiques démontrant leur échec jusqu'à ce jour (Clayton *et al.* 1991, Lee *et al.* 1982; Linne et Chiesa, 1987; Ekama *et al.* 1996; Gabb *et al.* 1991; Gabb *et al.* 1996a, b; Gabb *et al.* 1996; Lakay *et al.* 1999; Musvoto *et al.* 1999).

1. INTRODUCTION

Les stations de traitement des eaux usées municipales et industrielles traitent des millions de mètres cube d'eaux usées de par le monde. Ainsi, des boues activées sont produites et ce, en deux étapes, l'une biochimique (en bassin aéré) et l'autre physique (clarificateur secondaire). Dans le bassin aéré, l'élimination du carbone organique, de l'ammonium et des phosphates se fait grâce à divers microorganismes dans les boues (constitués de virus, de bactéries, de protozoaires, de champignons et d'algues) (Jenkins *et al.* 1993). Les bactéries sont donc conservées dans les boues activées sous un environnement contrôlé, ce qui permet d'enlever efficacement la matière organique et les nutriments des eaux usées et permet par la même occasion d'améliorer la décantation et la déshydratation des boues. Les clarificateurs secondaires facilitent la séparation des matières solides (par simple décantation gravitaire) et le soutirage des boues décantées.

Les boues d'épuration sont produites en grandes quantités (Hébert, 2004; USEPA, 1999). Ces rejets constituent de ce fait un réel problème environnemental; leur traitement et leur élimination représentent 50% des coûts des procédés de traitement des eaux usées (Leblanc, 2005). Les boues produites sont généralement réutilisées et/ou recyclées.

2. LES PROBLÈMES RENCONTRÉS DANS LES USINES DE TRAITEMENT DES EAUX USÉES

La déshydratation des boues dans une usine de traitement des eaux usées (UTEU) est un problème bien connu dû à la mauvaise décantation et à au phénomène de foisonnement. Ce phénomène de foisonnement est causée par des organismes filamenteux ou non-filamenteux (*Zooglea*), présents dans les boues (Martins *et al.* 2004; Peng *et al.* 2003; Donaldson, 1932; Krhutkova *et al.* 2002).

Les substances polymères extracellulaires (SPE) produites par les souches bactériennes non filamenteuses sont responsables du foisonnement entre les cellules microbiennes et les particules solides des boues influençant ainsi la formation, la décantation et la capacité de rétention de l'eau des flocs de boues activées (Figure 1) (Sanin *et al.* 1994; Salehizadeh *et al.* 2000; Wang *et al.* 2007). Cependant, certaines souches microbiennes telles que *Zoogloea* et *Thauera* sont connues pour leur production excessive de SPE, responsables de la formation de flocs. Lajoie *et al.* (2000) ont démontré une corrélation négative entre l'abondance des flocs de *Zoogloea* et la déshydratabilité des boues. En effet, une production excessive de SPE microbiennes pouvant contenir plus de 99% d'eau est susceptible d'inhiber la déshydratation des boues (Houghton *et al.* 2001; Lajoie *et al.* 2000).

De même, une croissance excessive des microorganismes filamenteux est responsable du phénomène de foisonnement des boues (Figure 2). La longueur totale des filaments microbiens dans un gramme de boue peut dépasser les 30 km (Lee *et al.* 1983; Ekama et Marais, 1984). Les masses boueuses deviennent extrêmement volumineuses ce qui rend difficile leur décantation puisque les boues agglomérées décantent plus lentement que les boues normales. La liste des microorganismes responsables du foisonnement des boues figure dans la première partie du chapitre 2.

Les microorganisme peuvent se développer selon trios types de croissance: (i) Croissance dispersée (bactéries libres les unes par rapport aux autres), (ii) Croissance floculée (bactéries regroupées en amas très souvent autour d'un support organique ou minéral), et (iii) Croissance filamenteuse (multiplication cellulaire incomplète). En effet, lors de la croissance filamenteuse, la séparation de cellules mères et filles n'a pas lieu en totalité, les cellules mères et filles restent en contact, voire partage une paroi cellulaire (formantion de filament). La présence d'une proportion importante de bactéries libres ou fixés traduit habituellement une bonne qualité de décantabilité

des boues, alors qu'un faible nombre de celle-ci est souvent associé à des problèmes de croissance dispersée. La microflore de base des flocs comprend une multitude de bactéries dont *Achromobacter, Alcaligenes, Arthrobacter, Citromonas, Flavobacterium, Pseudomonas* et *Zooglea*. Plusieurs de ces bactéries, dont *Zooglea*, excrètent des polymères extracellulaires jouant un rôle dans la floculation des boues activées. Dans les boues activées, les flocs ont des dimensions variant approximativement entre 5 et 1 000 µm. Ces particules sont composées de deux types de particules: une composante biologique consistant en une agglomération de microorganismes (bactéries, mycètes, protozoaires et métazoaires) et une composante de particules organiques et inorganiques.

D'une manière générale, Si l'âge des boues est trop faible, il y a un risque de développement de bactéries filamenteuses, provoquant une mauvaise décantabilité des boues (IVB élevé) et une concentration eleve de MES dans l'effluent. L'indice de volume des boues (IVB) ou indice de Molhman sert à évaluer l'aptitude des boues à décanter. L'indice de boue représente le volume occupé par un gramme de boue aprés 30 minutes de décantation statique dans une éprouvette d'une litre. Il peut être calculé comme suit

$$IVB = \frac{VD_{30}}{[MES]}$$

Avec VD_{30} = volume de boues décantées en trente minutes (en mL/L) et [MES] = concentration des matiéres en suspension dans l'éprouvette (en g/L). Le IVB s'exprime donc en mL/g. Des valeurs comprises entre 80 et 120 mL/g, voire 40 et 150 mL/g sont généralement déclarées satisfaisantes (WPCF, 1985).

En plus des facteurs associés à la croissance microbienne, les phénomènes de décantation et de déshydratation des boues d'épuration dépendent de leurs différentes propriétés physicochimiques (Jorand *et al.* 1998; Liao *et al.* 2001; Vogelaar *et al.* 2005; Whitchurch *et al.* 2002). Le foisonnement des boues est souvent due à une mauvaise optimisation des paramètres de

traitement tels que l'excès de nutriments, le taux d'alimentation, le ratio C/N, la température ou encore le taux d'oxygène dissout. Ces paramètres affectent la croissance des bactéries et par conséquent, altèrent les caractéristiques des SPE formées et la structure des flocs.

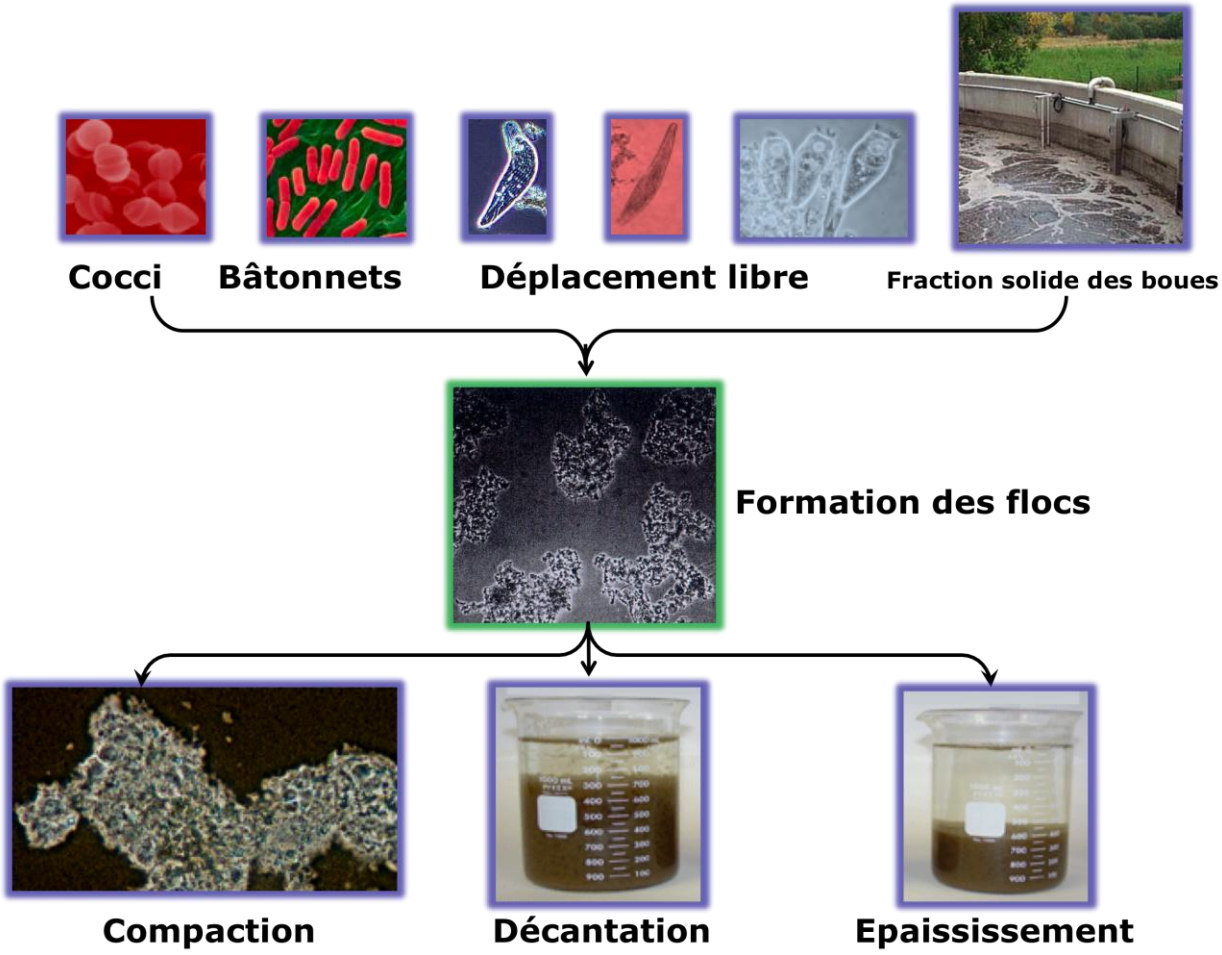

Figure 1 : Représentation schématique de la décantation des boues. Les flocs se constituent entre les microbes, les SPE et les fractions solides des boues et aboutissent à la compaction, la décantation et l'épaississement des boues.

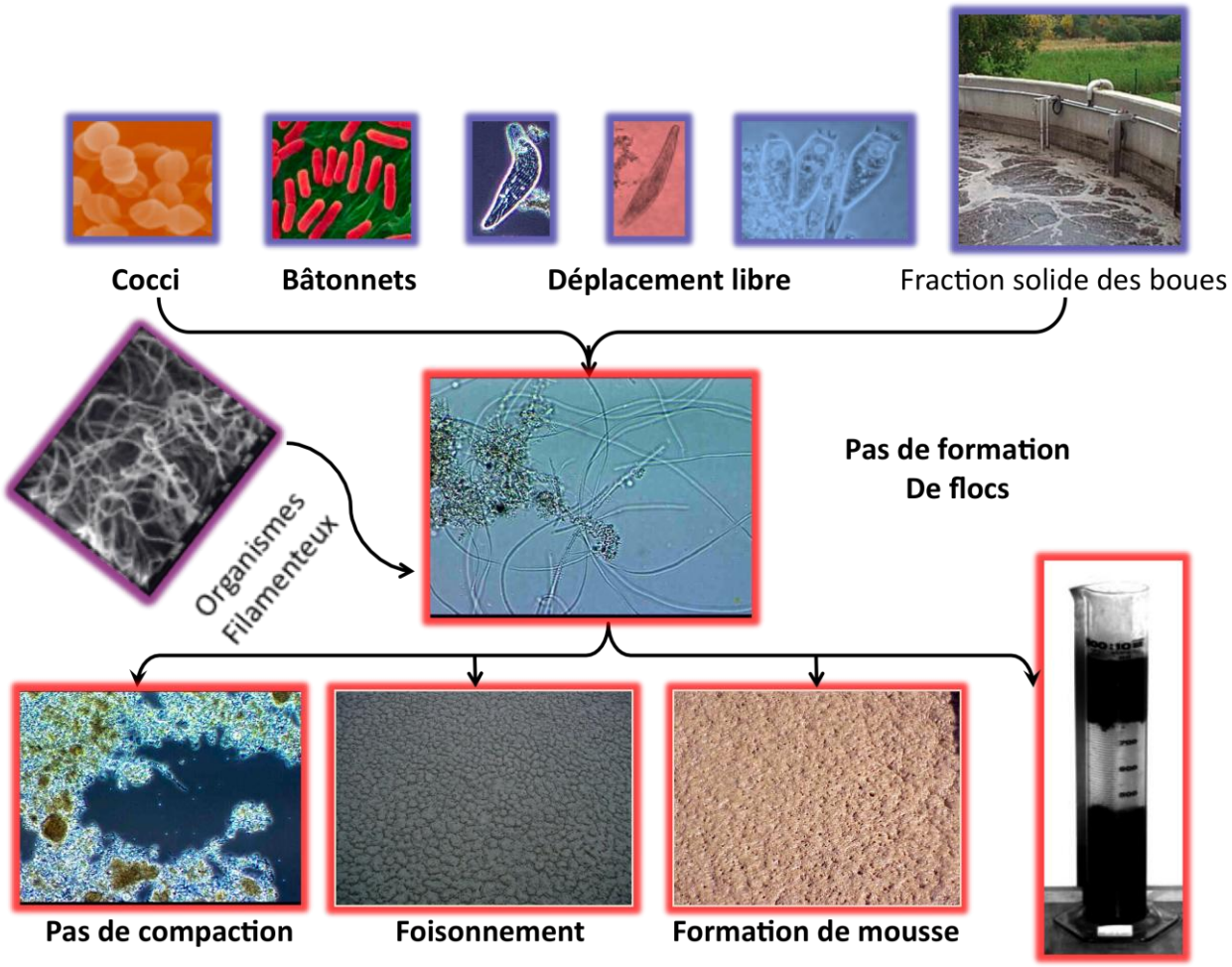

Figure 2 : Représentation schématique du phénomène de foisonnement et de formation de mousse causé par une croissance incontrôlée des microbes filamenteux. Les filaments empêchent la compaction des boues d'où une mauvaise décantabilité.

3. QUELQUES SOLUTIONS POUR EMPÊCHER LE PHÉNOMÈNE DE FOISONNEMENT DES BOUES

La décantation et la déshydratation des boues dans les procédés de traitement des eaux usées sont généralement réalisées par addition de polymères chimiques suivie d'une séparation physique des solides de la phase liquide (Yin *et al.* 2004). Les boues sont généralement chargées négativement. On utilise de ce fait des polymères cationiques synthétiques (CSP) ou des polymères synthétiques anioniques couplés à des cations (Al^{3+}, Fe^{3+}) pour neutraliser la charge à la surface des boues afin de faciliter leur floculation et leur décantation (Higgins et Novak, 1997a). Le principal avantage des polymères chimiques est qu'ils sont actifs sous différentes conditions de pH et induisent rapidement la formation de flocs. Cependant, ils sont polluants pour l'environnement et leur coût revient trop cher (Wang *et al.* 2005; Chu et Lee 2001; Chang *et al.* 2005).

Aux USA, on estime la consommation de polymères entre 25 et 50 million kg par an (correspond environ à 130 millions de dollars/an). En 2002, la province de Québec a généré 218 000 tonnes de boues (poids sec) (Hébert, 2004), avec une consommation de polymères d'environ 3 à 7 kg/tonne de boue ce qui revient à un coût allant de 7,6 à 10,6 million $CAD/an.

Nous avons estimé le coût nécessaire pour les polymères chimiques utilisés pour le conditionnement des boues avant déshydratation mécanique dans diverses stations d'épuration municipales et industrielles (Québec, Canada). Une industrie d'amidon sise à Montréal (ADM, Québec) consomme environ 28,8 tonnes de CSP / an (équivalent à 4,82 million $/an). Une petite industrie consomme et rejette de grandes quantités de polymères dans l'environnement. De même, une station d'épuration des eaux usées municipale au Québec [Communauté Urbaine du Québec (CUQ)] traite environ 200 000 m^3/jour d'effluent aqueux. Pour le traitement des boues, elle utilise en moyenne 600 ppm d'aluminium (Al^{3+}) et 0,1 % de polymères anioniques (nom commercial «Magnofloc» : copolymère d'acrylamide et d'acrylate coutant 5$/kg). Les monomères

d'acrylamide sont connus pour leur neurotoxicité et leurs effets carcinogènes (Chang *et al.* 2002). La consommation de CSP dans une autre station d'épuration à Victoriaville (traitant environ 31,000 à 46,000m³/jour) est de 12 tonnes/an (correpondant à 6,25$/kg).

Table 1: Inconvénients de l'utilisation des polymères synthétiques pour le traitement des boues.

Polymères synthétiques		
Problèmes	Descriptions	Réferences
Pollution de l'écosystème	Polymères non biodégradables en solution ne doivent pas être stockés plus que 24h. l'utilisation du sulfate d'Al entraîne la formation de boues gélatineuses nécessitant un prétraitement avant déshydratation.	Seka *et al.* 2001; Neyens *et al.* 2003; Bache et Papavasilopoulous, 2003
Effets néfastes sur la santé	Provoquent des irritations des yeux. Les monomères d'acrylamide sont des puissants agents carcinogènes et neurotoxiques.	Vanhoric et Moens, 1983; Dearfield et Ambermathy, 1988
	Il a été démontré que l'Al (constituant majeur du chlorure de poly-Aluminium) peut provoquer la maladie d'Alzheimer.	Masters *et al.* 1985; Chang *et al.* 2002
Odeurs	Les polymères utilisés pour l'épaississement des boues sont des sources d'amines odorantes. Ceci constitue une limitation de l'utilisation pour l'enrichissement des sols.	Chang *et al.* 2005
Corrosion	Les PS peuvent attaquer l'acier inoxydable et l'acier doux	Tramfloc Super Emulsion™

Les polymères synthétiques (PS) sont connus pour leurs effets sur les microorganismes du sol quand les boues déshydratées contenant ces polymères sont utilisées pour la fertilisation des terrains agricoles (Leem et Liu, 2000; Abu-Orf *et al.* 2001; Chang *et al.* 2001; Chang *et al.* 2002). De même, l'utilisation de boues déshydratées «chimiquement» comme matière première pour la production de biopesticides entraîne une diminution de l'entomotoxicité et du taux de croissance

des plantes, par comparaison aux boues décantées sans l'utilisation des polymères chimiques (Vidyarthi *et al.* 2002).

De plus, les boues déshydratées chimiquement affectent la flore microbienne naturelle présente dans de nombreux environnements tels que le compost et les terreaux utilisés en agriculture (Gagnon et Ziadi, 2004). Par conséquent, l'utilisation des PS pour la déshydratation des boues doit être réduite au maximum afin de minimiser le coût de traitement des boues et atténuer leurs effets toxiques (résumés dans le tableau 1) (Leem et Liu, 2000; Abu-Orf *et al.* 2001, Chang *et al.* 2001, Chang *et al.* 2002).

4. LA BIOFLOCULATION

La biofloculation est le procédé de floculation des boues sous l'action des microorganismes ou/et de leurs produits (SPE, polysaccharides, protéines, lipides, composés intracellulaires et les acides nucléiques) (Kampfer *et al.* 1996; Boessmann *et al.* 2003). La biofloculation est définie comme étant l'élimination des particules colloïdales de la biomasse avec ou sans biodégradation (Vogelaar *et al.* 2005).

5. DIFFÉRENTES APPROCHES DE BIOFLOCULATION DES BOUES (REVUE DE LA LITTÉRATURE)

Depuis 1876, le phénomène de la biofloculation a été décrit par plusieurs chercheurs (Pasteur, 1876; Bordet, 1899; Bloch, 1918; Butterfield, 1935). Le premier chercheur à avoir décrit le rôle des SPE dans la biofloculation était Mckinney (1956), ensuite Tenny et Verhoff (1973). L'historique, le mode d'action et les approches de la biofloculation sont décrites dans le chapitre 2.

Au cours des cinq dernières décennies, les chercheurs ont étudié le mécanisme et les paramètres affectant le processus de biofloculation. Diverses approches ont été étudiées dans le but d'améliorer la décantabilité et la déshydratabilité des boues. Ces approches sont basées sur 5 étapes

essentielles : (i) l'utilisation des biopolymères nonmicrobiens pour la biofloculation des boues; (ii) le contrôle en ligne des paramètres opérationnels; (iii) la caractérisation des SPE pour pouvoir comprendre leurs mécanismes d'action dans la biofloculation des boues; (iv) étude de la biofloculation des SPE sur des suspensions de kaolin, et; (v) la production de SPE/biofloculants provenant de divers microorganismes et leur application pour la biofloculation.

5.1 Biofloculation des boues par les polymères non microbiens

Huang et Chen (1996) ont étudié la coagulation de particules colloïdales dans l'eau utilisant les chitosanes. Ces derniers ont été synthétisés à partir de la chitine constituant la carapace des crabes.

Lee *et al.* (2001) ont utilisé des déchets de fruits de mer (coquilles de crevettes, huîtres et particules des os internes de calmar) comme additif dans la boue prétraitée (à l'alun ou au chlorure ferrique). Les résultats ont révélé que cet ajout a induit une amélioration de la déshydratabilité des boues. Ozacar et Sengil (2000) ont étudié l'effet d'un polyélectrolyte anionique naturel (tanin, extrait de plantes *Valonia*) sur la déshydratation des boues naturelles. Les résultats obtenus ont été comparés à ceux obtenus avec un polyélectrolyte anionique synthétique (AN913). La combinaison de tanin et d'$Al_2(SO_4)_3$ a démontré une meilleure déshydratabilité par rapport à la combinaison du polyélectrolyte synthétique avec $Al_2(SO_4)_3$ utilisé comme améliorant de la coagulation. De même, Diouri (2004) a démontré que les mucilages extraits de cactus mexicains pouvaient floculer les métaux organiques, les ions et les odeurs de l'eau et des eaux usées. Ghebremichael et Hultman (2004) ont étudié la déshydratation des boues en utilisant des biofloculants extraits de *Moringa oleifera*. Le biofloculant obtenu a montré une meilleure déshydratabilité que le polyélectrolyte de synthèse.

Afin de réduire au minimum la pollution et l'effet toxique des polymères synthétiques, Singh *et al.* (2000) ont étudié l'aptitude des polymères greffés à la biofloculation. Ils ont ainsi

constaté que l'amylopectine greffée était adaptée pour la floculation de divers effluents industriels. Wang *et al.* (2005) ont comparé l'action des biofloculants, des biopolymères greffés (copolymère d'acrylamide et de chitosane) et des polymères cationiques sur la granulation dans des réacteurs UASB (upward-flow anaerobic sludge blanket). Ils ont trouvé que les biofloculants augmentaient le processus de granulation en favorisant la multiplication microbienne et la biodégradation.

Parmar *et al.* (2001) se sont intéressés aux traitements enzymatiques (utilisant les protéases, les lipases et les cellulases) pour la réduction des boues solides et l'amélioration de leur décantabilité. Ils ont trouvé qu'en combinant l'action des protéases à celle des cellulases, il était possible de favoriser la réduction des boues solides. Parmi les différents traitements enzymatiques, l'utilisation des protéases a abouti à une meilleure décantabilité des boues par rapport aux lipases et aux cellulases. De même, Ayol (2005a, b) a étudié l'effet d'un traitement enzymatique contenant la protéase, la lipase, des bactéries anaérobies, *Aspergillus oryzae*, et des enzymes hydrolytiques (Enviro-Zyme 216) sur la déshydratabilité des boues. Il a suggéré que les boues traitées enzymatiquement nécessitaient une quantité moindre de polymères que les boues non traitées.

5.2 Contrôle en ligne des paramètres opérationnels des stations d'épuration des eaux usées

Une mauvaise décantation des boues conduit à une augmentation de la concentration de MES dans les eaux réceptrices. Des problèmes opérationnels tels que la mauvaise biofloculation des boues ou encore une défloculation de la biomasse sont à l'origine de cette mauvaise décantation. Ce genre de problème se produit en raison d'une diminution de la concentration de SPE naturelles qui favorise la formation biofloc (Seintuch *et al.* 1986). Le mécanisme de biofloculation est donc le résultat des interactions de ces polymères qui se sont accumulés pendant la croissance microbienne constituant ainsi un biofilm. Différents facteurs tels que la concentration en nutriments, le temps de rétention des boues, le pH, le ratio aliment/microorganismes et/ou

encore le temps de rétention hydraulique pourraient affecter la production de SPE (Hoa *et al.* 2003). Ainsi, l'optimisation des conditions opératoires des stations d'épuration a été étudiée par plusieurs chercheurs afin d'améliorer la décantabilité des boues (résumé ci-dessous).

Chen *et al.* (2001) ont fait état d'une nouvelle approche pour réduire l'excédent de boues produites et améliorer leur décantation. Cette approche comprend: (i) la modification du procédé classique de boues activées par l'insertion d'un réservoir de stockage des boues dans les boues de retour; (ii) la chloration (le chlore est utilisé pour remplacer l'ozone en raison de son coût élevé) de l'excès de boue de façon à minimiser la production de boues excédentaires et; (iii) l'utilisation perturbateur métabolique, le 3,3',4',5-tetrachlorosalicylanilide (TCS), afin de maximiser l'activité des microorganismes des boues permettant de réduire la croissance de la biomasse. Grâce à cette méthode, il a été possible de réduire les boues à 45-60%. En 2001, Chen *et al.* ont également étudié l'effet de l'acide et des surfactants sur la déshydratation des boues. En effet, une diminution du pH à 2,5 et l'ajout de 0,1 g d'agents surfactants a permis d'augmenter la déshydratabilité des boues jusqu'à 73,99%. Neyens *et al.* (2003) ont réussi à déshydrater et à réduire considérablement les boues par un procédé d'hydrolyse alcaline thermique.

Hoa *et al.* (2003), ont étudié l'effet des nutriments sur la production EPS et leurs effets sur les propriétés des boues. Ils ont constaté que certains éléments des SPE comme les protéines et les glucides ont rôle majeur dans la biofloculation. De même, Durmaz et Sanin (2001) ont étudié l'effet du ratio carbone sur azote (C/N) sur la composition des SPE. Ils ont découvert qu'à un rapport C/N de 5, la teneur des polymères en protéines est plus élevée que celle des hydrates de carbone. Pour un rapport C/N élevé (de 17,5 à 40), la teneur des hydrates de carbone augmente par rapport à celle des protéines. Le changement du débit d'alimentation peut réduire la croissance des bactéries filamenteuses et permettre une meilleure croissance des souches formatrices de flocs (Martins *et al.* 2003; Karapinar et Kargi, 1996). Poxon et Darby (1997) ont constaté que le

changement du débit d'alimentation entraîne des changements dans les caractéristiques des SPE. Ils ont aussi constaté que l'augmentation de la concentration en glucides du substrat entraîne une augmentation de la concentration en glucides des SPE. Martinez *et al.* (2004) ont étudié la relation entre la concentration en protéines des SPE et son effet sur la décantation des boues par l'indice du volume des boues (« sludge volume index » ou SVI). Ils ont constaté que l'augmentation de la teneur en protéines a augmenté la valeur du SVI. Houghton et Stephenson (2002) ont démontré qu'une concentration de SPE de 17,2 mg/g de MES est optimale pour la déshydratation. Les protéines et les glucides des polymères ne semblent pas affecter la déshydratabilité. Cette observation est contradictoire aux travaux précédents (Hoa *et al.* 2003; Durmaz et Sanin 2001, Liu *et al.* 2001, Martinez *et al.* 2004). Il faudrait donc approfondir davantage les investigations sur les propriétés physicochimiques des SPE pour obtenir le maximum de biofloculation.

Les effets du surfactant sur la stabilité des flocs microbiens (induits par *Acinetobacter johnsonii* S35 et *Oligotropha carboxidovorans* S23) ont été étudiés en utilisant le sodium dodécyl sulfate (SDS) (anioniques) et le Cetyltrimethylammonium bromure (CTAB) (cationiques) comme surfactants. La faible concentration d'agents de surface a permis d'accroître la stabilité des agrégats bactériens dans les boues d'épuration. À des concentrations plus élevées de surfactants (SDS et CTAB), on observe une défloculation des agrégats tandis que les flocs complexes des boues ont montré une défloculation seulement en présence de SDS (Malik *et al.* 2005). De même, Bott et Love (2004), ont détecté la présence de composés électrophiles dans les boues à l'origine de leur défloculation. Le groupe thiol des composés électrophiles induit l'exocytose des K^+ cytoplasmique. De ce fait, la concentration des ions K^+ dans les boues activées augmente ce qui entraîne la défloculation. La réduction microbienne des ions Fe (III) et Fe (II) par la bactérie *Shewanella alga* provoque la désintégration des flocs de boues activées (Nielsen et Keiding, 1998). La réduction du Fe (III) est susceptible d'atténuer la force du floc pouvant engendrer sa

désintégration par cisaillement sous l'action du pompage ou de l'agitation. La désintégration des boues activées à l'origine d'une mauvaise déshydratation des boues et la qualité des effluents.

Liu *et al.* (2001) ont étudié l'effet de l'ozonation sur l'amélioration des caractéristiques des SPE dans les boues activées. Ils ont trouvé que l'ozonation des boues n'induit pas l'augmentation de la sécrétion des SPE, tandis qu'elle provoque une détérioration du temps de succion capillaire (CST) et de la résistance spécifique à la filtration (SRF). Ils ont aussi montré que l'ozonation permet d'augmenter la demande en polyélectrolytes cationiques. Elle a des effets positifs sur la production de méthane. Martins *et al.* (2003), ont étudié l'effet de l'oxygène dissous (OD) sur la croissance des microorganismes filamenteux (à l'échelle du laboratoire). Une faible concentration d'OD ($\leq$1,1 mg/L O_2) a fortement entravé la décantation des boues et a engendré la prolifération des bactéries filamenteuses (*Thiothrix* spp). Par conséquent, une concentration d'OD au-dessus de 2,5 mg/L doit être maintenue pour assurer une bonne décantabilité dans le SBR. De même, l'effet de l'OD sur la biofloculation dans un filtre goutte à goutte a été étudié par Motta *et al.* (2003). Ils ont observé qu'une OD comprise entre 1,0 et 1,4 mg/L permettait une très bonne floculation et un effluent final de bonne qualité.

Morgan-Sagastume et Allen, (2005) ont démontré que l'augmentation de la température de 30 à 45 °C entraînait une défloculation des boues en raison de la solubilisation des SPE. La variation de la température a également réduit le métabolisme de microorganismes qui, à leur tour, dégradaient mal les boues solides ce qui entraînaient l'augmentation de la turbidité dans le surnageant, d'où la défloculation des boues. Vogelaar *et al.* (2005) a démontré que la présence de bactéries aérobies actives à 30°C permettait d'avoir une bonne biofloculation.

5.3 Les études sur les caractéristiques des SPE des boues

Plusieurs caractéristiques physiques, chimiques et biologiques jouent un rôle majeur dans la biofloculation et la biocoagulation des boues. Les paramètres physiques comme la température, le

temps de rétention des boues, la taille et la forme des flocs, la concentration en solides totaux, les MES des boues et le débit des eaux usées, contrôlent la décantabilité des boues. D'autre part, les paramètres chimiques tels que la concentration de substances organiques, inorganiques et des métaux ainsi que la présence de cations bivalents régulent la déshydratabilité des boues. Les paramètres biologiques incluent la nature des microorganismes et leurs substances polymériques extracellulaires (SPE). Certains de ces paramètres et leur rôle dans la déshydratation des solides sont décrits dans le chapitre 2, partie 1.

Trois principaux paramétres de SPE qui jouent un rôle important dans la bioflocculation sont: la concentration des SPE (Kang *et al.* 1989, Morgan *et al.* 1990; Urbain *et al.* 1993; Houghton *et al.* 2001; Keiding et Mikkelsen, 2002), la charge et l'hydrophobicité (Neyens *et al.* 2003; Zita et Hermansson, 1997; Wilen *et al.* 2003; Poxon et Darby, 1997). De même, les caractéristiques des flocs et la présence des cations influencent aussi la bioflocculation (Higgins et Novak, 1997; Shin *et al.* 2001, Zhang *et al.* 2007; Frølund *et al.* 1996; Sutherland, 2001; Tian *et al.* 2006; Bura *et al.* 1998; Whitchurch *et al.* 2002; Urbain *et al.* 1993; Zita et Hermansson, 1997; Jorand *et al.* 1998; Jin *et al.* 2003; Yu *et al.* 2004; Sobeck et Higgins, 2002; Bruss *et al.* 1992, Novak *et al.* 2001, Biggs *et al.* 2001; Higgins *et al.* 2004). Outre ces facteurs, la composition des SPE joue également un rôle majeur dans la biofloculation. Plusieurs chercheurs ont démontré que les protéines (Sharon et Lis, 1989; Elgavish et Shaanan, 1997; Bockelmann *et al.* 2002; Higgins *et al.* 1997, Nielsen et Jahn, 1999; Frolund *et al.* 1996; Wolfaardt *et al.* 1998; Houghton *et al.* 2001), les polysaccahrides (Droppo *et al.* 2004; Poxon et Darby, 1997; Horan et Eccles, 1986; Keiding & Mikkelsen, 2002; Steiner *et al.* 1976; Alm et Eriksson, 1991; Dudman et Wilkinson, 1956; Farrah et UNZ, 1976; Easson *et al.* 1987; Gerbsch et Buchholz, 1995; Bruus *et al.* 1992; McSwain *et al.* 2005) et d'autres composants des SPE (Whitchurch *et al.* 2002, Lawrence *et al.* 2003) sont d'une importance capitale dans la biofloculation des boues.

5.4 Biofloculation dans des boues synthétiques et des suspensions de kaolin

Plusieurs chercheurs ont isolé des microorganismes du sol producteurs de SPE (*Rhodococcus erythropolis, Klebsiella pneumoniae, Bacillus firmus; Sorangium cellulosum et B. mucilaginosus*) pour les utiliser dans les procédés de biofloculation (Kurane *et al.*, 1986 et 1994; Nakata et Kurane, 1999; Salehizadeh et Shojasosadati, 2002; Zang *et al.*, 2002; Deng *et al.*, (2003).

Fujita *et al.* (2001) ont produit des biofloculants en utilisant la souche *Citrobacter* TKF04 isolée de biofilms des déchets de cuisine. De même, Kumar *et al.* (2004) ont produit des biofloculants en utilisant des espèces de *Bacillus* isolées à partir d'échantillons d'eau de mer halo-alcalophilique (Corée). Gao *et al.* (2006), ont étudié la production de biofloculants de *Vagococcus* sp W31 isolés à partir d'échantillons d'eaux usées prélevés à partir de la Little Moon River (Beijing).

Tous les chercheurs ont utilisé différents milieux de culture synthétiques minéraux pour produire les biofloculants. Ces études ont été réalisées en mesurant l'activité de floculation du kaolin pour cibler les biofloculants produits par des microorganismes isolés de différents milieux. La méthode de coagulation du kaolin est utilisée pour le sélection des biofloculants de microorganismes, car ces derniers possèdent des propriétés similaires à celles des boues en termes de concentration en matières solides et de charge de surface constante (-30mV).

Cependant, le kaolin ne peut pas remplacer intégralement les boues d'épuration puisqu'ils ne possèdent pas les mêmes propriétés physiques. En effet, les boues sont constituées de nombreux composés complexes (ADN, ARN, métaux toxiques, substances humiques) et une flore microbienne diversifiée, qui jouent également un grand rôle important dans le mécanisme de floculation. Les boues possèdent naturellement une activité tampon (stabilisent le pH dans certaines conditions) ce qui fait que les boues synthétiques et les suspensions similaires (kaolin) sont loin de donner des résultats comparables à 100%. Ainsi, des études préliminaires pourraient

être entreprises en utilisant le kaolin comme contrôle, mais, en fin de compte, la décantation et la déshydratation des boues doivent être évaluées *in situ* sur des boues d'épuration fraîches.

De nombreuses études ont été menées sur la production de biofloculants à partir de souches bactériennes pures isolées des milieux naturels. Cependant, il n'y a pas beaucoup de références scientifiques concernant l'isolement de souches bactériennes isolées des boues d'épuration pour la production de SPE (Garnier *et al.* 2006). Aussi, le fait d'étudier seulement une seule souche microbienne ne fournira pas des conclusions scientifiques pertinentes pour un modèle standard pour la biofloculation de boues activées. Par conséquent, toutes les souches bactériennes produisant des SPE doivent être isolées pour étudier leur performance relative la biofloculation des boues.

5.5 Biofloculation des eaux usées et des boues

En agissant comme biofloculants, les microorganismes sont les principaux composants actifs jouant un rôle de premier plan dans la décantation des biosolides et des procédés de déshydratation. En général, on atteint une grande concentration de biomasse par autoflocculation dans un réacteur de type UASB (auto-immobilisation des cellules bactériennes). L'adhérence et la croissance floculée des bactéries sont associées aux SPE sécrétées par les souches microbiennes dans le système. Les SPE relient deux cellules bactériennes l'une à l'autre ainsi qu'à d'autres particules de matière inerte, l'ensemble constitue donc le floc. Wang *et al.* (1977) supposent que les SPE jouent un rôle important dans la granulation anaérobie dans les réacteurs UASB.

Les souches microbiennes filamenteuses sont appelées ainsi à cause de leurs formes enfilées. Bien qu'elles soient capables de former des flocs pour réduire efficacement la DBO, une croissance excessive des bactéries filamenteuses (par exemple *Nostocoida limicola*) peut dans certains cas être à l'origine d'une mauvaise décantation (Seka *et al.* 2001). En effet, elles exigent plus de temps pour décanter à cause de leur légèreté et de l'excédent de polymères libérés dans le

milieu provoquant de foisonnement des boues. De ce fait, ces espèces bactériennes ne sont pas très recherchées lors des procédés de traitement des eaux usées.

Norberg et Enfors (1982) ont étudié la production de polysaccharides extracellulaires chez *Zoogloea ramigera* ATCC 25935. La SPE produite était semblable à une capsule attachée à la cellule. Allen *et al.* (2004) ont isolé un exopolysaccharide de poids moléculaire d'environ 260 kDa à partir d'une bactérie isolée dans les flocs des eaux usées (*Thauer* sp. MZ1T).

Les contraintes rencontrées dans les procédés de déshydratation des boues chimiques (tannerie) ont été résolues en les mélangeant avec des boues activées (riches en SPE produites par les micro-organismes), à un ratio de 1:4 et de 1:8 (v/v) (Chang *et al.* 2001). Cette étude a démontré que la déshydratation des boues chimiques pourrait être améliorée par l'ajout de boues activées en raison de la présence de polymères extracellulaires et de microorganismes. Cette étude a révélé que les SPE des boues sont impliquées dans le processus naturel de biofloculation en raison de la présence de SPE naturelles.

Il y a très peu d'études (comme nous l'avons décrit ci-dessus) sur la biofloculation de boues utilisant des cultures pures de microorganismes. Ces études ont seulement fourni des approches scientifiques fondamentales pour comprendre le processus de biofloculation. En conséquence, il serait très intéressant d'isoler et d'étudier les souches microbiennes productrices de SPE pour la bioflocculation de la boue.

5.6 La biofloculation étudiée dans d'autres systèmes (autres que les boues)

Kaplan *et al.* (1987) ont étudié la chélation des métaux lourds en utilisant les SPE produits par *Chlorella* spp. Pinotti *et al.* (1997) ont, de leur côté, étudié la floculation dans une émulsion de déchets d'industries alimentaires en utilisant le chitosane comme polyélectrolyte. Jayachandran et Chandrasekaran (1998) se sont penchés sur la biocoagulation de latex en utilisant *Acinetobacter* sp, isolée par centrifugation à partir d'effluent de latex du caoutchouc naturel. Stoderegger et Hernd

(2001) ont travaillé sur la présence des SPE capsulaires dans les environnements océaniques. Vijayalakshmi et Raichur (2002) ont étudié la biofloculation de charbon indien hautement minéralisé par la souche *Paenibacillus polymyxa*. Avec cette souche, les auteurs ont été capables de floculer 60% des cendres. Mohan *et al.* (2002) ont analysé la biocoagulation et la biosorption de colorants azoïques provenant des effluents en utilisant des espèces de *Spirogyra*. Récemment, Yoon *et al.* (2003) ont utilisé des floculants bactériens dans le but d'accroître la récolte des cellules d'algues. Natarajan et Das (2003) se sont basés sur l'utilisation de bactéries du groupe *Acidithiobacillus* pour la floculation de minéraux. Toutes ces études nous mènent à dire qu'il y a un intérêt à utiliser des microorganismes capables de produire des SPE/biofloculants pour la décantation et la déshydratation des boues au cours des procédés de traitement des eaux usées.

6. ENJEUX ET ORIENTATION DE LA RECHERCHE

Comme mentionné ci-dessus, les trois facteurs les plus importants qui affectent la biofloculation sont la concentration, la charge à la surface et l'hydrophobicité des SPE. Il a été constaté que les SPE pouvaient être sécrétées avec une concentration optimale au cours du procédé de production des boues activées. La quantité d'EPS dans les boues varie en fonction de la méthode d'extraction utilisée et de la nature du des boues. Ainsi, plusieurs chercheurs donnent différentes concentrations de poids sec (PS) d'EPS par gramme de matières volatiles en suspension volatiles (MVS) : 16 à 284 mg de PS d'EPS par gramme de MVS (Comte *et al.* 2006), 20 à 318 mg de PS d'EPS par gramme de MVS (Comte *et al.* 2006), 26-165 mg PS d'EPS par gramme de MVS (Liu et Frang, 2002), 6-254 mg PS d'EPS par gramme de MVS (Liu *et al.* 2001) et 127-432 mg PS d'EPS par gramme de MVS (Frolund *et al.* 1996). Cependant, atteindre cette concentration en station d'épuration est pratiquement impossible. En outre, la charge et l'hydrophobicité des SPE de la boue sont des paramètres incontrôlables durant le cycle de traitement des eaux usées. Ce problème peut être résolu si l'on est capable d'identifier les

microorganismes produisant les SPE dans les boues ainsi que leurs caractéristiques. Cela permettra de sélectionner des SPE possédant les caractéristiques appropriées (concentration, charge et hydrophobicité) pour l'utiliser dans la déshydratation des boues. Deux approches ont été développées pour réaliser la biofloculation des boues : (i) en utilisant des SPE (biopolymères); et (ii) en utilisant des souches microbiennes formant des flocs. En ce basant sur ce fait, nous avons étudié ces deux approches et nous avons tenté de les mettre en œuvre pour réaliser la décantation et la déshydratation des boues.

Les substances polymériques synthétiques (SPE) aident à la formation de bioflocs dans les boues activées et influencent leurs structures, leurs charges en surface et leur décantabilité (Urbain *et al.* 1993, Houghton *et al.* 2001, Houghton et Stephenson, 2002). Les Bioflocs sont formés par interaction entre les agrégats microbiens, les souches bactériennes filamenteuses et les particules organiques et inorganiques maintenues ensemble par les SPE (Novak *et al.* 2001 et 2003; Butterfield, 1935; McKinney, 1952; Urbain *et al.* 1993; Bala Subramanian *et al.* 2008a&b).

Les SPE ont été identifiées comme l'un des principaux composants des bioflocs et des biofilms (Jian *et al.* 2006). Elles sont principalement constituées de polysaccharides, de protéines, d'acides nucléiques et d'autres composants cellulaires (Higgins et Novak, 1997b; Sobeck et Higgins, 2002; Hoa *et al.* 2003). Les SPE sont, soit liées aux cellules (SPE capsulaires), soit sécrétées à l'extérieur des cellules (dépôt gluant). Les SPE extracellulaires (ou dépôt gluant) sont généralement séparées de la matrice cellulaire au cours de la centrifugation, tandis que les SPE capsulaires sont stables et attachées à la paroi cellulaire des microorganismes tout au long du procédé de séparation.

De nombreux travaux se sont orientés vers l'étude de la biofloculation en utilisant un seul type d'EPS (dépôt gluant ou SPE capsulaires) provenant de différents microorganismes (Stoderegger et Herndl, 2001; Kumar *et al.* 2004). Il est rapporté que, même si les boues activées

contenaient suffisamment de SPE, ces dernières ne parviendraient pas à décanter spontanément les boues (Houghton *et al.* 2001). Jusqu'à présent, les études sur la biofloculation des boues au moyen de polymères bactériens n'ont pas été couronnées de succès pour différentes raisons : (i) il y a un manque de compréhension des interactions entre les SPE et les boues solides; (ii) les différents microorganismes produisant les SPE n'ont pas été isolés et identifiés; (iii) les caractéristiques des SPE microbiens ne sont pas encore connues et; (iv) le rôle actif des différents composants des SPE (protéines, lipides et des hydrates de carbone, de l'ADN, d'ARN et autres) dans la formation des bioflocs n'a pas encore été élucidé.

7. HYPOTHÈSES, OBJECTIFS ET ORIGINALITÉ

7.1 Hypothèses

En se basant sur la revue de littérature réalisée au cours de cette étude, les hypothèses suivantes ont été formulées :

- ✓ Les boues activées sont des sources potentielles de microorganismes et de substances polymériques extracellulaires (SPE) qu'ils sont susceptibles de produire. Ces deux acteurs sont impliqués dans la décantation et la déshydratation naturelles des boues. L'utilisation de souches microbiennes isolées et de leurs SPE pourrait aboutir à une biofloculation améliorée par rapport à celle observée dans les boues activées.
- ✓ Plusieurs chercheurs ont montré que la concentration, la charge, l'hydrophobicité et la composition biochimique des SPE des boues sont des facteurs importants conditionnant la biofloculation. Ces propriétés physicochimiques jouent un rôle similaire dans le cas des SPE produites à partir des souches bactériennes isolées des boues.
- ✓ Beaucoup de travaux ont demontré que les microorganismes filamenteux peuvent être des agents initiateurs de la biofloculation. Paradoxalement, d'autres chercheurs stipulent

qu’une croissance excessive de ces organismes peut être responsable du phénomène de foisonnement des boues (« bulking ») dans les bassins d’aération. La souche de champignon filamenteux isolée des boues possèderait une activité substantielle de biofloculation.

- ✓ Les boues d’épuration sont utilisées comme matières premières pour la croissance de plusieurs microorganismes et pour la production de substances à valeurs ajoutées. Ainsi, il est possible de cultiver la souche de champignon filamenteux isolée sous des conditions optimales afin d’améliorer la biofloculation.

7.2 Les objectifs de recherche

1. Isolation, criblage et identification des biofloculants (SPE) produits par les souches microbiennes isolées à partir des eaux usées municipales.
2. Production, caractérisation biochimique et évaluation des potentialités des SPE pour la formation de bioflocs.
3. Isolement de la (ou des) souche(s) de champignon(s) filamenteux à partir des boues pour les utiliser dans la biofloculation.
4. Utilisation du champignon filamenteux isolé des boues pour la dégradation de solides totaux, la réduction des pathogènes, la décantation et la déshydratation des boues.

7.3 L'originalité

Jusqu'à ce jour, aucune étude scientifique ne s’est intéressée à l’isolement et à l’identification des microorganismes des boues qui produisent des SPE. Les caractéristiques physicochimiques des SPE produites par différents microorganismes des boues sont mal connues. Dans cette recherche, nous avons extrait plusieurs SPE produites par différentes souches bactériennes isolées des boues. Trois types de SPE (dépôt gluant, capsulaires et combinaison des

deux) obtenus à partir des différentes souches ont été étudiées pour la biofloculation. Il s'agit de la première étude qui a permit d'isoler et d'identifier différentes souches microbiennes des boues pour les utiliser dans les procédés de biofloculation. Nous avons montré que certaines souches isolées possédaient une meilleure activité de biofloculation par comparaison à celle du consortium microbien (ensemble des microorganismes naturellement présent dans les boues).

Comme il a été décrit précédemment, l'implication des champignons filamenteux dans les procédés de décantation des boues semble contradictoire. Au cours de notre étude, nous avons pu montrer que la souche de champignon filamenteux isolée possédait une bonne activité de biofloculation des boues. De ce fait, il devient intéressant de l'utiliser dans les procédés de dégradation et de déshydratation des boues.

8. RÉSULTATS ET DISCUSSION

La thèse est présentée en trois parties : La partie 1 traite d'une revue de la littérature sur les biofloculants. La partie 2 présente les résultats des travaux réalisés sur la biofloculation et la déshydratation utilisant les souches bactériennes non filamenteuses produisant des SPE. La partie 3 présente les résultats des essais sur la biofloculation et la déshydratation utilisant les souches de mycètes filamenteux. Le résumé des résultats obtenus dans cette étude est présenté ci-après.

8.1 Partie 1. Revue et la littérature

8.1.1 Biofloculants

Les microorganismes peuvent produire des métabolites secondaires durant leur croissance. La biocoagulation, la biofloculation et la déshydratation des boues sont des traitements pouvant être assurés par des microbes. Ces procédés sont très importants pour la décantation et la déshydratation des boues dans les usines de traitement des eaux usées (UTEU). Les UTEU utilisent conventionnellement des substances chimiques (polymères) pour floculer les boues. Ces

substances sont très onéreuses et très polluantes pour l'environnement lorsque les boues déshydratées sont utilisées pour l'enrichissement du sol. Afin de minimiser l'utilisation de polymères chimiques, il convient d'explorer des voies plus écologiques et peu coûteuses pour produire des polymères biodégradables pouvant être utilisés pour la décantation des boues. D'un autre côté, une quantité substantielle de biopolymères ou de substances polymériques extracellulaires sont produites pendant le traitement des eaux usées et peuvent jouent un rôle important dans la floculation et la décantation. L'efficacité des procédés de biofloculation est basée sur les caractéristiques, les paramètres physiologiques et les propriétés des flocs. Les caractéristiques microbiennes peuvent être identifiées et utilisées pour la production d'agents biofloculants et biocoagulants. Cette approche écologique pourrait se substituer à l'ajout de polymères chimiques et assurer la décantation et la déshydratation des boues avec la même efficacité. Ces aspects sont rigoureusement discutés dans ce chapitre.

8.2 Partie 2 : Bioflocculation et de déshydratation utilisant EPS non-filamenteux produisant des souches bactériennes.

Les stations de traitement des eaux usées ont souvent des problèmes de sédimentation des flocs dus à l'agrégation des boues. Généralement, un polymère organique synthétique (Polydimethyl diallyl ammonium chloride) et / ou un coagulant inorganique (chlorure de fer, alun et chaux) sont utilisés pour faire sédimenter les boues. Ces produits chimiques sont très coûteux et dangereux pour l'environnement. Il existe aujourd'hui des biofloculants qui sont moins nocifs pour l'environnement et qui peuvent être utilisés pour la floculation des boues. Les SPE produites par les microorganismes présents dans les boues jouent un rôle essentiel dans la floculation des boues. L'étude présentée ici a analysé vingt-cinq SPE produites par différentes souches isolées d'une usine municipale de traitement de l'eau. Les souches retenues ont été sélectionnées selon leur capacité à produire des SPE sur milieu solide. Trois types de SPE (le biofilm, la capsule et un

mélange des deux) ont été récoltés et analysés pour déterminer leurs caractéristiques. La concentration en SPE (mesurée par le poids sec), la viscosité et la charge des échantillons ont également été mesurées (à l'aide d'un Zêtaphoremètre). La capacité des SPE obtenues à biofloculer a été évaluée par la mesure de l'activité de floculation du kaolin. Selon ces résultats, les six meilleures souches (BS2, BS8, BS9, BS11, BS15 and BS25) ont été sélectionnées pour des tests ultérieurs. Les SPE de biofilm se sont avéré de meilleurs biofloculants que les SPE capsulaires et le mélange des deux. Les SPE de biofilm extraits des six souches identifiées précédemment ont donc été étudiées pour leur capacité à floculer et à déshydrater les boues en déterminant respectivement l'indice de volume des boues (SVI) et le temps de succion capillaire (CST). Les biopolymères produits par les souches ont significativement amélioré la déshydratation. Les SPE de biofilms de ces six souches ont été partiellement caractérisées.

8.3 Partie 3 : Biofloculation et de déshydratation utilisant les souches de mycètes filamenteux

8.3.1 Une nouvelle souche de champignon formant des flocs : isolement, identification moléculaire et mesure de sa capacité à réduire, floculer et déshydrater simultanément les boues solides.

Les microorganismes filamenteux et non filamenteux peuvent provoquer de foisonnement des boues et la formation de mousse lors de la décantation et la déshydratation. Dans cette recherche, la dégradation et la biofloculation des boues ont été étudiées en utilisant des champignons formateurs de granules, isolés des boues d'épuration municipales. Pour comprendre le rôle de ces champignons dans la décantation et la déshydratation des boues, deux batch de boues, stérilisée et non stérilisée et contenant différentes concentrations de MES ont été inoculés par les spores et les granules de champignons. Nous avons ensuite effectué un certain nombre d'analyses telles que la mesure de la formation de bioflocs, la décantation et la dégradation des boues, la variation du pH dans le milieu, le potentiel zeta et les observations microscopiques sur les

bioflocs. Nous avons pu montrer que la concentration en MES diminuait après cinq jours d'incubation à cause de l'utilisation de la biomasse fongique. La souche fongique isolée s'est bien adaptée à la production de bioflocs et aux flores microbiennes et a montré un faible temps de succion capillaire, synonyme d'une bonne capacité de déshydratation des boues.

8.3.2 Utilisation de la souche filamenteuse Penicillium expansum *BS30, isolée à partir de boues d'épuration des eaux usées municipales, pour la réduction des agents pathogènes, la déshydratation et la décantation simultanées des boues solides (SSPRSD).*

Dans cette étude, un champignon filamenteux (*Penicillium expansum* BS30) isolé de boues activées d'usine de traitement des eaux a été utilisé afin de réduire les solides présents dans les boues, de réduire la prolifération de pathogènes et d'améliorer la floculation et la déshydratation des boues (SSPRSD). Ces essais ont été réalisés dans des fioles Erlenmeyer et dans un fermenteur de 10L. Le rôle de la souche fongique dans le processus de SSPRSD a été évalué à différentes températures et à différentes concentrations d'inoculum (spores). Les conditions optimales pour le procédé sont une température d'incubation de 25°C et une concentration d'inoculum de 10^6 spores/mL. Dans ces conditions, les solides totaux et les solides volatiles totaux ont été dégradés à plus de 50% et à plus de 53% respectivement. Le temps de succion capillaire (CST) (moins de 13s) est plus bas que le temps requis pour l'assèchement des boues (conventionnellement CST = 20s). Les populations de coliformes et de Salmonelle (indicateur de pathogènes) ont été réduites de 2 et de 4 unités log respectivement. Des études génétiques ont également été réalisées sur la biosynthèse des pénicillines, sur la ségrégation des gènes et sur la machinerie de dégradation des matières organiques toxiques (MOT). Il s'est avéré que le champignon porte effectivement des gènes de production de pénicillines, mais ne contient pas de gènes MOT, ce qui rend cette souche incapable de dégrader ce genre de substances.

9. CONCLUSIONS

Parmi les vingt-cinq souches bactériennes isolées à partir de boues d'eaux usées municipales, quinze d'entre elles ont été capables de produire des EPS capsulaires. Elles appartenaient, pour la plupart, au genre *Bacillus*. La quantité de SPE (dépôt gluant) (3.6-35.8 g / L) et de bouillon bactérien (5,0 - 36,4 g / L), produite par les souches isolées a été plus élevée que la quantité de SPE produite par le consortium de micro-organismes dans les boues. La caractérisation des suspensions de SPE par un zêtaphoremètre a montré qu'ils portent une charge négative. De plus, les SPE (dépôt gluant) ont révélé une meilleure activité de floculation du kaolin que les SPE capsulaires et les bouillons bactériens, avec et sans addition de Ca^{++}. Les SPE produites par la souche bactérienne BS8 (genre *Serratia*) ont montré la meilleure activité de décantation des boues d'épuration. Les valeurs de CST ont sensiblement augmenté avec l'ajout d'extraits de SPE provenant de souches isolées. De plus, nous avons remarqué que l'addition de cations a permis d'améliorer le procédé de déshydratation et de décantation des boues. Les SPE produits par six souches bactériennes sélectionnées et par un consortium de micro-organismes provenant de boues activées ont été caractérisées en termes de protéines (0.7-4%) et d'hydrates de carbone (4-7%). Les extraits de SPE sont très riches en éléments autres que les glucides totaux et les protéines totales (lipides, ADN, ARN) (89-94%).

Au cours de cette étude, nous avons pu isoler et caractériser une nouvelle souche de champignon formant des flocs provenant des boues d'épuration. Une identification moléculaire a montré que le champignon appartenait au genre *Penicillium expansum* BS30. La souche isolée a montré une bonne adaptation à la boue. La biomasse fongique est chargée négativement (charge globale anionique).

Il a été démontré que le champignon filamenteux isolé est valorisable pour les procédés de réduction et de décantation des boues. Nous avons enregistré un taux de dégradation maximal des

matières solides par la biomasse fongique de 68%, cultivée sur la boue fraîche (sans stérilisation). Ce taux est supérieur à la fois à celui du contrôle (dégradation à 22%) et à celui des procédés classiques de digestion aérobie. Toutefois, le pourcentage de déshydratation de ces boues était faible lorsque la concentration des matières en suspension (MES) dépassait 2,5 g/L. Nous avons obtenu une dégradation maximale des MES (64%) et une meilleure déshydratation des boues fongiques brutes (non stérilisées), inoculées avec le champignon cultivé, avec un temps de succion capillaire CST <20 s et à une concentration initiale en MES de 15 g/L. Les boues brutes inoculées avec les spores du champignon ont montré une réduction substantielle de la MES tandis que le taux de déshydratation a diminué pour une concentration initiale en MES de 15 g/L. L'ajout de Ca^{++} a permis d'améliorer le taux de déshydratation des boues fongiques stérilisées mais non pas celui des boues brutes.

La souche de champignon isolée possède des gènes de biosynthèse de la pénicilline, mais ne contient pas les gènes codant pour la dégradation des composés organiques toxiques. L'étude de la reproductibilité du procédé, menée dans un bioréacteur de 10L, a conduit à un taux de réduction des MES important de 54%, à une réduction de 2-4 unités log et à un meilleur taux de déshydratation des boues (CST <20s par rapport au contrôle).

REFERENCES

Abu-orf, M. M., Griffin, P. and Dentel, S. K. 2001. Chemical and physical pre-treatment of ATAD biosolids for dewatering. Wat. Sci & Technol. 44 (10): 309-314.

Allen, M.S., Welch, K.T., Prebyl, B.S., Baker, D.C., Meyers, A.J., and Sayler, G.S. (2004). "Analysis and glycosyl composition of the exopolysaccharide isolated from the floc-forming wastewater bacterium Thauera sp. MZ1T." Environ. Microbiol. , 6(8), 780–90.

Ardern E, Lockett WT. Experiment on the oxidation of sewage without the aids of filters. J Soc Chem Ind 1914;33:523–39

Ayol A (2005). Enzymatic treatment effects on dewaterability of anaerobically digested biosolids-I: performance evaluations. Proc. Biochem. 40(7): 2427-2434.

Ayol, A. and Dentel, S.K. (2005) Enzymatic treatment effects on dewaterability of anaerobically digested biosolids-II: laboratory characterizations of drainability and filterability. Process Biochemistry 40(7), 2435-2442.

Bache DH. and Papavasilopoulos EN. (2003). Dewatering of alumino-humic sludge: impacts of hydroxide. *Water Research*, 37, 3289–3298.

Bala Subramanian S, S. Yan, R. D. Tyagi and R. Y. Surampalli. 2008a. A New Pellet Forming Fungal Strain: Its Isolation, Molecular Identification, and Performance for Simultaneous Sludge Solids Reduction, Flocculation, and Dewatering. *Water Environmental Research*, 80 (9): 840-852.

Bala Subramanian S, S. Yan, R. D. Tyagi, R. Y. Surampalli and BN Lohani. 2008b. Isolation and Molecular Identification of Extracellular Polymeric Substances (EPS) Producing Bacterial Strains for Sludge Settling and Dewatering. *Journal of Environmental Science and Health, Part* A, 43(13): 1495-1503.

Biggs, CA, Ford, AM., Lent, PA. (2001). Activated sludge flocculation: direct determination of effect of Ca^{++} ions. Water sci & tech: 43(11): 75-80.

Bloch, M. (1918). "Beitrag zur Untersuchung über die *Zoogloea ramigera* (Itzigsohn) auf Grund von Reinkulturen." *Zbl. Bakt*., II. Abt. 48, 44–62.

Bockelmann U, Manz W, Neu T R & Szewzyk U, (2002) A new combined technique of fluorescent in situ hybridization and lectin binding analysis (FISH-LBA) for the investigation of lotic microbial aggregates, *J Microbiol Meth*, 49: 75-87.

Boessmann M, Staudt C, Neu T R, Horn H, & Hempel D C, (2003) Investigation and modeling of growth, structure and oxygen penetration in particle supported bioflms, *CET Chem Eng Technol*, 26 219-222.

Bordet, J. (1899). "Le mechanisme de l' agglutination." Ann. Inst. Pasteur., 13, 225–250.

Bott, CB and Love, NG. (2004). Implicating degluthionine gate K^+ efflux system as a cause of electrophile induced activated sludge deflocculation. Appl. Env. Microbiol. 70(9): 5569-78.

Bowen P.T., J.E. Hendrick, T.A. Woodward, L.S. Mitchell et M. Lahlou (1989) Sludge treatment, utilization, and disposal. Water Environment Res. 61(6), 821-829.

Bruus J H, Nielsen P H, & Keiding K, (1992) On the stability of activated sludge flocs with implications to dewatering, *Water research* 26: 1597-1604.

Butterfield, C.T. (1935). "A Zoogloea-forming bacterium isolated from activated sludge, studies of sewage purification (II).” Public Health Rep., 50, 671–681.

Chang G R, Liu J C & Lee D J, (2001) Co-Conditioning And Dewatering Of Chemical Sludge And Waste Activated Sludge, Water Res, 35: 786-794.

Chang J S, Abu-Orf M, & Dentel S K, (2005) Alkylamine odors from degradation of flocculant polymers in sludges, *Water Res*, 39, 336-3375.

Chang L. L., Bruch M.D., Griskowitz N. J. and Dentel S. K. (2002). NMR spectroscopy for determination of cationic polymer concentrations. *Water Research,* 36, 2255–2264.

Chen Y, Yang H & Gu G, Effect of acid and surfactant treatment on activated sludge dewatering and settling, *Water Res*, 35 (2001) 2615-2620.

Chu C P, & Lee D J,, Experimental analysis of centrifugal dewatering process of polyelectrolyte flocculated waste activated sludge, *Water Res*, 35, 10 (2001) 2377-2384.

Clayton AJ, Wentzel MC, Ekama GA, Marais GvR. Denitrification kinetics in biological nitrogen and phosphorus removal systems treating municipal wastewaters. Water Sci Technol 1991; 23(4-6):567–76.

Comte, S., Guibaud, G., and Baudu, M. 2006. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties Part I. Comparison of the efficiency of eight EPS extraction methods. Enzyme and Microbial Technology. 38: 237–245.

Dearfield KL, Ambermathy CO. (1988) Acrylamide: its metabolism, developmental and reproductive effects, genotoxicity and carcinogenicity. Mutat Res;195:45–77.

Deng S. B, Bai, R. B, Hu, X. M. and Luo, Q. (2003) Characteristics of a bioflocculant produced by Bacillus mucilaginosus and its use in starch wastewater treatment, *Appl Microbiol Biotechnol*, 60, 588–593.

Diouri, M. (2004). Study of natural flocculating agent for water and wastewater treatment. Report submitted to the research experience for undergraduate, University of south Florida, USA.

Donaldson W. (1932) Use of activated sludge increasing. Civil Eng; 2(3):167–9.

Droppo, I.G., G.G. Leppard, S.N. Liss and T.G. Milligan. (2005). *Flocculation in Natural and Engineered Environmental Systems.* CRC Press, Boca Raton, FL. pp. 438.

Dudman W F., and Wilkinson J F, (1956) The composition of extracellular polysaccharides of aerobacter-Klebsiella strains, *Biochem . J*, 62, 289.

Durmaz and Sanin (2001). Effect of carbon to nitrogen ratio on the composition of microbial extracellular polymers in activated sludge. Water Science & Technology, 44 (10): 221–229.

Ekama GA, Wentzel MC, Casey TG, Marais GvR. Filamentous organism bulking in nutrient removal activated sludge systems. Paper 6: review, evaluation and consolidation of results. Water SA 1996; 22(2): 147–52.

Ekama, GA and Marais, GvR (1984) Two improved activated sludge settleability parameters. *IMIESA* 9(6) 20-27.

Elgavish S & Shaanan B, (1997) Lectin-carbohydrate interactions: different folds, common recognition principles, *Trends Biochem Sci*, 22: 462-467.

Eriksson L, & Alm B, (1991) Study of flocculation mechanisms by observing effects of complex agent on activated sludge properties, *Water Sci Technol*, 24: 21-28.

Frolund B, Griebe T & Nielsen P H, (1996) Extraction of extracellular polymers from activated sludge using a cation exchange resin, *Water Res*, 30: 1749-1758.

Fujita M, Ike M, Jang J H, Kim S M & Hirao T, (2001) Bioflocculation production from lower-molecular fatty acids as a novel strategy for utilization of sludge digestion liquor, *Water Science & Technology*, 44: 237-243.

Gabb DMD, Ekama GA, Jenkins D, Wentzel MC, Casey TG, Marais GvR. (1996a) Filamentous organism bulking in nutrient removal activated sludge. Paper 4: system configurations and operating conditions to develop low F/M filament bulking sludges at laboratory-scale. Water SA, 22(2): 127–137.

Gabb DMD, Ekama GA, Jenkins D, Wentzel MC, Casey TG, Marais GvR. (1996b) Filamentous organism bulking in nutrient removal activated sludge. Paper 5: experimental examination of aerobic selectors in anoxic–aerobic systems. Water SA, 22(2): 139–146.

Gabb DMD, Still DA, Ekama GA, Jenkins D, Marais GvR. (1991) The selector effect on filamentous bulking in long sludge age activated sludge systems. Water Sci Technol, 23: 867–877.

Gao Jie, B. H.-Y., Xin Ming-Xiu, Liu Yuan-Xia, Li Qian, Zhang Yan-Fen (2006). "Characterization of a bioflocculant from a newly isolated Vagococcus sp. W31." Journal of Zhejiang University SCIENCE B 7(3): 186-192.

Garnier C., Gorner T., Lartiges B. S., Abdelouhab S. and Donato P. (2005). Characterization of activated sludge exopolymers from various origins: A combined size-exclusion chromatography and infrared microscopy study. *Water Research*, 39**,** 3044–3054.

Ghebremichael, AK. and Hultman, B. (2004). Alum sludge dewatering using *Moringa oleifera* as a conditioner. Water, Air and soil pollution. 158: 153-167.

Hébert M., Valorisation des boues municipales comme matières fertilisantes au Québec, *Vecteur Environnement* 37 (5) (2004), pp. 38–41 <http://www.mddep.gouv.qc.ca/matieres/articles/valorisation.htm>.

Higgins M. J. and Novak J. T. (1997a). Characterization of exocellular protein and its role in bioflocculation. *J. Environ. Eng*. 123, 479–485.

Higgins M. J. and Novak J.T. (1997b). The effect of cations on the settling and dewatering of activated sludges: laboratory results. *Water Environ. Res*. 69, 215–224.

Higgins, MJ., Sobbeck DC., Owins, SJ and Szabo, LM. (2004). Case study II: application of the divalent cations bridging theory to improve biofloc properties and industrial activated sludge system performance-using alternative to sodium based chemicals. Water environmental federation. 76(4): 353-359.

Hoa PT, Nair L and Visvanathan C. (2003) The effect of nutrients on exocellular polymeric substance production and its influence on sludge properties. *Water SA*, 29, 437-442.

Horan N J, & Eccles C R, (1986) Purification and characterization of extracelular polysaccharide from activated sludge, *Water Research*, 20: 1427-1432.

Houghton, J.I. J. Quarmby, T. Stephenson, (2001) Municipal wastewater sludge dewaterability and the presence of microbial extracellular polymer, Water Sci. Technol. 44 (2-3): 373-379.

Houghton, JI and Stephenson, T. (2002). Effcte of influent organic content on the digested sludge extracellular polymer content and dewaterability. Wat. Res. 36:3620-3628.

Huang, C and Chen, Y. (1996). Coagulation of colloidal particles in water by chitosan. J. of chemical. Bitechnol. 66: 227-232.

Irvine RL, Busch AW. 1979 Sequencing batch biological reactors — an overview. J Water Pollut Control Fed, 51(2):235–43

Irvine RL, Ketchum Jr LH. 1989 Sequencing batch reactors for biological wastewater treatment. CRC Crit Rev Environ Control, 18(4):255–94;

Irvine RL, Wilderer PA, Flemming H-C. 1997Controlled unsteady state processes and technologies—an overview. Water Sci Technol, 35(1):1–10.

Jayachandran K & Chandrasekaran M, (1998) Biological coagulation of skim latex using *Acinetobacter* sp. isolated from natural rubber latex centrifugation effluent, *Biotechnology letters*, 20: 161-164.

Jenkins, D., M. G. Richard and G.T. Daigger, 1993. Manual on the causes and control of activated sludge bulking and foaming, 2nd Ed., Lewis publishers, Boca Raton, FL.

Jian, Z., Zhi-gao, W and Wen-chao, J. 2006. Effects of extra-cellular polymeric substances on organic pollutants biodegradation kinetics for A-step of adsorption-biodegradation process. Journal of Central South University of Technology. 13(3): 229-233.

Jorand F., Bigne F B., Block J.C. and Urbain V. (1998). Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. *Water science and technology,* 37, 307–315.

Kampfer P, Erhart R, Beimfohr C, Bohringer J, Wagner M, & Amann R, (1996) Characterization of bacterial communities from activated sludge: culture dependent numerical identification versus in situ identification using group- and genus- specific rRNA targeted oligonucleotide probes. *Microb Ecol*, 32: 101-121.

Kang, S.-M., Kishimoto, M., Shioya, S., Yoshida, T., Suga, K.-I., and Taguchi, H. (1989). "Dewatering characteristics of activated sludges and effect of extracellular polymer." *J. Ferment. Bioeng.*, 68(2), 117-122.

Kaplan, D., Christien, D and Arad, S. (1987) Chelating properties of extracellular polysaccharides from chlorella spp. Appl and Env. Microbiol. 53(12): 2953-2956

Karapinar and kargi, 1996, Effect of wastewater composition on treatment performance of a column bioreactor with recycle Bioprocess Eng, 14: 245-248.

Krhutkova O, Ruzickova I, Wanner J. (2002) Microbial evaluation of activated sludge and filamentous population at eight Czech nutrient removal activated sludge plants during year 2000. Water Sci Technol, 46(1/2):471–478

Kumar, G., Joo, HS., Kavali, R., Choy, JW, Chang, CS. (2004). Characterization of an extracellular biopolymer flocculant from a haloalkalophilic *Bacillus* Isolate. World j. Microbiol & Biotechnol. 20: 837-843.

Kurane, R., Ahatamochi, K., Kakuno, T., Kiyohira, M., Kawaguchi, K. (1994). Characterization of liquid bioflocculant produced by *Rhodococcus erythropolis*. Biosci. Biotechnol. Biochem. 58: 1977-1982.

Kurane, R., Takeda, K., Suzuki, T., 1986. Screening and characteristics of microbial flocculants. *Agric. Biol. Chem.*, 50:2301-2307.

Lajoie, C.A. A.C. Layton, I.R. Gregory, G.S. Sayler, D.E. Taylor, A.J. Meyers, (2000) Zoogleal clusters and sludge dewatering potential in an industrial activated sludge wastewater treatment plant, Water Environ. Res. 72, 56–64.

Lakay MT, Hulsman A, Ketley D, Warburton C, de Villiers M, Casey TG, Wentzel MC, Ekama GA. (1999) Filamentous organism bulking in nutrient removal activated sludge systems. Paper 7: exploratory experimental investigations. Water SA, 25(4): 383–96.

Lawrence J R, Swerhone, G D W, Leppard G G, Araki T, Zhang X, West M M, & Hitchcock A P, (2003) Scanning transmission x-ray, laser scanning and transmission electron microscopy mapping of the expolymeric matrix of microbial biofilms, *Appl Environ Microbiol*, 69: 5543-5554.

Leblanc, R.J., 2005. The need for the establishment of Canadian biosolids partnerships. Environmental Science & Engineering. On line: <htpp://www.esemag.com> – July 2005.

Lee S-E, Koopman BL, Jenkins D, Lewis RF. (1982) The effect of aeration basin configuration on activated sludge bulking at low organic loading. Water Sci Technol,14(6/7): 407–27.

Lee, D.Y., S. R. Jing and Y. F. Lin, (2001). Using seafood waste as sludge conditioners. Wat Sci & Tech. 44(10): 301-307.

Lee, Se, Koopman, B, Bode, H and Jenkins, D (1983) Evaluation of sludge settleability indices. *WaterRes*. 17: 1421-1426.

Leem C. H. and J. C. Liu. 2000. Enhanced Sludge Dewatering by Dual Polyelectrolytes Conditioning. Wat. Res. 34(18): 4430-4436.

Liao B. Q., Allen D. G., Droppo G., Leppard G. G. and Liss S. N. (2001). Surface properties of sludge and their role in bioflocculation and settleability. *Water Research*, 35, 339–350.

Linne SR, Chiesa SC. 1987. Operational variables affecting performance of the selector-complete mix activated sludge process. J Water Pollut Control Fed, 59(7): 716–21.

Liu, JC, Lee, CH, Lai JY, Wang KC., Hsu, YC., Chang, BV. (2001). Extracellular polymers of ozonized waste activated sludge. Wat. Sci & technol. 44(10): 137-142.

Liu H. and Fang, H. (2002). Extraction of extracellular polymeric substances (EPS) of sludges. J Biotechnol. 95: 249–56.

Liu Y, Lam MC, Fang H. (2001). Adsorption of heavy metals by EPS of activated sludge. Water Sci Technol. 43: 59–66.

Malik A, Kimchhayarasy P & Kakii K, (2005) Effect of surfactants on stability of *Acinetobacter Johnsonii* S35 and *Oligotropha carboxidovorans* S23 coaggregates, *FEMS Microbiol Ecol*, 51: 313-321.

Martinez, F., Lema, J., Mendez, R., Curvo-lopez, F. and Gomez, J (2004). Role of exopolymeric protein on the settleability of nitrifying sludge. Bioresource technol. 94: 43-48.

Martins, A. M. P., Pagilla, K., Heijnen, J. J. and Van Loosdrecht, M. C. M. 2004. Filamentous bulking sludge – a critical review. Water Res. 38, 793-817.

Martins, AMP., Heijen, JJ., Van-loosdrecht, MCM. (2003). Effcet of DO concentration on sludge settleability. Appl. Microbiol. Technol. 62: 586-593.

Masters CL, Multhaup G, Simms G, Pottgiesser J, Martins RN, Beyreuther K (1985) Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. EMBO J 4:2757–2763.

McKinney R E, (1952) A fundamental approach to the activated sludge process II. A proposed theory of floc formation. *Sewage Ind Wastes,* 24: 280-287.

Mckinney, R.E. (1956). "Biological flocculation." In: Biological Treatment of Sewage and Industrial Wastes, vol. 1, Reinhold, New York, pp. 88–117.

McSwain, BS. Irvine, RL., Hausner, M., Wilderer, PA., (2005). Composition and distribution of extracellular polymeric substances in aerobic and granular sludge. Appl. Env. Microbiol. 71(2): 1051-1057.

Mikkelsen L. H. and Keiding K. (2002). Physico-chemical characteristics of full scale sewage sludges with implications to dewatering. *Water research,* 36, 2451-2462.

Mohan S V, Bhaskar Y V & Karthikeyan J, (2004) Biological decolourisation of simulated azo dye in aqueous phase by algae *Spirogyra* species, *International Journal of Environment and Pollution,* 21: 211-222.

Morgan J W, Forster C F, & Evison, L, 1990. A comparative study of the nature of biopolymer extracted from anaerobic and activated sludges, Water res, 24, 6, 743-750,

Morgan-Sagastume, F and Allen, DG. (2005). Activated sludge deflocculation under temperature upshift from 30-45° C. Wat Res. 39: 1061-1074.

Motta E J L, Jimenez J A, Josse J C & Manrique M, (2003) The effect of air-induced velocity gradient and dissolved oxygen on bioflocculation in the trickling filter/solids contact process, *Advances in Environmental Research,* 7: 441-451.

Musvoto EV, Lakay MT, Casey TG, Wentzel MC, Ekama GA. (1999) Filamentous organism bulking in nutrient removal activated sludge systems. Paper 8: the effect of nitrate and nitrite. Water SA, 25(4):397–407

Nakata K and Kurane R. (1999). Production of an extracellular polysaccharide bioflocculant by *Klebseilla pneumoniae*. Biosci. Biotechnol. Biochem. 63: 2064-2068.

Natarajan K A & Das A, (2003) Surface chemical studies on '*Acidithiobacillus*' group of bacteria with reference to mineral flocculation, *Int J Miner Process*, 72: 189-198.

Neyens E, Baeyens J & Creemers C, (2003) Alkaline thermal sludge hydrolysis, *Journal of Hazardous Materials* B97: 295-314.

Nielsen P H & Jahn A, Extraction of EPS. (1999) *In microbial extracellular polymeric substances. Characterization, structure and function*, edited by W. Calmano and U. Forstner Springer-Verlag, Berlin, Germany, 143-154.

Nielsen, PH and Keiding, K. (1998). Disintegration of activated sludge flocs in presence of sulphide. Wat. Res. 32(2): 313-320.

Norberg, A. B. and Enfors, S. (1982) Production of extracellular polysaccharide by *Zoogloea ramigera*. *Appl. Environ. Microbiol.* 44(5), 1231-1237.

Novak J T, Muller C D and Murthy S N, (2001) Floc structure and role of cations, *Water Sci Technol*, 44, 209-213.

Novak, J.T., Sadler, M.E., and Murthy, S.N. (2003) Mechanisms of Floc Destruction During Anaerobic and Aerobic Digestion and The Effect on Conditioning and Dewatering of Biosolids. *Water Res*., 37(13), 3136–3144.

Ozacar, M and Sengil, A. (2000). Effectiveness of tannin obtain from *valonia* as a coagulant aid or dewatering of sludge. Wat res, 34(4): 1407-12.

Parmer N, Singh A & Ward O P, (2001) Enzyme treatment to reduce solids and improve settling of sewage sludge, *J Industrial Microbiology and Biotechnology*, 26: 383-386.

Pasteur, L. (1876). Etudes Sur La Bierre, Gauthier Villars, Paris.

Pasveer A. (1969) A case of filamentous activated sludge. J Water Pollut Control Fed 41(7):1340–52

Peng, Y.-Z., J.-F. Gao, S.-Y. Wang and M.-H. Sui. 2003. Use of pH as fuzzy control parameter for nitrification under different alkalinity in SBR process. 47 (11): 77–84.

Pinotti, A., Bevilacqua, A and Zaritzky, N. (1997). Optimization of the flocculation stage in a model system of a food emulsan waste using chitosan as a polyelectrolytes. J. food eng. 32: 69-81.

Poxon, TL and Darby JL. (1997). Extracellular polyaninins in digested sludge measurement and relationship to sludge dewaterability. Wat. Res. 31(4): 749-758.

Salehizadeh H and Shojasosadati SA. (2002). Isolation and characterization of a bioflocculant produced by *Bacillus firmus.* Biotechnolgy letters. 24: 35-40.

Salehizadeh, H. M. Vossoughi, I. Alemzadeh, (2000) Some investigations on bioflocculant producing bacteria, Biochemical Engineering Journal. 5 (1): 39-44.

Sanin, F.D. P.A. Vesilind, (1994) Effect of centrifugation on the removal of extracellular polymers and physical properties of activated sludge, Water Sci. Technol. 30: 117–27.

Seintuch M., O. Lev, P. Einav and E. Rubin. 1986, Role of exocellular polymer in the design of activated sludge. Biotechnol and Bioeng., 28: 1564-1576

Seka A M, Wiele T V D & Verstraete W, (2001) Feasibility of a multi-component additive for efficient control of activated sludge filamentous bulking, *Water Res*, 35: 2995-3003.

Sharon N & Lis H, (1989) Lectins as cell recognition molecules, *Science*, 246 227.

Singh RP, Tripathy T, Karmakar GP, Rath, SK, Karmakar, NC Pendey, SR, Kannan, K, Jain, SK., and Lan, NT. (2000) Novel biodegradable flocculants based on polysaccharides. Current science, 79(7): 798-803.

Sobeck D. C. and Higgins M. J. (2002). Examination of Three Theories for Mechanisms of Cation-Induced Bioflocculation. *Water Research*, 36, 527-538.

Steiner AE, McLaren DA, Foster CF. (1976) The nature of activated sludge floc. Water Res, 10: 25–30.

Stoderegger, KE. and Herndl, JG. (2001). Visualization of the the exopolysaccharide bacterial capsule and its distribution in oceonic environment. Aquatic Microbiol ecology. 26: 195-199.

Tenny M W, & Verhoff F H, (1973) Chemical and auto flocculation of Microorganisms in biological wastewater treatment, *Biotechnol Bioengrg*, 15: 1045-1073.

Tramfloc Super Emulsion™. http://www.flocculants.info/polymers-for-solids-and-sludge.html

Urbain V., Block J. C. and Manem. J. (1993). Bioflocculation in activated sludge: An analytical approach, *Water Res*, 27, 829-838.

USEPA (Environmental Protection Agency), 1999. EPA Biosolids Generation and Uses, EPA 530R-99-009, September 1999.

Vanhoric M, Moens W. (1983) Carcinogen of acrylamide. Carcinogenesis, 4:1452–63.

Vidyarthi, A.S., R.D. Tyagi, J.R. Valéro and R.Y. Surampalli (2002). Studies on the production of *B. thuringiensis* based biopesticides using wastewater sludge as raw material. Water Res. 36, 4850-4860.

Vijayalakshmi S P & Raichur A M, (2002) Bioflocculation of high-ash Indian coals using *Paenibacillus polymyxa*, Int. J. Miner. Process. 67: 199-210.

Vogelaar J C T, Keizer A D, Spijker S & Lettinga G, (2005)Bioflocculation of mesophilic and thermophilic activated sludge, *Water Research,* 39: 37-46.

Wang J S, Hu Y Y, & Wu C D, (2005) Comparing te effect of bioflocculants with synthetic polymers on enhancing granulation in UASB reactors for low strength wastewater treatment, *Water SA*, 31 (2): 177-182

Wang, S.G. W.X. Gong, X.W. Liu, L. Tian, Q.Y. Yue, B.Y. Gao, (2007) Production of a novel bioflocculant by culture of *Klebsiella mobilis* using dairy wastewater, Biochemical Engineering Journal, 36 (2): 81-86.

Water Pollution Control Federation (WPCF) et Association Québécoise des Techniques de l'Eau (AQTE) (1985) Exploitation des stations d'épuration des eaux usées. Environnement Canada, Ottawa, Ontario, Canada.

Whitchurch C B, Tolker N T, Ragas P C, & Mattick J S, (2002) Extracellular DNA required for bacterial biofilm formation, Science, 295: 1487.

Wilderer PA, Irvine RL, Goronszy MC. Sequencing batch reactor technology. Scientific and Technical Report No. 10. Great Britain: IWA Publishing; 2001.

Wilen B M, Jina B & Lant P, (2003) The influence of key chemical constituents in activated sludge on surface and flocculating properties, *Water Research*, 37: 2127-2139.

Wolfaardt G M, Lawrence J R, Robarts R D, & Caldwell D E, (1998) In situ characterization of biofilm exopolymers involved in the accumulation of chlorinated organics, *Microb Ecol*, 35: 213-223.

Yin, X., Pingfang Han, Xiaoping Lu, Yanru Wang. 2004. A review on the dewaterability of bio-sludge and ultrasound pre-treatment. Ultrasonics Sonochemistry 11; 337–348.

Yoon, JH., Oh, HM., Yoon, BD., Kang, KH. and Park, YH. (2003). *Paenibacillus kribbensis* sp. Nov. and *Paenibacillus terre sp.* nov. bioflocculants for efficient harvesting of algal cells. Int.J.syst.and Evol.Microbiol. 53:295-301.

Zang, J., Wang, R, Ziang, P., Liu, Z. (2002). Production of an exopolysaccharides bioflocculant by So*rangium cellulosum.* Lett in Appl. Microbiol. 34: 178-181.

Zhang, Z.Q., Lin, B., Xia, S.Q., Wang, X.J., amd Yang, A.M. (2007). "Production and application of a bioflocculant by multiple-microorganism consortia using brewery wastewater as carbon source." *J. Environ. Sci.-China*, 19, 660–666.

Zita, A and Hermansson, M. (1997). Effect of bacterial cell surface structure and hydrophobicity an attachment to activated sludge flocs. Appl. Env. Microbiol. 63(3):1168-1170.

CHAPITRE 2.

REVUE DE LA LITTÉRATURE

PART I

BIOFLOCCULANTS

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CHAPITRE 3.

BIOFLOCCULATION ET DE DÉSHYDRATATION UTILISANT EPS NON-FILAMENTEUX PRODUISANT DES SOUCHES BACTÉRIENNES

PART I

SCREENING OF EXTRACELLULAR POLYMERIC SUBSTANCES (EPS) PRODUCING MICROORGANISMS FOR SLUDGE SETTLING

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PART II

EXTRACELLULAR POLYMERIC SUBSTANCES (EPS) PRODUCING BACTERIAL STRAINS OF MUNICIPAL WASTEWATER SLUDGE: ISOLATION, MOLECULAR IDENTIFICATION, EPS CHARACTERIZATION AND PERFORMANCE FOR SLUDGE SETTLING AND DEWATERING

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RÉSUMÉ

Les stations de traitement des eaux usées ont souvent des problèmes de sédimentation des flocs dus à l'agrégation des boues. Généralement, un polymère organique synthétique (Polydimethyl diallyl ammonium chloride) et / ou un coagulant inorganique (Chlorure de Fer, alun et chaux) sont utilisés pour faire sédimenter les boues. Ces produits chimiques sont très dispendieux et sont dangereux pour l'environnement. Il existe aujourd'hui des bioflocculants qui sont moins dommageable pour l'environnement et qui peuvent être utilisés pour la floculation des boues. Les exopolysaccharides (EPS) produit par les microorganismes présents dans les boues jouent un rôle essentiel dans la floculation des boues. L'étude présentée ici a analysé vingt-cinq EPS produits par différentes souches qui ont été isolées d'une usine municipale de traitement d'eau. Les souches retenues ont été sélectionnées selon leur capacité à produire des EPS sur milieu solide. Trois types d'EPS (le biofilm, la capsule et un mélange des deux) ont été récoltés et analysés pour déterminer leurs caractéristiques. La concentration en EPS (poids sec), la viscosité et la charge (à l'aide d'un Zetaphoremètre) des échantillons ont également été mesurées. La capacité des EPS obtenus à biofloculer a été mesurée par l'activité de floculation de kaolin. Selon ces résultats, les six meilleures souches (BS2, BS8, BS9, BS11, BS15 and BS25) ont été sélectionnées pour des tests ultérieurs. Les EPS de biofilm sont de meilleurs biofloculants que les EPS de capsules et que le mélange des deux. Les EPS de biofilm extraits des six souches identifiées précédemment ont donc été étudié pour leur capacité à floculer [indice de volume des boues (SVI)] et à déshydratation [temps de succion capillaire (CST)] les boues. Les biopolymères produits par les souches ont significativement amélioré la déshydratation. Les EPS de biofilm de ces six souches ont été partiellement caractérisées.

ABSTRACT

Wastewater treatment plants often face the problems of sludge settling mainly due to sludge bulking. Generally, synthetic organic polymer and/or inorganic coagulants (ferric chloride, alum and quick lime) are used for sludge settling. These chemicals are very expensive and further pollute the environment. Where as, the bioflocculants are environment friendly and may be used to flocculate the sludge. Extracellular polymeric substances (EPS) produced by sludge microorganisms play a definite role in sludge flocculation. In this study, twenty-five EPS producing strains were isolated from municipal wastewater treatment plant. Microorganisms were selected based on EPS production properties on solid agar medium. Three types of EPS (slime, capsular and bacterial broth mixture of both slime and capsular) were harvested and their characteristics were studied. EPS concentration (dry weight), viscosity and their charge (using a Zetaphoremeter) were also measured. Bioflocculability of obtained EPS was evaluated by measuring the kaolin clay flocculation activity. Six bacterial strains (BS2, BS8, BS9, BS11, BS15 and BS25) were selected based on the kaolin clay flocculation. The slime EPS was better for bioflocculation than capsular EPS and bacterial broth. Therefore, extracted slime EPS (partially purified) from six bacterial strains was studied in terms of sludge settling [sludge volume index (SVI)] and dewatering [capillary suction time (CST)]. Biopolymers produced by individual strains substantially improved dewaterability. The extracted slime EPS from six different strains were partially characterized.

Keywords: Slime, EPS, Zeta potential, Microorganisms, bioflocculation and sludge.

INTRODUCTION

Wastewater sludge is produced in large quantities (Hébert, 2004; USEPA, 1999). Sludge disposal is an increasing environmental problem and may be onerous; treatment and disposal alone represent 50% of wastewater utility costs (Leblanc, 2005). The produced sludge must be recycled and/or reused for economical disposal.

In a wastewater treatment plant (WWTP) sludge settling and dewatering are well-known problem, mainly due to poor settling associated with sludge bulking. Sludge bulking is caused by both filamentous and non-filamentous (Zoogloea) organisms present in sludge (Martins *et al.*, 2004). Sludge settling and dewatering in WWTP is conducted with the aid of chemical polymers followed by physical separation of solids and liquids (USEPA, 1999). Sludge is typically negatively charged (approximately –30 mV). Hence, cationic-synthetic polymers (CSP) or anionic synthetic polymers in combination with cations (Al^{3+}, Fe^{3+}) are typically used to neutralize the sludge-surface charge, facilitating flocculation and settling (Higgins and Novak, 1997a). It is estimated that 25–50 million kg of polymers ($2–4 per kg CSP with cost of $130 million/year) are utilized annually in the United States for sludge conditioning (Chang *et al.*, 2002).

The synthetic polymers (SP) are known to affect soil microorganisms, when dewatered sludge containing these polymers is applied to agricultural land (Hébert, 2004). The chemically dewatered sludge when used as a raw material for production of value added products (biopesticides), decreased yield (entomotoxicity) and bacterial growth rate have been observed compared to the sludge settled without chemical polymers (Vidyarthi *et al.*, 2002). Furthermore, chemically dewatered sludge affects the natural microbial community present in many environments such as: composting, in the receiving soil environments during agriculture spreading, soil rehabilitation and sheet cover (Gagnon and Ziadi, 2004). Hence, use of SP for sludge

dewatering must be minimized to reduce the cost of sludge handling and their toxic effect (Chang *et al.*, 2002). To overcome this problem, WWTP can use environment friendly biopolymers/biocoagulants/bioflocculants that are produced by microorganisms. The objective of this study was to achieve bioflocculation of sludge; using ecofriendly, less expensive and biodegradable bioflocculants.

In general, bacterial extracellular polymeric substances (EPS) help in formation of bioflocs in activated sludge and contribute to its structural, surface charge and settling properties (Urbain *et al.*, 1993; Houghton *et al.*, 2001; Houghton and Stephenson, 2002). Bioflocs are formed with interaction among microbial aggregates, filamentous bacterial strains, organic and inorganic particles, which are held together by EPS (Urbain *et al.*, 1993; Novak *et al.*, 2001; Bala Subramanian *et al.*, 2008a, b). EPS have been identified as one of the major components in bioflocs/biofilms (Urbain *et al.*, 1993). They mainly consist of polysaccharides, proteins, nucleic acids and other cellular components (Higgins and Novak, 1997b; Sobeck and Higgins, 2002). EPS is either cell bound (capsular EPS) or secreted outside the cell (slime EPS). Slime EPS is generally washed out from the cell during centrifugation/harvesting, and the capsular EPS is stable and remain attached on the cell wall of microorganisms during the separation process. Many bioflocculation studies were carried out using only one type of EPS (either slime or capsular EPS) from individual microorganism (Hirst *et al.*, 2003). It is observed that even though activated sludge has enough EPS, it fails to settle the sludge naturally (Houghton *et al.*, 2001).

So far bioflocculation of sludge using biopolymers or EPS produced by bacterial strains is not successful due to: (i) the individual sludge microorganisms producing EPS have not been isolated and identified; and (ii) the characteristics of EPS produced by individual sludge microorganisms are not known.

Therefore, specific objectives of this study were focused on: (i) isolation and screening of bioflocculants (EPS) producing microbial strains from municipal wastewater sludge; (ii) growing the isolated strains separately to produce EPS of desired characteristics for sludge bioflocculation; and (iii) evaluating the sludge bioflocculation potential of individual EPS (slime, capsular and their combination) produced by individual strains.

MATERIALS AND METHODS

Sludge Sample Collection and isolation of EPS producing strains

Wastewater sludge samples were collected from Communauté Urbaine du Québec (CUQ, Québec, Canada). Microbial isolation was attempted on plate count agar (PCA) and Sabouraud's dextrose agar (SDA) media using serial dilution techniques. Plates were incubated at 25°C for 48–72 hrs. Isolated bacterial strains were named as BS1 to BS25. These EPS producing microorganisms were selected based on their mucoidal colony (which displayed viscous or sticky growth due to production of large quantities of EPS) formation on the growth medium and string forming ability by touching with inoculating loop. Congo red staining was carried out to identify the presence of extracellular polysaccharides (slime) in the microbial EPS (Neu, 2000). Capsule staining was employed to identify the presence of capsular EPS on the surface of bacterial strains using crystal violet and 20% $CuSO_4$ aqueous solution (Cain *et al.*, 2009). Isolated bacterial strains were identified using 16S rRNA gene sequencing as described in the molecular identification of bacterial strains section.

EPS Production and Harvesting

To produce EPS, isolated strains were grown individually as pure cultures and consortium (0.1% V/V secondary sludge used as inoculum) as well in a reported mineral medium. The composition of mineral medium utilized for the biopolymer production was as follows; 25 g/L glucose, 0.2 g/L $MgSO_4$ $7H_2O$, 2 g/L K_2HPO_4, 1 g/L KH_2PO_4, 1 g/L NH_4Cl and 0.01 g/L yeast extract. The initial pH of media was adjusted to 7.0. Glucose and $MgSO_4$ were sterilized separately and mixed aseptically with other ingredients before inoculation. Bacterial strains were inoculated in the mineral medium from the PCA slants, and mineral medium was incubated in an orbital shaker at 250 rpm for 3 days at 25°C. At the end of 3 days, the broth became highly viscous (discussed below).

After incubation (3 days), the medium was centrifuged at 6,000 g for 15 minutes at 4°C to obtain slime EPS (in centrifuged supernatant) and capsular EPS (in bacterial pellet or microorganisms) (Zhang *et al.*, 1999 and 2002). The culture (bacterial) broth containing both slime and capsular EPS was also used in this study. The three types of harvested EPS were then stored at 4°C for further studies. Bioflocculation characteristics were measured in terms of flocculation activity of kaolin clay suspension to investigate the performance of EPS produced by isolated microorganisms; slime EPS, capsular EPS and culture (bacterial) broth [combination of both slime and capsular EPS (total EPS)].

To induce the production of positively charged biopolymers from the isolated bacterial strains, the initial pH (4, 7 and 10) of the EPS production media was varied. Alkaline condition was achieved using 4N NaOH and acidic condition was achieved by adding 4N H_2SO_4. Medium preparation and inoculation were carried out as mentioned above. The zeta potential of bacterial broth produced at different pH (4, 7 and 10) was also measured as described below.

Dry weight of crude EPS

Dry weight of the crude slime EPS, capsular EPS and bacterial broth (combined slime and capsular EPS) were measured by drying at 105°C to a constant weight (APHA, 2005).

Viscosity

Viscosity measurement was carried out only for the EPS produced bacterial broth after 3 days of fermentation. Viscosity of EPS produced culture (bacterial) broth was measured using viscometer (DV-II+PRO, Brookfield); 18 mL of culture broth was used to measure the viscosity using ULA S 34 spindle, at 60 rpm and room temperature. The viscosity is related to concentration of the biopolymer produced in the broth.

Zeta potential [ζ]

The charges of three types of EPS (produced from medium initial pH 7) were determined by adding 50 to 1000 μL volume of EPS samples in 100 mL of deoinzed water. The zeta potential of bacterial broth (combined slime and capsular EPS) was measured from individual bacterial grown media (non-centrifuged media) after 3 days of fermentation. To measure zeta potential of individual EPS (slime and capsular), the fermented broth was centrifuged. The supernatant obtained was used to measure zeta potential of slime EPS. The capsular EPS (bacterial pellet) was re-suspended in deionized water with volume equal to that of the production media followed by measurement of zeta potential. The surface charge (ζ) measurements were carried out using three types of EPS obtained from all 25 bacterial strains and consortium as well. Characterization of charge (zeta potential) was implemented using Zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France) with the application of the Smoluckowski equation. Surface charge of the wastewater sludge was also measured. The zeta potential values were obtained from

the average of around 24 measurements, the average values are presented with its half-width confidence interval at 95% confidence level.

Kaolin Flocculating activity of different EPS

Kaolin clay suspension was used as test material to determine the flocculating activity as described by Kurane *et al.* (1986) with minor modification. Kaolin clay mimics wastewater sludge in terms of its charge (-32 mV), which is similar to the surface charge of sludge. Using kaolin can provide more reproducible results than the sludge, whose characteristics are found to vary every time it latter is sampled. Kaolin clay was suspended in deionized water at a concentration of 5.0 g/L. Kaolin suspension was dispensed into a 15 mL test tube in the following manner: 9 mL kaolin clay suspension, with three types of EPS with and without addition of 0.25 mL of $CaCl_2$ solution (100 mg $CaCl_2$/L). In order to obtain comparable results exact quantity of biopolymers (produced at pH 7) were used to measure kaolin clay flocculation activity. Biopolymers produced at pH 7 were only used to measure the bioflocculation activity, which is due to the fact that, most of the biopolymers [by 4 (BS1, BS2, BS4 and BS16) out of 25 bacterial strains] produced at pH 7 revealed less negative charge than those produced at pH 4 and 10. However, EPS surface charge is not directly related to sludge settling and dewatering. EPS concentrations were varied in the range of 5, 10, 20, 30, and 50 at both μg/10 mL and/or mg/10 mL level. The effect of calcium ions in kaolin bioflocculation was examined due to the fact that divalent cations are known to improve the biofloc formation and increase the biofloc size through interacting as a bridging agent between negatively charged sludge solids and microbial polymer (Higgins and Novak, 1997b; Sobeck and Higgins, 2002; Novak *et al.*, 2001). Deionized water, instead of bacterial culture broth, was used as a control under similar conditions. After short vortexing, the suspension was allowed to flocculate for 5 min. Then the upper phase of settled kaolin suspension was used to measure the absorbance using UV spectrophotometer at 550 nm. Flocculating activity was calculated using the formula

[(*B*−*A*)/*B*]×100% as mentioned by Gao *et al.* (2006), in which *A* and *B* were the absorbance at 550 nm for sample and control, respectively.

Chemical characterization of Slime EPS produced from selected six bacterial strains

Slime EPS Extraction

Slime EPS extraction from selected six pure cultures strains (which revealed highest kaolin flocculation activity) and consortium was studied. EPS extraction was carried out using a modified protocol from Zhang *et al.* (1999 and 2002). Bacterial culture broth was centrifuged at 6000 g for 15 min at 4°C to remove bacterial cells. The supernatant was precipitated with 2.2 volumes of absolute chilled ethanol by incubating the mixture at -20°C for 1 h. Precipitated EPS was collected by centrifugation at 6000 g for 15 min at 4°C. The supernatant was discarded and the pellet containing slime EPS was dried at room temperature in a laminar hood for 6 h. Dry weight of the extracted EPS (biopolymer) was measured (APHA, 2005). Prior to dry weight measurement, precipitated EPS was suspended in water to conduct sludge settling and dewatering studies. EPS extraction from fresh sludge was also conducted. Sludge EPS was extracted as described in Urbain *et al.* (1993). Collected secondary sludge was concentrated to total solids of 37.40 g/L, suspended solids of 30.35 g/L and dissolved solids of 7.05 g/L. Concentrated 100 mL sludge sample (in duplicate) was sonicated thrice using a sonicator (Ultrasonic processor, CV33, Cole Parmer, USA) at 50W for 15 s with a time interval of 10 s. The sonicated sludge samples were well mixed with an equal volume of Milli-Q water and then the mixture was allowed to stand for 45 min at 4°C. Well mixed samples were centrifuged at 14000 g for 20 min at 4°C. Then collected supernatant containing sludge EPS was precipitated and extracted with ethanol by following a similar EPS extraction procedure as used for pure bacterial strains and consortium. The dry weight of the extracted polymer was measured as described above.

Chemical characterization of slime EPS

The total carbohydrates (TC) content of extracted slime EPS was determined by the phenol-sulfuric acid method (Dubois *et al.*, 1956). The total protein (TP) content of the extracted EPS was investigated by the Bradford (1976) method with bovine serum albumin as a standard.

Sludge settling and dewatering studies using slime EPS produced from six selected strains

Sludge Volume Index

The purified/extracted slime EPS (at concentration of 10% v/v) from six individual bacterial strains (BS2, BS8, BS9, BS11, BS15 and BS25) was used to measure the SVI and capillary suction time (CST). In our earlier study (Bala Subramanian *et al.*, 2006), it was found that SVI (sludge settling) and CST (dewatering) values did not change appreciably at slime EPS concentration (crude slime EPS) higher than 10% v/v. Therefore, 10% v/v concentration of purified/extracted biopolymer (slime EPS) was used in this study. The sludge settling efficiency of biopolymers was compared with that of cationic synthetic polymer (CSP) at 0.01% (W/V) [0.1 g/L]. To measure SVI, fresh sludge sample collected from municipal wastewater treatment plant (CUQ, Quebec) was mixed thoroughly. The well-mixed sludge (sample volume 1 L) was transferred into beakers (1 L in each beaker), followed by the addition of slime EPS (test beaker) and another beaker served as control (no addition of EPS). After addition of EPS, the samples were mixed in two different stages; at 117 rpm for 5 min followed by 10 rpm for 10 min. First stage mixing enables the biopolymer to mix and brings it in contact with sludge solids, second stage mixing enables flocs to form before sludge settling. Each mixed sludge sample was then transferred into a 1 L graduated measuring cylinder for SVI measurement. Sludge settling efficiency in each cylinder was monitored at intervals of 5, 10, 20 and 30 min, respectively.

Capillary suction time

CST was measured (Vesilind and Örmeci, 2000) to ascertain dewatering properties of sludge using slime EPS produced from six bacterial strains (BS2, BS8, BS9, BS11, BS15 and BS25). The samples for CST were prepared in a similar way as described in the SVI section. CST was carried out using standard protocols as outlined by the manufacturer (Triton electronics, model 304M CST, Dunmow, Essex). The effect of Ca^{++} ions on CST was measured by adding 100 mg $CaCl_2$/L to the sludge prior to performing the CST test. To check the reproducibility of the CST results, 10 samples were measured simultaneously.

Molecular Identification of EPS Producing Bacterial Strains

Genomic DNA isolation

DNA was extracted from all 25 EPS producing bacterial strains (BS1 to BS25) isolated from municipal wastewater sludge. Genomic DNA isolation procedure was used as described by Bala Subramanian *et al.* (2008b). The extracted DNA was resuspended in appropriate volume of Tris-EDTA (TE) buffer and stored at -20^{o}C. Agarose gel (0.7%) was made in 0.5 X Tris Borate EDTA (TBE) buffer and run at 60 V for 1 h at 10^{o}C in an electrophoretic apparatus. The fractionated DNA bands were visualized under UV Transilluminator (UVP, 3UV benchtop transilluminator, Canada) and compared with known DNA molecular weight markers. Genomic DNA showed above 23 kb band of λ DNA Hind III digest in agarose gel electrophoresis (Horizontal Midi-gel systems, C.B.S. Scientific company, Inc. Del Mar, CA).

Polymerase Chain Reaction (PCR)

The genomic DNA isolated from BS1 to BS25 was used for direct amplification of 16S rRNA gene portions (Widmer *et al.*, 1998). Universal primers were used to amplify the full-length 16S rRNA gene from the rrn operons of 25 bacterial isolates (Weisburg *et al.*, 1991). Primers were custom synthesized from Eurogen (Eurogen, San Diego, California, USA) and

primers for 16S rDNA had the following sequence: Forward primer 5' <agagtttgatcatggctcag> 3' and Reverse primer 5' <aaggaggtgatccarccgca> 3'. PCR was performed for a 100 μL reaction volume contained in 1 X PCR amplification buffer (MBI Fermentas Inc, Amherst, NY), 200 μmol of each deoxynucleotides, 100 picomoles of each oligonucleotide primers and template DNA (0.25 μg of purified DNA from 1 μg/μL of stock solution) of 25 different bacterial isolates (BS1 to BS25). Amplification was carried out in a thermalcycler (Eppendorf Mastercycler® ep Systems, North America) with heated lid (104°C) facility and was run with block temperature control (thermal regulation by 6°C/s). Denaturation of template DNA was done for 5 min at 94°C. After the hot start, 2U of Taq DNA polymerase (MBI Fermentas, Amherst, NY) was added. PCR was performed for amplification of 16S rRNA gene under a specific thermal profile as follows; denaturation at 94°C for 60 s, annealing at 54°C for 2 min and polymerization at 72°C for 3 min for 36 cycles followed by final extension at 72°C for 10 min. Five μL of amplified PCR product was resolved by electrophoresis on 1.2% agarose gel (Amresco, Ohio, USA) and observed on a UV transilluminator (UVP, 3UV benchtop transilluminator, Canada). The remaining PCR product was stored at –20°C for further work.

Purification of Amplified products

Amplified product was fractionated on 0.7% low melting agarose gel (Amresco, Ohio). PCR fragments were gel cleaved and purified using spin-columns from Qiagen gel extraction Kit (Qiagen, Canada). Finally DNA was eluted with elution buffer EB (10 mM Tris-Cl; pH-8.5) and the purified products were stored at -20°C. The product purity was ascertained by the presence of a single amplified band in agarose gel electrophoresis and subsequently used for sequencing.

Gene sequencing and Homology search

DNA sequencing was carried out using a DNA sequencer (ABI 3730xl DNA Analyzer, Applied biosystems, USA). The obtained 16S rDNA sequences were aligned using DNASIS bioinformatics software (Hitachi Software Japan, Tokyo). 16S rDNA sequences were blasted into the internet (http://www.ncbi.nlm.nih.gov/BLAST/) for a similarity search. Isolated bacterial strains 16S rRNA gene sequences were submitted to GenBank, USA and their assigned accession numbers are from EU031751 to EU031772, EU017381, EU017383 and FJ604109.

RESULTS AND DISCUSSION

Isolation and characterization of EPS producing bacterial strains

Twenty-five bacterial strains were isolated from the municipal wastewater sludge. These microorganisms were selected based on their mucoidal colony formation on the growth medium and string forming ability by touching with inoculating loop (Figure 1a). All these 25 bacterial strains (named BS1 to BS25) were screened to study their potential for EPS production. Thirteen out of 25 bacterial strains were identified as gram positive bacteria and the remaining 12 were identified as gram negative microorganisms (Table 1, Figure 1b, c). Capsule staining showed the presence of bacterial capsular EPS; and 15 out of 25 bacterial strains were found to have capsular EPS (Table 1). The production of capsular and/or slime type of EPS by the isolated bacterial strains was observed. The bacterial strains BS1, BS7, BS11, BS13, BS15, BS20-23 and BS25 were capable of producing only slime EPS in the medium and each of them could form an EPS string longer than 2 cm. However, capsular EPS producing strains failed to produce string with a length of more than 2 cm. Slime EPS producing bacterial strains produced different lengths of EPS string, which were related to the concentration and chemical characteristics of the EPS produced.

In general, slime and capsular EPS are produced by bacterial cells to protect them against unfavorable environmental conditions such as: desiccation, presence of toxic compounds, low temperatures or high osmotic pressures, and may contribute to the uptake of metal ions (Hirst *et al.*, 2003). The presence of EPS also favors the interaction between sludge solids and bacterial cells through physical supports, resulting in the appearance of bioflocs/biofilms (Higgins and Novak, 1997a, b).

The 16S rDNA sequence data were subjected to a BLASTn search. The homology search results of BS1-BS25 bacterial strains resembling (96-99%) with existing DNA sequence database were identified. Based on 16S rDNA sequence homology, the bacterial strains were identified and presented in Table 1. It was found that out of twenty-five bacterial strains, eleven bacterial strains belonged to *Bacillus* genus, three were *Serratia,* two were *Pseudomonas*, two were *Enterobacter*, two were *Yersinia,* two were *Microbacterium* and one strain was from each genus of *Pantoea, Photorhabdus* and *Pectobacterium*.

EPS Production and characteristics

Dry weight

EPS (biopolymers) concentrations, produced by individual 25 bacterial strains and consortium, at the end of 3 days of fermentation are presented in Figure 2. The quantity of slime EPS produced by individual strains was in the range of 3.6 to 35.8 g/L. Similarly, the concentration of total EPS produced in bacterial broth (slime and capsular EPS and microorganisms) was in the range of 5.0 to 36.4 g/L. The concentration of slime EPS produced by the bacterial consortium was 4.6 g/L and total EPS (slime + capsular) in bacterial broth was 7.2 g/L. The sludge microorganisms also produced similar a concentration of slime EPS (4.0 g/L).

The concentration of EPS, produced by the consortium of different microbial strains grown in mineral media, was lower than the EPS quantity produced by individual strains. This was due to complex interaction and competition among different strains of the consortium (also wastewater sludge characteristics keeps on changing every time and their operating parameters are also challenging for the microbial community); whereas, individual microbial strains grow in a non-competitive environment and thus produce higher EPS concentration (slime EPS and in bacterial broth). The capacity of individual bacterial strains to produce different concentrations of EPS demonstrated their different metabolic activity.

EPS produced by different bacterial strains also possesses different properties (zeta potential and viscosity of the culture broth). This variation in EPS concentration could affect the chemical characteristics of EPS produced by individual strains, and subsequently could affect sludge bioflocculation, as discussed in the following sections.

Viscosity

Viscosity measurement was carried out, only for individual bacterial broths, after 3 d of fermentation. The viscosity of the collected bacterial culture broth increased, from 100 mPas of the medium before growth to 105 - 179 mPas, after growth due to production of EPS and bacterial growth during the incubation period (Table 1). The reason for variation in viscosity among bacterial strains was due to different final cell concentration (to a lesser extent) and the different amount of EPS produced (to a greater extent) which in turn is related to individual cell metabolism.

Zeta Potential (ζ) value of Biopolymers

The zeta potential value of slime EPS, capsular EPS and bacterial broth ranged between –9.28±5.15 and –47.40±4.22, –3.48±0.53 and –66.81±2.12, and –6.76±0.89 and –55.36±3.26 mV, respectively (Figure 3). The variation of charge, on different EPS produced by different strains,

was due to two reasons: (i) different microorganisms produced EPS in different concentrations due to differences in their metabolic rates and/or routes, and (ii) different microorganisms may produce EPS of different chemical composition (different functional groups) and properties. The differences in the properties of polysaccharides and proteins of EPS can lead to different charges. For example, the presence of higher quantity of basic amino acids in EPS gives higher positive charge (or less negative charge); while the existence of higher acidic amino acids in the protein (part of EPS) gives higher negative charge. Moreover, the presence of capsule on a cell surface can reduce the negative surface charge of microorganisms to a certain extent.

Surface charge of the fresh sludge (–77.27±4.71 mV) used in this study revealed a higher negative charge than the biopolymers (EPS from bacterial strains) (Figure 3). The possible reason for higher sludge surface charge may be due to the fact that the sludge, in addition to EPS (proteins, carbohydrates, lipids, DNA and RNA), is also composed of various other components including microorganisms, humic substances and solids. These components may contribute to higher anionic charge.

The EPS produced from 25 bacterial strains, in bacterial broth at different pH are also anionic in nature (Figure 4). The zeta potential value of EPS (in bacterial broth) produced at pH 4, pH 7 and pH 10 ranged between –18.36 and –53.00, –6.76 and –50.82, and –35.52 and –79.40 mV, respectively (Figure 4). The EPS produced (in bacterial broth) at pH 7 had relatively less negative charges than (with the exception of bacterial strains BS1, BS2, BS4 and BS16) the EPS produced at pH 4 and pH 10.

It should be mentioned here that exracted slime EPS (in supernatant) could contain ions from the production medium (left after growth of individual strains), which could have also imparted a role in the measured zeta potential value. Since, slime EPS in supernatant was used to measure kaolin clay flocculation activity, therefore, zeta potential of slime EPS was also measured

in the same form. Similarly, capsular EPS suspended in water was used to measure the kaolin flocculation activity, therefore, zeta potential was also measured in the same form.

Further, capsular EPS was separated by centrifugation and resuspended in deionized water (where phosphate and other possible ions left after growth of EPS production media were absent). The zeta potential of the resuspended EPS also revealed negative value. Similarly, slime EPS was precipitated and partially purified (as described in results in section 3.7). The extracted slime EPS was then resuspended in deionized water (absence of phosphate and other possible ions) and zeta potential measured. The negative value of zeta potential revealed by the extracted slime EPS are presented in Table 4. These observations clearly established that the EPS produced by sludge isolated bacterial strains were anionic in nature.

It is known that pH plays a major role in determining zeta potential of colloidal particles (Sobeck and Higgins, 2002; Hirst *et al.*, 2003). To achieve bioflocculation, cationic biopolymer can be added to neutralize overall net negative charge of the sludge. Though the biopolymers possess certain positively charged groups, the produced biopolymers are negatively charged (Bala Subramanian *et al.*, 2006 and 2008a, b). In general, cations are supplemented (externally) to achieve bioflocculation in combination with anionic polymers.

Kaolin flocculation activity

Kaolin flocculating activities of 3 types of EPS (slime, capsular and broth), produced at pH 7, by 25 bacterial strains and their consortium (employing different EPS concentrations) varied in the following order: slime EPS >capsular EPS >EPS in bacterial broth. The results revealed enhanced flocculation activity with addition of Ca^{++} ions (Tables 2 and 3).

The trend of kaolin clay flocculation activity upon addition of slime EPS 0.5, 1, 2, 3 and 5 g/L demonstrated four different patterns (Tables 2 and 3). Ten bacterial strains (BS2, BS4, BS7, BS8, BS10, BS11, BS12, BS15, BS18 and BS22) and the consortium, revealed better kaolin

flocculating activity between 2-3 g/L (20-30 mg/10mL) of slime EPS addition (Tables 2 and 3). Two bacterial strains (BS9 and BS21) demonstrated better flocculating activity at 1 g/L (10 mg/10 mL) of slime EPS. Nine bacterial strains (BS1, BS3, BS5, BS6, BS13, BS14, BS16, BS20 and BS25) displayed increased flocculation activity with the biopolymer concentration, whereas four strains (BS17, BS19, BS23 and BS24) revealed a decreasing trend with increasing slime EPS concentration. This trend was more or less similar with other biopolymers (Capsular EPS and bacterial broth) with and without Ca^{++} addition. Six bacterial strains (BS2, BS8, BS9, BS11, BS15 and BS25) revealed highest flocculating activity of 77.4 to 83.7% (Tables 2 and 3). Hence, these six bacterial strains were selected for further study on sludge settling and dewatering, and bio-chemical characterization of their slime EPS.

Slime EPS of individual strains were better than capsular and bacterial broth EPS, irrespective of concentration used (Tables 2 and 3). The reason for better bioflocculation property of slime EPS than the other two EPS (capsular EPS and bacterial broth) was, probably due to the fact that slime EPS were long polymeric chain biopolymers compared to the capsular EPS. Slime EPS may also possess more active sites (than capsular EPS and EPS in broth) to bind with the colloidal particles and other microorganisms. Capsular EPS could hinder the efficiency of slime EPS when two EPS are together in broth; the flocculation activity of broth was lower than slime EPS (in supernatant). Moreover, the variation of kaolin flocculating activity using EPS from different isolated bacterial strains, could be due to variation of their chemical characteristics and zeta potential values. However, for better understanding of sludge flocculation, chemical characterization of EPS (i.e., free functional groups, molecular size, overall charge and hydrophobicity) generated by individual bacterial strains, rather than their quantity, should be studied in future.

Concentration of slime EPS extracted from six bacterial strains

Slime EPS concentration, extracted from six pure bacterial strains culture broth, varied from 2.1 to 3.2 g/L (Table 4). This variation was due to different capacity of individual strains to synthesize different amounts of EPS emanating from their different metabolic activities. The concentration of slime EPS extracted from culture broth of bacterial consortium was 2.4 g/L. The concentration of EPS, extracted directly from sludge, was 2 g/L (Table 4). Isolated individual bacterial strains produced up to 3.2 g/L of slime EPS (strain BS25), but when they grew in consortium, they failed to produce an equivalent quantity of EPS.

Sludge Settling

Slime EPS exhibited better kaolin flocculating activity than other type of EPS. Therefore, slime EPS was used to estimate sludge settling and dewatering characteristics.

In all cases, the SVI value was below 150 mL/g (Figure 5a), which is required for a good sludge settling (APHA, 2005). Addition of cations, slightly improved the SVI results, including the control. This phenomenon was due to the reduction of negative surface charge of the sludge particles or compression of electrical double layers surrounding the sludge particles by Ca^{++}, which can reduce the repulsive force hindering the agglomeration between the sludge particles and the EPS. In addition, divalent cations such as Ca^{++}, possessing two positively charged groups on their structure, can act as a bridge to link two negatively charged molecules. This may be either in between the anionic charged EPS and EPS or in between anionic charged EPS and sludge solids and/or microorganisms.

Bacterial strain BS8 displayed the lowest SVI value of 60 mL/g at EPS concentration of 10% v/v [or 0.3 g/L of extracted slime EPS (Table 4)]. The cationic synthetic polymer (CSP), at concentration of 0.01% w/v (0.1 g/L), revealed better sludge settling (SVI of 56 mL/g) than the biopolymers produced from bacterial strains (SVI of >60 to 75 mL/g). Addition of cations with

CSP, the SVI values remained unchanged (56 mL/g). The bacterial strain BS8 revealed almost similar SVI values (54 mL/g) as that of CSP with addition of cations. Thus, biopolymers (biodegradable), produced by pure bacterial strains, were effective in sludge settling at a very low concentration (0.3 g/L).

Sludge dewatering

The CST value was found to decrease substantially (from 158 s in the control to 45 s with EPS from BS15 strain), with the addition of extracted slime EPS to sludge (Figure 5b) in comparison to control. The EPS produced by individual strains was more effective in lowering the CST value than EPS produced by the consortium of bacterial strains. Fortification with Ca^{++} ions, along with individual slime EPS or that produced by the consortium, further improved the sludge dewaterability (lowered the CST value). However, the CST value observed, in all cases, was higher than the CST value required (<20 s), for good sludge dewaterability. The slime EPS, of bacterial strains BS8 (CST value of 48.4 s) and BS15 (CST value of 45 s), displayed the lowest CST value [at EPS concentration of 10% v/v (0.30 for BS8 and 0.21 g/L for BS15)] without cations additon. Fortification of sludge, with EPS of bacterial strains BS8 (CST value of 36.4 s) and BS25 (CST value of 29.4 s), revealed lowest CST values, near the required value for good dewaterability with addition of cations (Figure 5b). Addition of CSP (0.1 g/L) with and without addition of cations revealed a better dewaterability (CST values ~15 s) than the biopolymers. Even though, the EPS requirement is 2-3 times higher (0.30 and 0.21 g/L), to achieve CST values near to that of CSP (0.1 g/L), the slime EPS is biodegradable and nontoxic. Therefore, use of slime EPS could be a possible option to achieve good sludge settling and dewatering. Slime EPS and CSP could be combined, the optimum ratio should be determined, to achieve desired sludge settling and dewatering. This could provide better sludge flocculation, and ultimately reduce the CSP consumption, toxicity (ecofriendly) and cost. These studies are being conducted in our laboratory.

EPS concentration, EPS charge and EPS hydrophobicity are three important factors that govern bioflocculation. Some authors reported that, sludge requires an optimal EPS concentration (Urbain *et al.*, 1993), whereas others reported that sludge requires high EPS concentration (Mikkelsen and Keiding, 2002), for better sludge settling. However, results presented here, based on kaolin flocculation activity, clearly establish that there was an optimum concentration of slime EPS (of each individual strains), to achieve maximum settling and dewatering (Table 2).

The zeta potential (surface charge) values of extracted slime EPS, from selected six bacterial strains, ranged from -54.26 to -35.67 mV (Table 4). The zeta potential of slime EPS, of bacterial strain BS8, was lowest (-54.26 mV). However, BS8 strain demonstrated better kaolin flocculation activity (Table 2) as well as one of the lowest CST value (48.4 s), without addition of Ca^{++} (Figure 5b). Thus, EPS charge is not related to floc formation tendency or sludge dewatering capability, which is concurrent with the reported literature (Eriksson *et al.*, 1992).

In fact, the sludge settling/dewatering (and its relation to properties and concentration of EPS) has been studied (Urbain *et al.*, 1993; Houghton *et al.*, 2001), but most often the results are incoherent and there is no clear explanation. These reported studies were carried out on EPS, produced by consortium of natural sludge microbial flora extracted directly from the fresh sludge, and the extracted EPS in these cases was expected to be a mixture of many EPS (carrying different charges, molecular weights and hence affecting differently the sludge settling and dewaterability), produced by different sludge dwelling microorganisms. However, the present study was conducted employing individual slime EPS (crude or partially purified), produced by isolated individual strains (from sludge) and mixed slime EPS (produced by consortium of similar sludge microorganisms). It is clear, from these results that EPS, produced by a consortium of microorganisms, revealed poor dewaterability (higher CST values) than EPS from individual strains (Figure 5b). Also, these values varied with different individual EPS. Thus, incoherent

results of sludge settling/dewaterability with slime EPS extracted from sludge, as observed by other researchers, should be construed due to the presence of different EPS in different concentration, produced by different strains proliferating in a specific sludge.

In the present laboratory study we used the slime EPS concentration of 10% v/v of sludge. This certainly appears to be a large volume to increase the volume of residual water (after bioflocculation). However, it must be pointed that the actual concentration of slime EPS used in the study was 0.2 to 0.3 g/L (Table 4, Figures 5a, b). Further, this study demonstrates the effectivity of the slime EPS produced for bioflocculation. In real practice a production process of slime EPS using specified bacterial strain(s) should be developed and this is possible through the production process parameter optimization. Sludge could be used as a raw material for the production of EPS. The produced EPS could be concentrated by several available technologies, for example centrifugation or ultrafiltration etc. The concentrated EPS could be used for sludge flocculation. And, thus the volume of residual water after bioflocculation could be reduced.

Slime EPS (partially purified) bio-chemical characteristics

In all cases, except the bacterial consortium, the TC concentration in EPS was higher than TP (Figure 6), and probably played a dominant role in sludge settling compared to the proteins fraction. This fact was also in concurrent with reported literature that polysaccharides (carbohydrates) play a major role in sludge flocculation, due to their capacity to form bridges between their negatively charged groups and the divalent cations available in sludge (Higgins and Novak, 1997a, b). However, in many studies, proteins were found to be the main component for sludge floc formation (Urbain *et al.*, 1993; Hirst *et al.*, 2003). The protein contribution to flocs binding strength, is explained by hydrophobic interactions and polyvalent cation bridging, both enhancing the stability of the biopolymer network (Jorand *et al.*, 1998).

It was found that EPS, produced by pure culture strains, mainly consist of other components (89-94%) such as lipids, DNA and RNA etc (Table 4); and is in agreement with Garnier *et al.* (2005), who found higher concentration of other components in EPS, extracted from sludge of different origins. EPS, produced by pure bacterial strains, possesses higher TC/TP ratio than those produced by the consortium and EPS extracted from sludge (Table 4). Further, the role of these individual compounds, in floc formation and sludge settling, needs to be studied. In addition, the biopolymer composition may change, if produced in sludge and/or different medium, as described above, and may result in different sludge settling and dewatering characteristics.

Therefore, the individual EPS and its major components should be purified (in terms of carbohydrates, proteins and lipids) and subsequently the purified components should be studied for sludge settling and dewatering. This study will explain why EPS of pure bacterial strains lead to better results than those of consortium and sludge extracted EPS. Also, molecular characterization of extracted biopolymers (in terms of their molecular weight, chemical nature and active groups) needs to be further studied. This may result in further improving the bioflocculation process using the purified polymers.

In the future, molecular identification of *eps* genes (genes responsible for synthesis and production of EPS in bacterial strains) should be studied, using DNA probes/specific PCR primers, to identify the *eps* gene possessing organisms. Cloning of *eps* gene, may be carried out, to produce a high quantity of EPS and the required biopolymers, which could help to enhance bioflocculation. Medium composition can also change the charge and composition of the produced EPS. Addition of divalent or trivalent cations, in the production media, could yield/enhance the production of extracellular proteins (in EPS) (Higgins and Novak, 1997a). Similarly, varying the protein and carbohydrate ratio of the medium, one could obtain the desired nature of EPS composition with less negative charge. Since addition of Ca^{++} in combination of individual slime EPS was observed

to improve sludge dewaterability, use of other cations such as alum and $FeCl_3$ should be investigated for its impact on sludge dewaterability.

CONCLUSIONS

Among twenty-five bacterial strains isolated from municipal wastewater sludge, 15 of them were capable of producing capsular EPS. Most of the EPS producing bacterial strains belonged to genus *Bacillus*. The quantity of slime EPS (3.6 - 35.8 g/L) and bacterial broth (5.0 - 36.4 g/L), produced by individual strains, was higher than EPS produced by the consortium of sludge microorganisms. Increase in viscosity of the individual bacterial broths (105-179 mPas), after 3 days fermentation, confirmed EPS production by individual strains. Characterization results, using a zetaphoremeter, revealed that EPS was negatively charged in nature, but it was less negative than the EPS present in fresh sludge. In addition, slime EPS revealed better performance, in terms of kaolin flocculation activity and sludge settling, than capsular EPS and bacterial broth, with and without Ca^{++} addition. Slime EPS, from bacterial strain BS8 (genus *Serratia)*, displayed the best performance on sludge settling. Sludge dewaterability were significantly improved, with addition of extracted slime EPS from individual strains. Addition of cations increased the sludge settling and dewatering capacity. The slime EPS, produced by selected six bacterial strains and consortium of activated sludge microorganisms, were characterized in terms of proteins (0.7-4%) and carbohydrates (4-7%), repectively. Extracted EPS contains major portions (89-94%) of components (lipids, DNA and RNA) other than total carbohydrates and total proteins.

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REFERENCES

APHA. (2005). Standard methods for the examination of water and wastewater. 21st ed. Washington, DC.

Bala Subramanian, S., Yan, S., Lai, K.C.K., Tyagi, R.D., Surampalli, R.Y., 2006. Screening of Extracellular Polymeric Substances (EPS) Producing Microorganisms for Sludge Settling. *IWA Specialized Conference - Sustainable Sludge Management: state of the art, challenges and perspectives*, May 29-31, 2006; Crocus Expo Exhibition Center, Moscow, Russia, 188-195.

Bala Subramanian, S., Yan, S., Tyagi, R.D., Surampalli, R.Y., 2008a. A New Pellet Forming Fungal Strain: Its Isolation, Molecular Identification, and Performance for Simultaneous Sludge Solids Reduction, Flocculation, and Dewatering. Water Environ Res. 80 (9), 840-852.

Bala Subramanian, S., Yan, S., Tyagi, R.D., Surampalli, R.Y., Lohani, B.N., 2008b. Isolation and Molecular Identification of Extracellular Polymeric Substances (EPS) Producing Bacterial Strains for Sludge Settling and Dewatering. J Environ Sci Heal-A. 43(13), 1495-1503.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(1-2), 248-254.

Cain, D., Hanks, H., Weis, M., Bottoms, C., and Lawson, J. (2009) Microbiology Laboratory Manual. Collin County Community College District, McKinney, TX.

Chang, L.L., Bruch, D.M., Griskowitz, N.J., Dentel, S.K. 2002. NMR spectroscopy for determination of cationic polymer concentrations. Water Res. 36, 2255–2264.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal Chem. 28, 350-356.

Eriksson, L., Steen, I., Tendaj, M., 1992. Evaluation of sludge properties at an activated sludge plant. Water Sci Technol. 25, 251-265.

Gagnon, B., Ziadi, N., 2004. Value of paper mill sludge in agriculture: Crop yield, soil properties, and environmental impacts. Rec Res Develop Crop Sci. 1(1), 1-10.

Gao, J., Ying, B.H., Xiu, X.M., Xia, L.Y., Qian, L., Fen, Z.Y., 2006. Characterization of a bioflocculant from a newly isolated Vagococcus sp. W31. J. Zhejiang Univ. Science B. *7*(3), 186–192.

Garnier, C., Gorner, T., Lartiges, B.S., Abdelouhab, S., Donato, P., 2005. Characterization of activated sludge exopolymers from various origins: A combined size-exclusion chromatography and infrared microscopy study. Water Res. 39, 3044–3054.

Hébert, M., 2004. Valorisation des boues municipales comme matières fertilisantes au Québec, Vecteur Environnement. 37 (5), 38–41.

Higgins, M.J., Novak, J.T., 1997a. Dewatering and settling of activated sludge: The case for using cation analysis. Water Environ Res. 69, 225–232.

Higgins, M.J., Novak, J.T., 1997b. Characterization of Exocellular Protein and Its Role in Bioflocculation. J. Environ. Eng. 123, 479-485.

Hirst, C.N., Cyr, H., Jordan, I.A., 2003. Distribution of Exopolymeric Substances in the Littoral Sediments of an Oligotrophic Lake. Microb Ecol. 46, 22–32.

Houghton, J.I., Quarmby, J., Stephenson, T., 2001. Municipal wastewater sludge dewaterability and the presence of microbial extracellular polymer, Water Sci Technol. 44(2-3), 373-379.

Houghton, J.I., Stephenson, T., 2002. Effcte of influent organic content on the digested sludge extracellular polymer content and dewaterability. Water Res. 36, 3620-3628.

Jorand, F., Boué-Bigné, F., Block, J.C., Urbain, V., 1998. Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. Water Sci Technol. 37, 307–315.

Kurane, R., Takeda, K., Suzuki, T., 1986. Screening and characteristics of microbial flocculants. Agric. Biol. Chem. 50, 2301-2307.

Leblanc, R.J., 2005. The need for the establishment of Canadian biosolids partnerships. Environmental Science & Engineering. On line: <htpp://www.esemag.com> – July 2005.

Martins, A.M.P., Pagilla, K., Heijnen, J.J., Van Loosdrecht, M.C.M., 2004. Filamentous bulking sludge – a critical review. Water Res. 38, 793-817.

Mikkelsen, L.H., Keiding, K., 2002. Physico-chemical characteristics of full scale sewage sludges with implications to dewatering. Water Res. 36(10), 2451–2462.

Neu, T. R. 2000. In situ cell and glycoconjugate distribution in river snow studied by confocal laser scanning microscopy. Aquat Microb Ecol. 21: 85-95.

Novak, J.T., Muller, C. D., Murthy, S.N., 2001. Floc structure and role of cations, Water Sci Technol. 44, 209-213.

Sobeck, D.C., Higgins, M.J. 2002. Examination of Three Theories for Mechanisms of Cation-Induced Bioflocculation. Water Res. 36, 527-538.

Urbain, V., Block, J.C., Manem, J., 1993. Bioflocculation in activated sludge: An analytical approach, Water Res. 27, 829-838.

US.EPA (Environmental Protection Agency), 1999. EPA Biosolids Generation and Uses, EPA 530R-99-009, September 1999.

Vesilind, P. A., and Örmeci, B., 2000. A modified capillary suction time apparatus for measuring the filterability of super-flocculated sludges. Water Sci Technol. 42 (9), 135-139.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., 2002. Studies on the production of *B. thuringiensis* based biopesticides using wastewater sludge as raw material. Water Res. 36 (19), 4850-4860.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 173(2), 697-703.

Widmer, F., Seidler, R.J., Gillevet, P.M., Watrud, L.S., Di Giovanni, G.D. 1998. A highly selective PCR protocol for detecting 16S rRNA gene of the genus *Pseudomonas* (Sensu stritcto) in environmental samples. Appl Environ Microbiol. 64 (7), 2545-2453.

Zhang, X., Bishop, P.L., Kinkle, B.K., 1999. Comparison of extraction methods for quantifying extracellular polymers in biofilms. Wat Sci Tech. 39 (7), 211–218.

Zhang, J., Wang, R., Ziang, P., Liu, Z., 2002. Production of an exopolysaccharides bioflocculant by So*rangium cellulosum.* Lett in Appl. Microbiol. 34, 178-181.

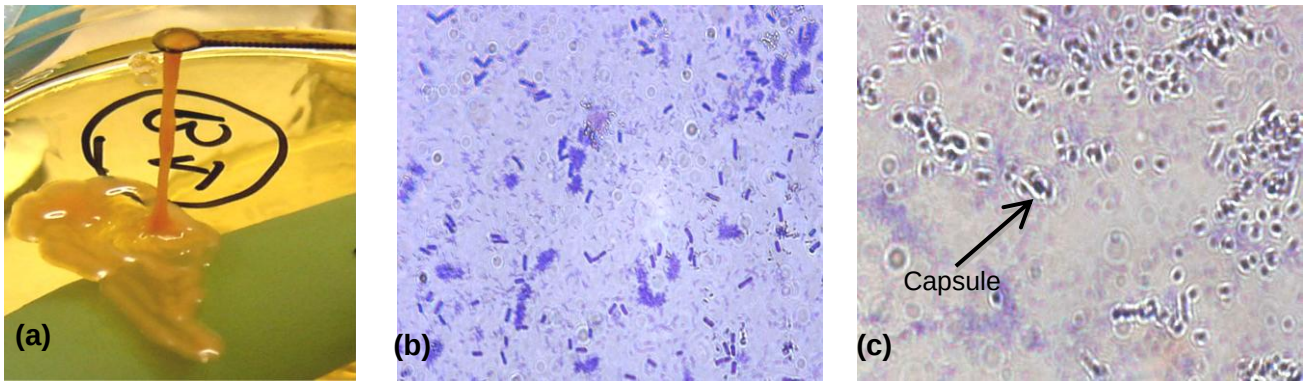

Figure 1 : (a) Slime EPS producing bacterial strain (BS1) showing string of secreted biopolymer on PCA agar plate, **(b)** Capsular staining results revealed the absence of cellular bound EPS in BS1, **(c)** and the presence of capsular EPS in BS2.

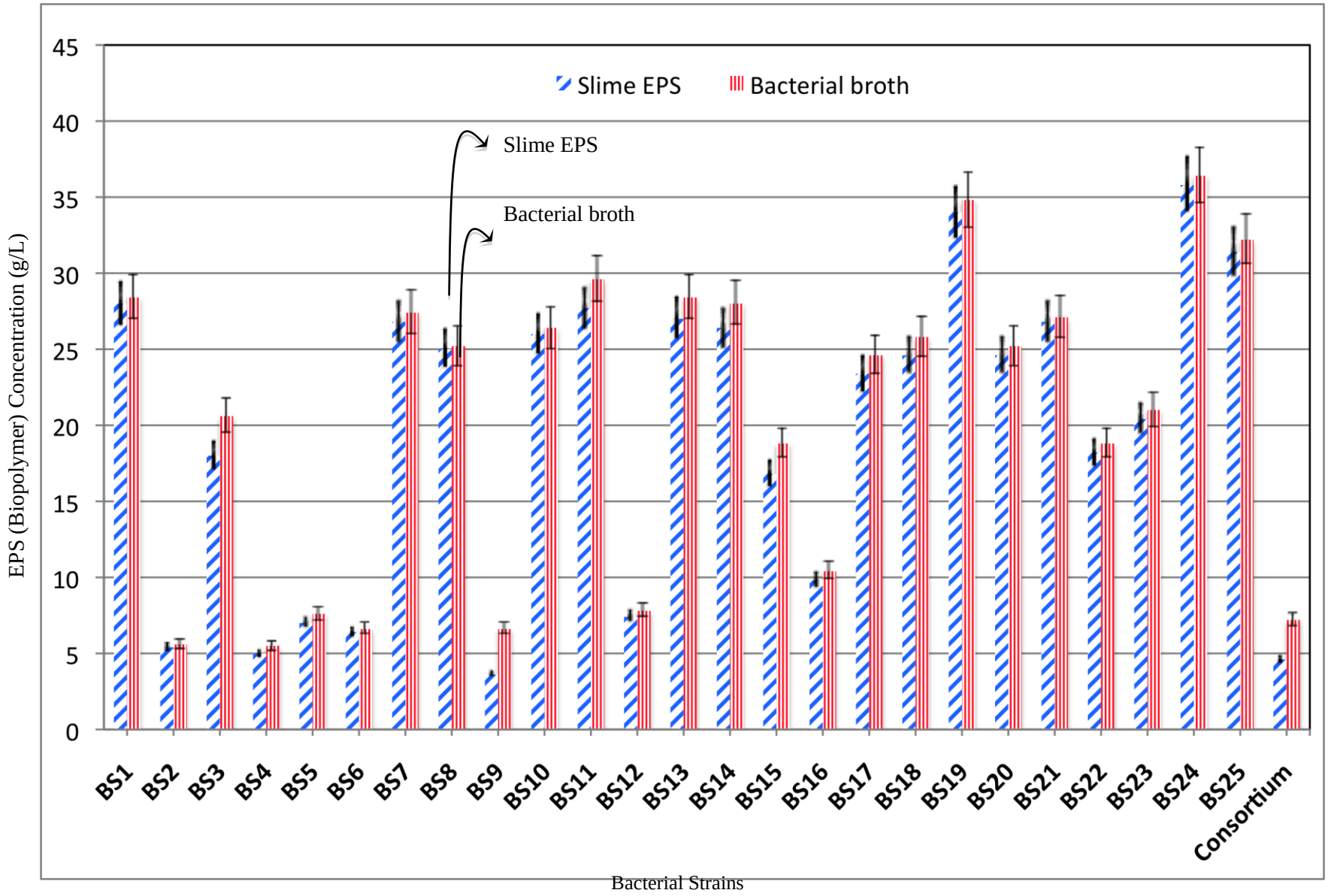

Figure 2: EPS concentrations produced by 25 bacterial strains and consortium at the end of 3 days of fermentation at initial pH 7.0.

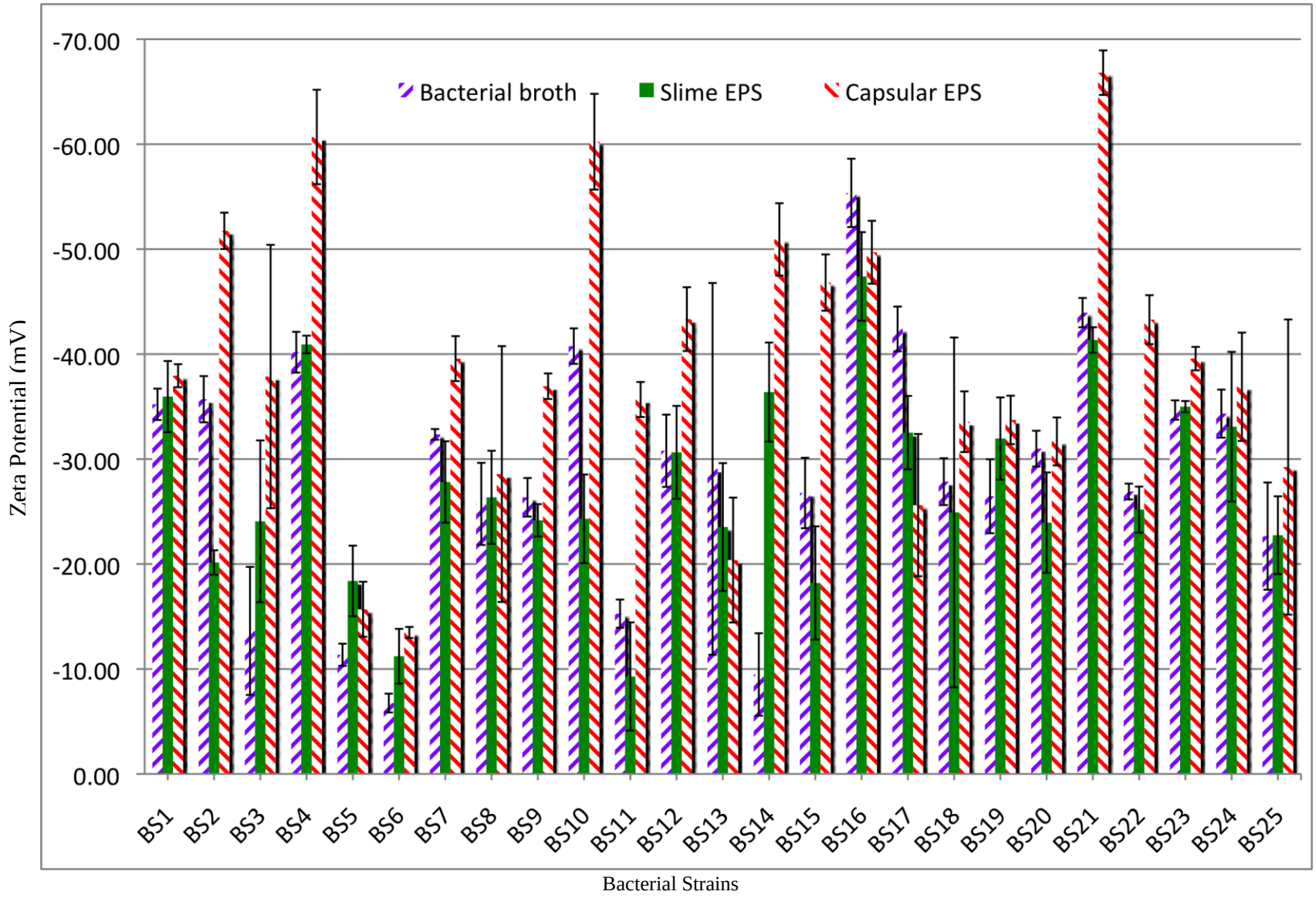

Figure 3: Zeta potential values of slime EPS, capsular EPS and bacterial broth produced by 25 bacterial strains at initial pH 7.0.

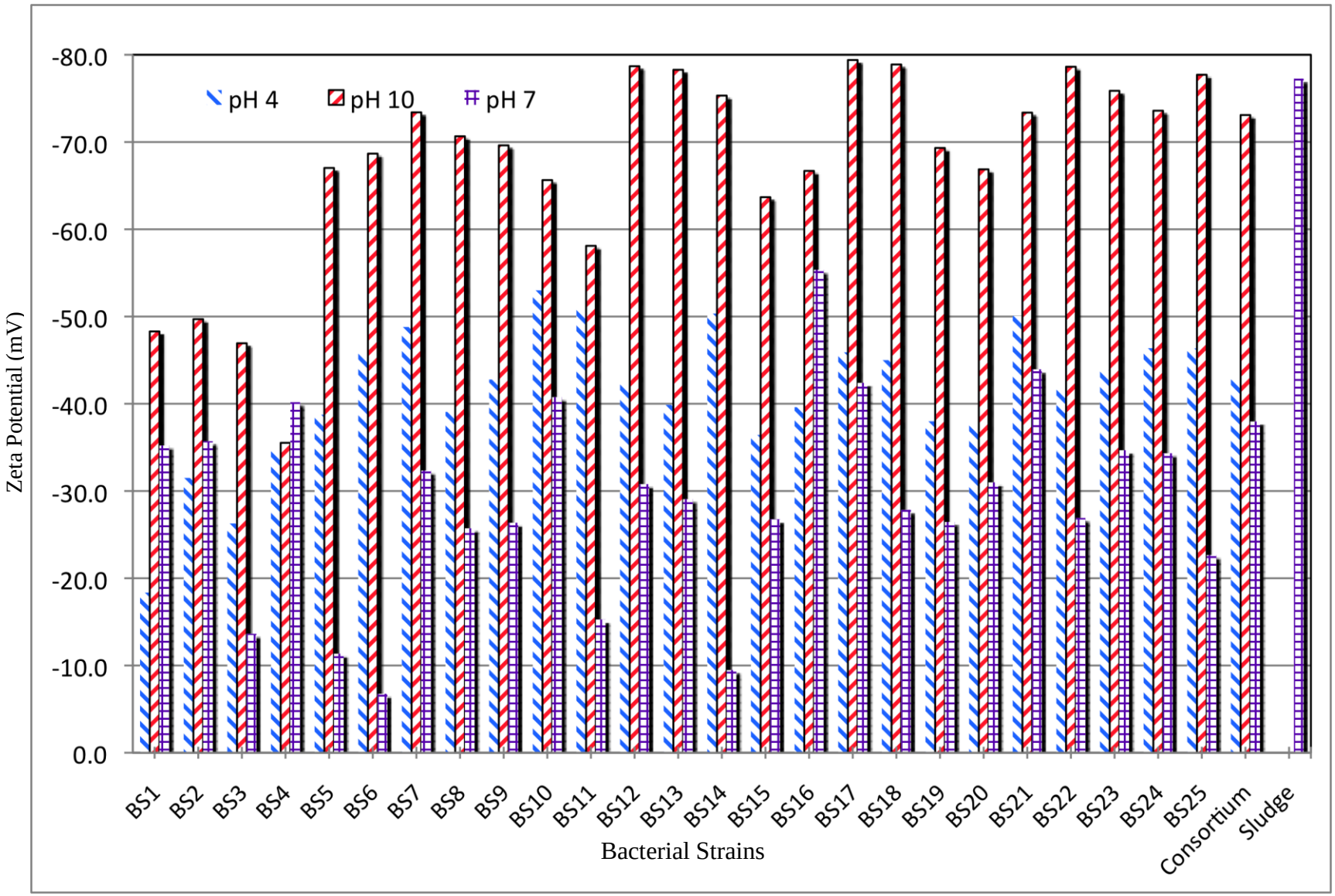

Figure 4: Zeta potential values of bacterial broth obtained from 25 bacterial strains and consortium at different pH (4, 7 & 10).

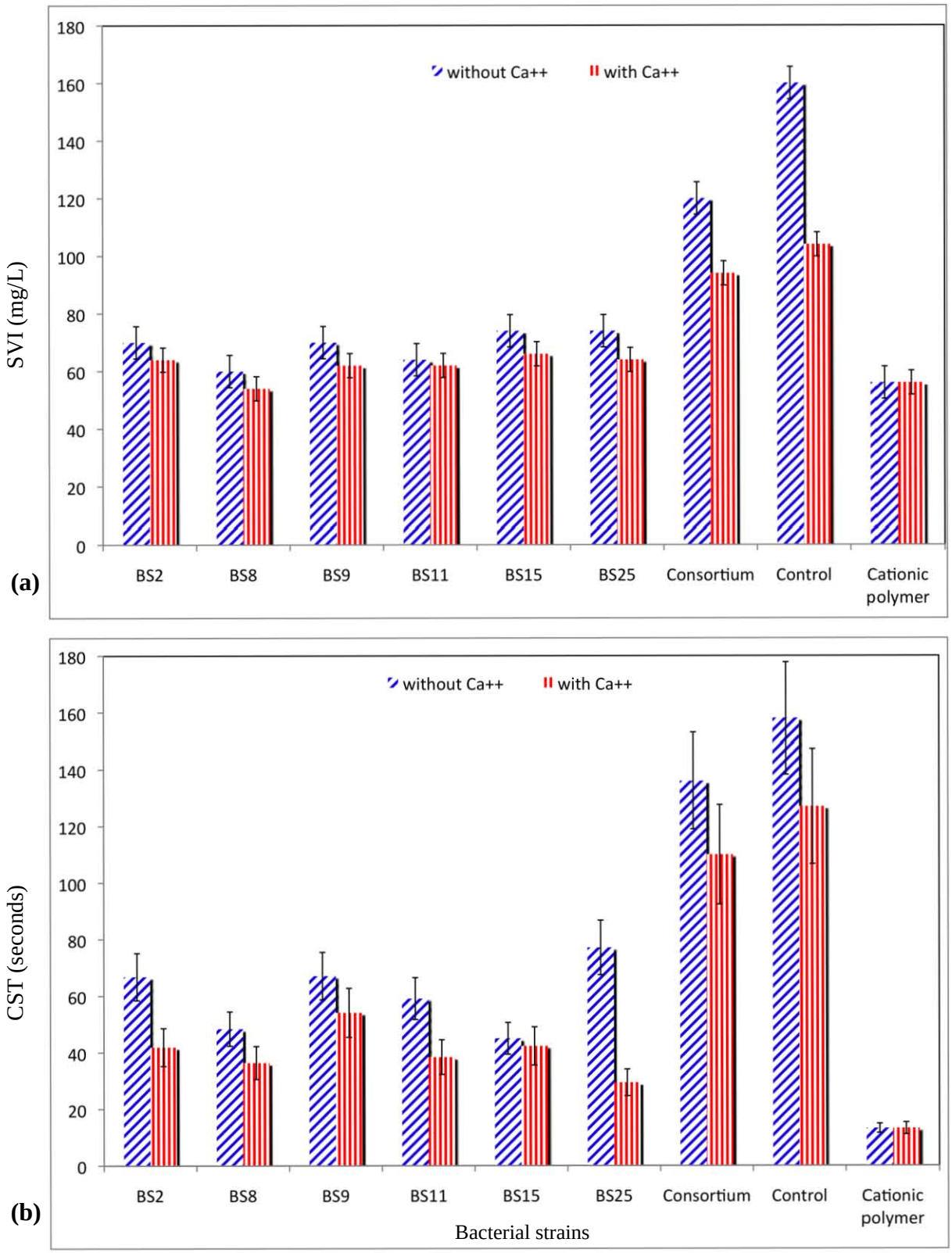

Figure 5: Sludge settling and dewatering efficiency of slime EPS [10 % V/V - (0.23, 0.30, 0.24, 0.25, 0.21 0.32 and 0.24 g/L)] of bacterial strains BS2, BS8, BS9, BS11, BS15, BS25 and consortium, respectively with and without cations. (a) Sludge volume index, and (b) Capillary suction time.

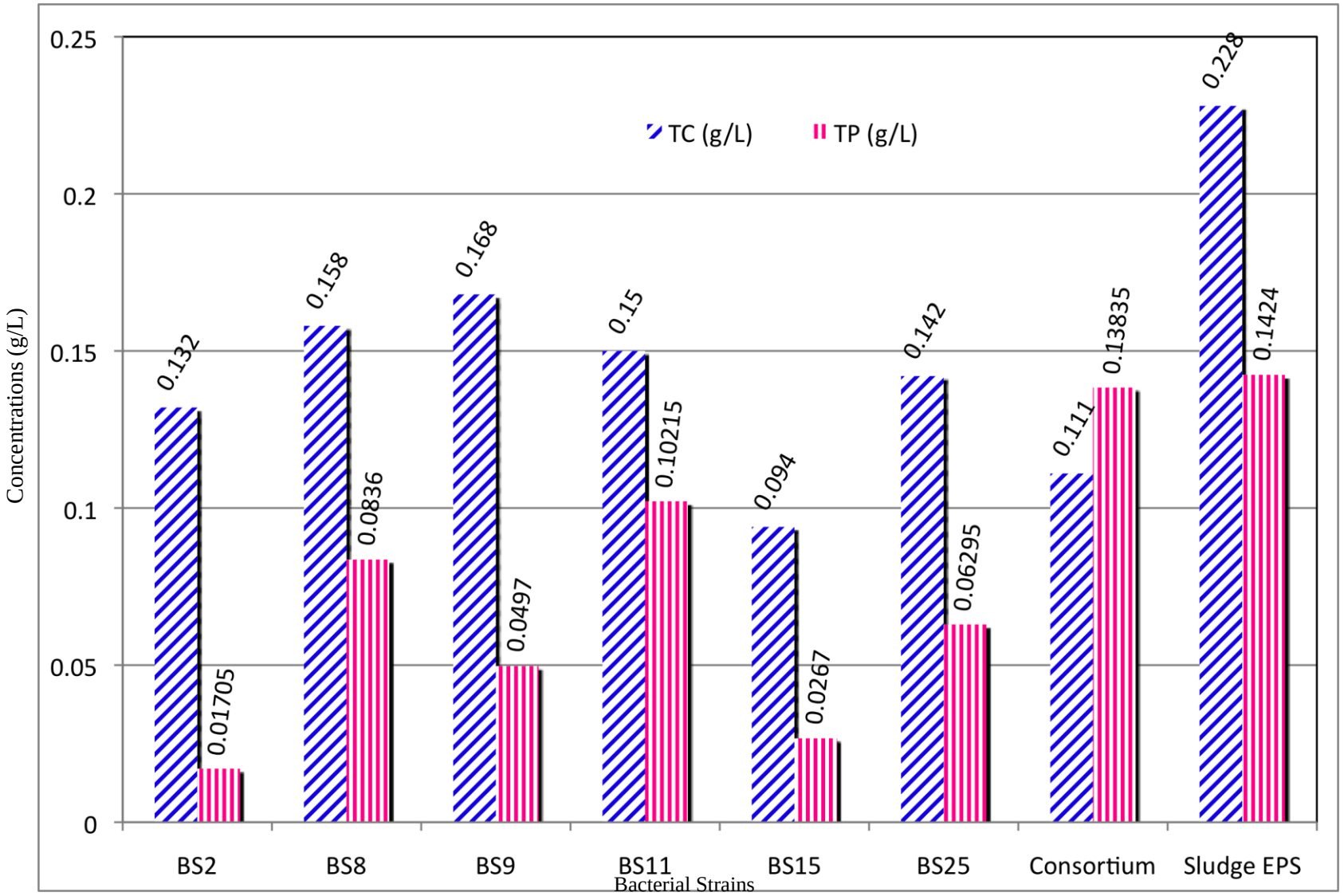

Figure 6: Extracted slime EPS total carbohydrates (TC) and total protein (TP) concentrations from six selected pure bacterial strains, consortium and fresh sludge.

Table 1: Molecular identification and microbiological characterization of EPS producing bacterial strains isolated from municipal wastewater sludge. Viscosity of various bacterial broth after 3 day of fermentation.

S. No	Bacterial strains code	Staining results			Bacterial Identification results	Accession number	Viscocity (mPas) Bacterial broth
		Slime EPS	Capsular EPS	Gram's			
1	BS1	+	–	Negative	*Pseudomonas* strain BS1	EU031751	147
2	BS2	+	+	Negative	*Pseudomonas* strain BS2	EU031752	149
3	BS3	+	+	Positive	*Bacillus* strain BS3	EU031753	179
4	BS4	+	+	Positive	*Bacillus* strain BS4	EU031754	134
5	BS5	+	+	Positive	*Bacillus* strain BS5	EU031755	169
6	BS6	+	+	Positive	*Bacillus* strain BS6	EU031756	179
7	BS7	+	–	Negative	*Pantoea* strain BS7	EU031757	131
8	BS8	+	+	Negative	*Serratia* strain BS8	EU031758	118
9	BS9	+	+	Positive	*Bacillus* strain BS9	EU031759	134
10	BS10	+	+	Positive	*Bacillus* strain BS10	EU031760	164
11	BS11	+	–	Negative	*Yersinia* strain BS11	EU031761	145
12	BS12	+	+	Positive	*Bacillus* strain BS12	EU031762	159
13	BS13	+	–	Negative	*Yersinia* strain BS13	EU031763	160
14	BS14	+	+	Positive	*Bacillus* strain BS14	EU031764	171
15	BS15	+	–	Positive	*Microbacterium* strain BS15	EU031765	122
16	BS16	+	+	Positive	*Bacillus* strain BS16	EU031766	132
17	BS17	+	+	Positive	*Bacillus* strain BS17	EU031767	147
18	BS18	+	+	Negative	*Serratia* strain BS18	EU031768	155
19	BS19	+	+	Positive	*Bacillus* strain BS19	EU031769	125
20	BS20	+	–	Negative	*Enterobacter* sp. BS20	EU017381	110
21	BS21	+	–	Negative	*Photorhabdus* strain BS21	FJ604109	135
22	BS22	+	–	Positive	*Microbacterium* strain BS22	EU031770	149
23	BS23	+	–	Negative	*Pectobacterium* strain BS23	EU031771	173
24	BS24	+	+	Negative	*Serratia* strain BS24	EU031772	120
25	BS25	+	–	Negative	*Enterobacter* sp. BS25	EU017383	105
26	Consortium						160
27	Synthetic media						100
28	Water						100

Table 2: Kaolin clay flocculation activity obtained using 3 different EPS without cations addition.

Bacterial strains	% of kaolin clay flocculation using various concentration (g/L) of EPS (without Ca^{++})														
	Slime EPS (g/L)					Bacterial broth (g/L)					Capsular EPS (mg/L)				
	0.5 g	1 g	2 g	3 g	5 g	0.5 g	1 g	2 g	3 g	5 g	0.5 mg	1 mg	2 mg	3 mg	5 mg
BS1	15.8	18.1	22.6	24.3	26.0	42.9	42.9	48.5	57.2	57.2	52.3	54.5	61	61	57
BS2	72.7	75.5	78.0	78.0	76.0	64.1	64.1	64.1	66.2	66.3	52.1	56.8	65.4	65.4	55
BS3	33.1	34.8	35.0	36.5	36.5	42.9	50.1	57.2	54.1	49.2	52.3	54.3	59.5	59.5	62.8
BS4	50.4	49.5	51.3	52.1	49.9	28.7	31.5	31.5	28.7	24.4	31.2	49.1	45.2	45.2	46.9
BS5	14.3	17.3	21.5	28.6	37.6	21.3	21.4	21.5	35.8	35.8	48.8	53.4	43.2	43.2	23.5
BS6	23.5	33.1	39.1	39.9	41.0	35.8	35.8	22.0	22.0	25.8	17.1	20.1	36.1	36.1	22.6
BS7	62.6	64.7	69.9	71.4	68.9	57.2	42.9	35.0	34.1	27.3	44.5	53.6	52.3	52.3	36.2
BS8	74.6	73.8	78.1	77.4	70.0	52.7	62.9	65.3	66.0	56.9	63.6	64.6	71.3	74.4	63.2
BS9	75.2	78.2	76.4	74.7	68.1	48.4	51.8	56.9	56.9	49.8	54.7	57.4	66.6	66.6	27.7
BS10	24.5	28.1	34.4	33.8	31.3	35.5	31.3	27.0	27.0	25.3	13.2	15.4	16.8	16.8	26
BS11	75.4	76.7	81.2	83.7	80.9	45.5	56.9	64.1	68.3	68.2	64.7	61.9	60.9	60.9	53
BS12	39.1	42.1	44.4	47.6	46.3	57.2	42.9	41.2	42.9	45.0	12.7	17.9	12.6	12.6	12.4
BS13	47.4	47.4	51.1	53.9	55.1	22.9	28.7	25.0	21.3	22.7	33.3	36.1	54.9	54.9	51.5
BS14	59.4	60.9	62.7	64.3	64.3	34.1	38.4	42.5	42.7	42.7	29.9	42.5	45	45	49.3
BS15	71.4	74.4	81.2	78.4	75.4	56.9	56.9	52.8	49.8	42.7	43.7	54.3	68.3	68.3	56.8
BS16	33.0	43.4	47.2	48.3	48.6	37.3	37.0	34.1	37.7	32.3	37.5	38.7	45.1	45.1	41.5
BS17	28.8	27.1	27.5	24.3	23.3	50.1	50.1	42.9	35.8	35.8	24.9	25.3	27.3	28.4	13.6
BS18	42.1	50.4	56.9	56.9	55.2	43.3	44.4	42.4	38.7	36.7	31.3	28.7	19.8	19.8	15.8
BS19	63.9	61.6	59.1	54.6	52.6	35.5	49.8	42.7	28.4	28.4	17.2	22	12.5	12.5	3.9
BS20	42.9	44.4	52.6	56.4	60.9	28.7	14.4	17.3	17.3	15.7	41.8	49.4	53.1	50.1	21.4
BS21	25.3	29.3	26.3	25.3	26.0	28.7	24.3	21.5	21.5	28.7	41.2	45.1	55	54.9	43.3
BS22	54.9	57.5	58.6	58.9	56.7	49.8	49.8	35.5	21.3	14.1	28.9	35.8	45.1	43.4	41
BS23	49.4	44.7	43.2	43.7	42.9	14.4	18.0	24.0	25.0	27.0	49.6	53.6	61.3	64.4	43.2
BS24	68.4	65.4	63.2	63.2	56.4	31.3	34.4	38.5	42.7	42.0	45.2	56.1	50.6	45.1	45.3
BS25	63.9	68.9	71.1	73.3	76.1	50.5	56.9	60.5	64.1	51.3	64.1	68.5	64	64	41.1
Consortium	65.0	63.0	66.0	66.0	64.0	28.7	25.0	27.0	27.3	24.2	53.9	56.3	56.3	56.3	52.1

Table 3: Kaolin clay flocculation activity obtained using 3 different EPS with cations addition.

Bacterial strains	% of kaolin clay flocculation using various concentration (g/L) of EPS (with Ca^{++})														
	Slime EPS (g/L)					Bacterial broth (g/L)					Capsular EPS (mg/L)				
	0.5 g	1 g	2 g	3 g	5 g	0.5 g	1 g	2 g	3 g	5 g	0.5 mg	1 mg	2 mg	3 mg	5 mg
BS1	17.8	19.1	25.6	25.3	28.0	19.8	20.3	13.1	13.2	11.4	57.3	59.5	63	63	59
BS2	72.7	75.5	81.0	82.3	78.0	61.3	62.4	69.8	68.8	66.8	58.1	62.8	70.4	70.4	59
BS3	42.1	50.4	56.9	56.9	55.2	45.4	47.5	49.8	52.0	49.7	57.3	59.3	63.5	63.5	63.8
BS4	63.9	61.6	59.1	54.6	52.6	26.3	28.2	34.4	33.5	34.7	36.2	55.1	49.2	49.2	51.9
BS5	42.9	44.4	52.6	56.4	60.9	27.3	34.0	37.6	48.0	46.0	51.7	55.3	47.2	47.2	29.5
BS6	25.3	29.3	26.3	25.3	26.0	39.0	38.6	49.4	50.8	52.6	23	28.1	41	41	29.6
BS7	63.6	64.7	70.9	73.4	71.9	57.3	50.0	46.5	46.5	49.0	47.5	57.1	57.3	57.3	46.2
BS8	77.6	76.8	79.1	81.4	76.0	57.5	61.2	66.0	67.4	64.6	67.6	69.6	75.3	75.3	67.3
BS9	77.2	79.2	79.9	81.7	78.1	59.7	64.3	63.4	61.2	62.7	57.7	59.4	69.3	69.3	47.7
BS10	25.5	29.1	33.4	32.8	32.3	33.6	31.5	32.7	34.4	36.3	15.2	17.2	19.8	19.8	29.1
BS11	76.4	77.7	84.2	85.7	78.9	51.7	52.9	59.5	65.8	59.1	67.6	64.3	64.9	64.9	56.2
BS12	41.2	43.8	45.4	48.6	45.3	45.4	47.6	43.9	42.6	41.2	15.9	19.9	15	15	15.7
BS13	48.4	49.4	54.1	54.9	56.1	45.0	28.4	16.4	18.3	20.3	35.3	39.1	57.3	57.3	54.3
BS14	62.4	62.9	64.7	64.3	65.3	16.2	17.3	18.1	18.1	26.8	31	45.5	47	47	51
BS15	73.4	76.4	84.2	84.4	77.4	60.5	57.8	53.6	50.9	43.6	44.5	56.3	70.3	70.3	59.3
BS16	33.1	34.8	35.0	36.5	36.5	37.5	39.0	42.7	47.8	48.6	39.5	41.7	47.1	47.1	43.6
BS17	50.4	49.5	51.3	52.1	49.9	49.5	55.8	53.5	55.2	59.8	27.9	27.3	29.3	31.4	15.8
BS18	42.1	53.4	57.9	58.9	55.2	46.7	48.9	44.3	39.6	38.3	24.3	24.7	21.8	21.8	17.4
BS19	63.9	62.6	65.1	66.6	62.6	45.7	46.3	54.6	49.5	46.2	19.2	23	17.5	17.5	11.4
BS20	42.9	47.4	54.6	57.4	62.9	38.2	37.5	43.6	41.3	25.5	42.8	51.2	54.1	51.9	45.5
BS21	25.3	31.2	32.3	35.2	28.3	26.3	25.1	26.8	24.1	23.5	44.3	67.2	57	57	47
BS22	54.9	59.3	60.6	61.3	59.3	48.9	42.3	42.8	41.7	40.4	31.9	36.8	47.1	46.4	43.2
BS23	51.0	45.0	46.3	45.5	43.4	22.7	21.8	29.4	28.3	29.6	51.6	55.6	64.3	67.5	51.2
BS24	66.4	66.4	64.2	65.2	61.4	41.7	42.3	44.4	46.8	54.0	48.2	57.1	53.1	47.1	47
BS25	67.9	70.9	75.1	76.3	75.1	52.5	58.9	63.5	66.1	65.3	67.2	71.5	66	66	56
Consortium	69.0	73.0	76.0	76.0	74.0	40.7	39.3	39.0	37.5	33.8	55.9	60	60	59.4	57

Table 4. Partial characterization of extracted/purified slime EPS from six selected pure bacterial strains, consortium and sludge (EPS concentrations, zeta potential value, TC (%), TP (%) and TC/TP ratio).

EPS source	Dry weight (g/L)	(10% v/v) EPS* (g/L)	Zeta potential (mV)	Chemical characteristics			
				TC/TP ratio	TC (%)	TP (%)	Other components (%)
BS2	2.3	0.23	-36.68	7.740	5.739	0.741	93.5
BS8	3.0	0.30	-54.26	1.890	5.267	2.787	91.9
BS9	2.4	0.24	-38.75	3.380	7.000	2.071	90.9
BS11	2.5	0.25	-45.46	1.468	6.000	4.086	89.9
BS15	2.1	0.21	-36.86	3.520	4.476	1.271	94.2
BS25	3.2	0.32	-35.67	2.255	4.438	1.967	93.6
Consortium	2.4	0.24	-37.97	0.802	4.625	5.765	89.6
Sludge EPS	2.0	0.20	-35.00	1.601	11.400	7.120	81.5

* - Slime EPS concentration used in SVI and CST studies.

PART III

16S RIBOSOMAL RNA GENE SEQUENCE ANALYSIS OF EPS PRODUCING MICROBIAL COMMUNITY ISOLATED FROM MUNICIPAL WASTEWATER SLUDGE

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ABSTRACT

The EPS-producing bacterial community structure of municipal wastewater sludge from CUQ was studied using 16S rRNA gene sequence analysis. The 16S rDNA sequence data were subjected to a BLASTn search. The 16S rDNA homology search results of BS1-BS25 bacterial strain resembling 96-99% with existing DNA sequence database were found. Based on 16S rDNA sequence homology, the bacterial strains were identified. It was found that out of twenty five bacterial strains, eleven bacterial strains belonged to *Bacillus* genus, three were *Serratia*, two were *Pseudomonas*, two were *Enterobacter*, two were *Yersinia*, two were *Microbacterium* and there was one strain of each genus *Pantoea*, *Photorhabdus* and *Pectobacterium*. The obtained gene sequences were further analyzed using bioinformatics software to understand the similarity among the isolated EPS producing microbial community and within their genus level. Phylogenetic tree, secondary structure of RNA and multiple sequence alignment among the same genus were obtained. The bioinformatics analysis revealed that isolated 25 bacterial strains are significantly different from each other. Further genus level gene sequence analysis bacterial strains revealed that gene sequences were significantly different from each other. This confirmed the importance of molecular identification of bacterial strains and the necessity of gene sequences for further classification and characterization of microbial strains. This method of 16S rRNA gene sequencing analysis of EPS producing microbial strains in activated sludge could provide a regular tool to obtain meaningful and quantitative information.

INTRODUCTION

The rrn operon or rDNA sequence is known to encode for 16S, 23S and 5S rRNA (Guasp *et al.*, 2000). The 16S rRNA molecule is having a chain length of approximately 1542 nucleotide bases (Achouak *et al.*, 2000; Anzai *et al.*, 2000; Rainey *et al.*, 1998). It is reported that this 16S rRNA folds into a four-domain cloverleaf structure in all organisms (Anzai *et al.*, 1997; Ochi, 1995; Seal *et al.*, 1993; Tan and Chen, 1997) and also shows highly conserved regions for a particular genus (Sahin *et al.*, 2000; Lee *et al.*, 2000; Yoon and Park, 2000). Therefore, the 16S rrn operon showing conserved region has been selected for the molecular identification of EPS producing bacterial strains.

Total microbial community analysis from waste activated sludge, upflow sludge anaerobic blanket and a conventional anaerobic digestion process was analysed in detail (Snaidr *et al.*, 1997; Liu *et al.*, 2005; Kurisu *et al.*, 2002; Zhang *et al.*, 2005; Kees *et al.*, 2005). But, gene sequences of the EPS producing microbial community from a particular wastewater sludge have not been analyzed in detail. Therefore, in this study EPS producing microbial strains gene sequences were analyzed using bioinformatic software to obtain valuable informations from their gene sequences.

MATERIALS AND METHODS

Sampling

Wastewater sludge samples were collected from Communauté Urbaine du Québec (CUQ, QC). Microbial isolation was attempted on plate count agar (PCA) and Sabouraud's dextrose agar (SDA) media using serial dilution techniques. Plates were incubated at 25°C for 48–72 h. Isolated bacterial strains were named BS1 to BS25. These EPS producing microorganisms were selected

based on their slime EPS and capsular EPS production. Isolated bacterial strains were identified using 16S rRNA gene sequencing.

DNA extraction

DNA was extracted from all 25 EPS producing bacterial strains (BS1 to BS25) isolated from municipal wastewater sludge. Genomic DNA isolation procedure was used as described by Bala Subramanian *et al.* (2008).

Polymerase chain reaction (PCR)

The extracted genomic DNA from 25 different EPS producing bacterial strains isolated from municipal wastewater sludge (BS1 to BS25) was used for direct amplification of 16S rRNA gene portions (Widmer *et al.,* 1998). Universal primers were used to amplify the full length 16S rRNA gene from rrn operons of 25 bacterial isolates (Weisburg *et al.,* 1991).

Gene sequencing

DNA sequencing was carried out using a DNA sequencer (ABI 3730xl DNA Analyzer, Applied biosystems, USA). The obtained 16S rDNA sequences were aligned using DNASIS bioinformatics software (Hitachi Software Japan, Tokyo). 16S rDNA sequences were blasted into the internet (http://www.ncbi.nlm.nih.gov/BLAST/) for a similarity search.

Nucleotide sequence accession numbers

EPS producing bacterial strains partial 16S rRNA gene sequences were submitted to GenBank, USA. The accession numbers were as follows EU031751 – EU031772, EU017381, EU017383 and FJ604109.

Sequence analysis

Using the gene sequence information, the multiple alignment of gene sequences, phylogenetic tree (neighbourhood joining method), pairwise comparison and RNA secondary structures were predicted using CLC Bio software, version 5.1.

RESULTS AND DISCUSSION

DNA characteristics

Isolated bacterial genomic DNA was visualized in agarose gel (0.7%) electrophoresis and compared with known molecular weight markers. All bacterial genomic DNA bands were seen $\geq$ 23 kb marker band (data not shown). Genomic DNA was gel purified and used for the direct PCR amplification of 16S rRNA gene portion (Figure 1). The obtained amplicons were sequenced and bacterial strains were identified; the gene sequences are presented in Table 1.

The molecular identification of bacterial strains was carried out based on 16S rDNA gene sequences, which revealed 97-99% homology with the existing gene sequences. Based on this the microbial strains were identified and the phylogenetic tree was drawn, only six selected EPS (BS2, BS8, BS9, BS11, BS15, BS25) producing bacterial strains dendrograms are shown in Figures 2a to 2f. Phylogenetic tree produced based on rrn operon gene sequences of bacterial strains using the basic local alignment search tool (BLAST) pairwise alignments and neighbor-joining method. Sequences with less than 0.05 difference were used.

The similarity analysis among all 25 EPS producing bacterial trains was carried out by drawing a dendrogram using the neighbourhood joinging method. Multiple alignment of gene sequences was done followed by aligned sequences which was treated for phylogenetic tree construction (Figure 3), which revealed the relatedness among the microbial strains.

The gene sequences similarities and differences are clearly indicated in Table 2. All the 11 *Bacillus* strains showed significant relatedness among them compared to other bacterial genus. Similarly, other bacterial strains belonging to different strains were indicated distant from other strains.

The predicted RNA secondary structures obtained from the partial gene sequences of bacterial strains revealed the unique folding patterns of each bacterial strains (Figure 4). This confirmed that each strain is significantly different from each other.

The multiple alignment of gene sequences was conducted for all bacterial strains belonging to the same genus (within 11 *Bacillus*, 3 *Serratia*, 2 *Pseudomonas*, 2 *Enterobacter* and 2 *Yersinia*) isolated from wastewater sludge. Though the isolated strains belong to the same genus they are significantly different from each by their gene sequence analysis (Figures 5-9). Therefore, the isolated strains produced a different concentration, charge and characteristics of EPS, and displayed different patterns in kaolin flocculation, sludge settling and dewatering.

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REFERENCE

Achouak, W., L. Sutra, T. Heulin, J. M. Meyer, N. Fromin, S. Degraeve, R. Christen, and L. Gardan. 2000. *Pseudomonas* brassicacearum sp. nov.and *Pseudomonas* thivervalensis sp. nov; two root associated bacteria isolated from Brassica napus and Arabidopsis thaliana. Int. J. Syst. Evol. Microbiol. Jan; 50 Pt 1: 9-18.

Anzai, Y, Y. Kudo, and H. Oyaizu. 1997. The phylogeny of the genera chryseomonas, flavimonas and *Pseudomonas* supports synonymy of these three genera. Int. J. Syst. Bacteriol. Apr; 47(2): 249-251.

Anzai, Y., H. Kim, J. Y. Park, H. Wakabayashi, and H. Oyaizu. 2000. Phylogenetic affiliation of the Pseudomonads based on 16S rRNA sequence. Int. J. Syst. Evol. Microbiol. Jul; 50 Pt 4: 1563-1589.

Bala Subramanian S, S. Yan, R. D. Tyagi, R. Y. Surampalli and BN Lohani. 2008b. Isolation and Molecular Identification of Extracellular Polymeric Substances (EPS) Producing Bacterial Strains for Sludge Settling and Dewatering. *Journal of Environmental Science and Health, Part* A, 43(13): 1495-1503.

Guasp, C., E. R. Moore, J. Lalucat, and A. Bennasar. 2000. Utility of internally transcribed 16S-23S rRNA spacer regions for the definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species. Int J Syst Evol. Microbiol. Jul; 50 Pt 4: 1629-1639.

Kees, R., Hans G. H. J., Hauke, S., Willem M.D., Alfons J. M.S., Antoon D. L.A. (2005) Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater. Systematic and applied microbiology. 28(2): 175-185.

Kurisu, F., Satoh, H., Mino, T., and Matsuo, T. (2002) Microbial community analysis of thermophilic contact oxidation process by using ribosomal RNA approaches and the quinone profile method. Water Research. 36 (2): 429-438.

Lee, S. D., E. S. Kim, and Y. C. Hah. 2000. Phylogenitic analysis of the genera Pseudonocardia and Actinobispora based on 16S ribosomal DNA sequences. FEMS Microbiol.Lett. Jan 1; 182(1): 125-129.

Liu, Y., Zhang, T., and Fang, H.H.P. 2005. Microbial community analysis and performance of a phosphate-removing activated sludge. Bioresource Technology. 96 (11): 1205-1214.

Ochi, K. 1995. Comparative ribosomal protein sequence analyses of a phylogenetically defined genus, *Pseudomonas*, and its relatives. Int. J. Syst. Bacteriol. Apr; 45(2): 268-273.

Rainey, F. A., N. W. Rainey, C. G. Gliesche, and E. Stackebrandt. 1998. Phylogenetic analysis and intrageneric structure of the genus Hyphomicrobium and the related genus Filomicrobium. Int. J. Syst. Bacteriol. Jul;48 Pt 3: 635-639.

Sahin, N., K. Isik, A. U. Tamer, and M. Goodfellow. 2000. Taxonomic position of "*Pseudomonas* oxalaticus" strain ox 14T (DSM 1105T) (Khambata and Bhat, 1953) and its description in the genus Ralstonia as Ralstonia comb. nov. Syst. Appl. Microbiol. Jun; 23(2): 206-209.

Seal, S. E., L. A. Jackson, J. P. Young, and M. J. Daniels. 1993. Differentiation of *Pseudomonas* solanacearum, *Pseudomonas* syzygii, *Pseudomonas* pickettii and the blood Disease Bacterium by partial 16S rRNA sequencing: Construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. J Gen Microbiol. Jul; 139 (Pt 7): 1587-1594.

Snaidr, J., Amann, R., Huber, I., Ludwig, W., and Schleifer, K.H. 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl. Microbiol. Environ. 63(7): 2884-2896.

Tan, Z., and W. Chen. 1997. Sequencing the 16S rDNA of representative strain of new rhizobial group and determining of its phylogenetic relationship. Wei Sheng Wu Xue Bao. Dec; 37 (6): 411-416.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 173(2): 697-703.

Widmer, F.; Seidler, R.J.; Gillevet, P.M.; Watrud, L.S.; Di Giovanni, G.D. A highly selective PCR protocol for detecting 16S rRNA gene of the genus *Pseudomonas* (Sensu stritcto) in environmental samples. Applied Environmental Microbiology. 1998, *64* (7), 2545-2453.Weisburg, W.G.; Barns, S.M.; Pelletier, D.A; Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology. 1991, *173* (2), 697-703.

Yoon, J. H., and Y. H. Park. 2000. Phylogenetic analysis of the genus Thermoactinomyces based on 16S rDNA sequences. Int. J. Syst. Microbiol. May; 50 Pt 3: 1081-1086.

Zhang, Y., Aiyuk, S., Xu, H., Chen, G., Verstraete, W. 2005. Study of microbial community structures in UASB sludge treating municipal wastewater by denaturing gradient gel electrophoresis of 16S rDNA. Science in China. Series C, Life sciences. 48(1): 128-35.

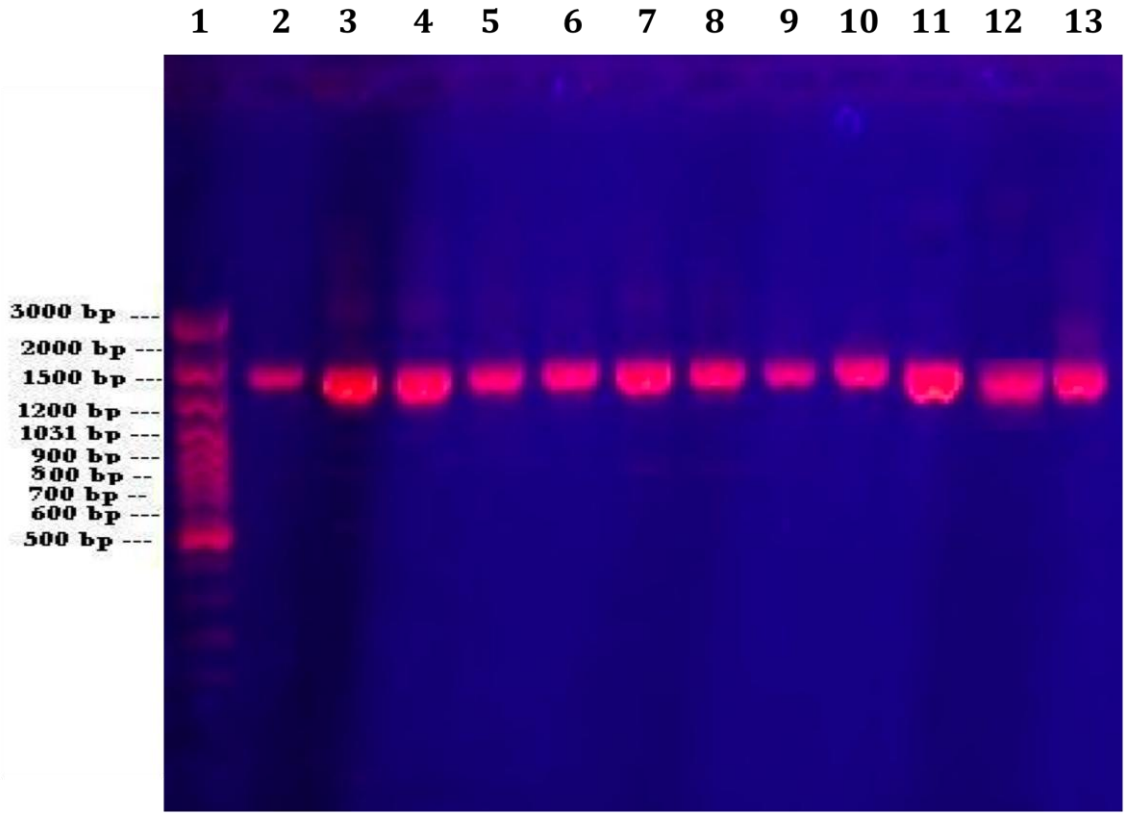


Figure 1: PCR amplified 16S rDNA gene portion revealed ~1.5 kb (kilo base) size. Lane 1: standard PCR markers and Lane 2-13: Amplicons from EPS producing bacterial strains (BS1-BS12). The photograph of amplicons for BS13-B25 were not shown.

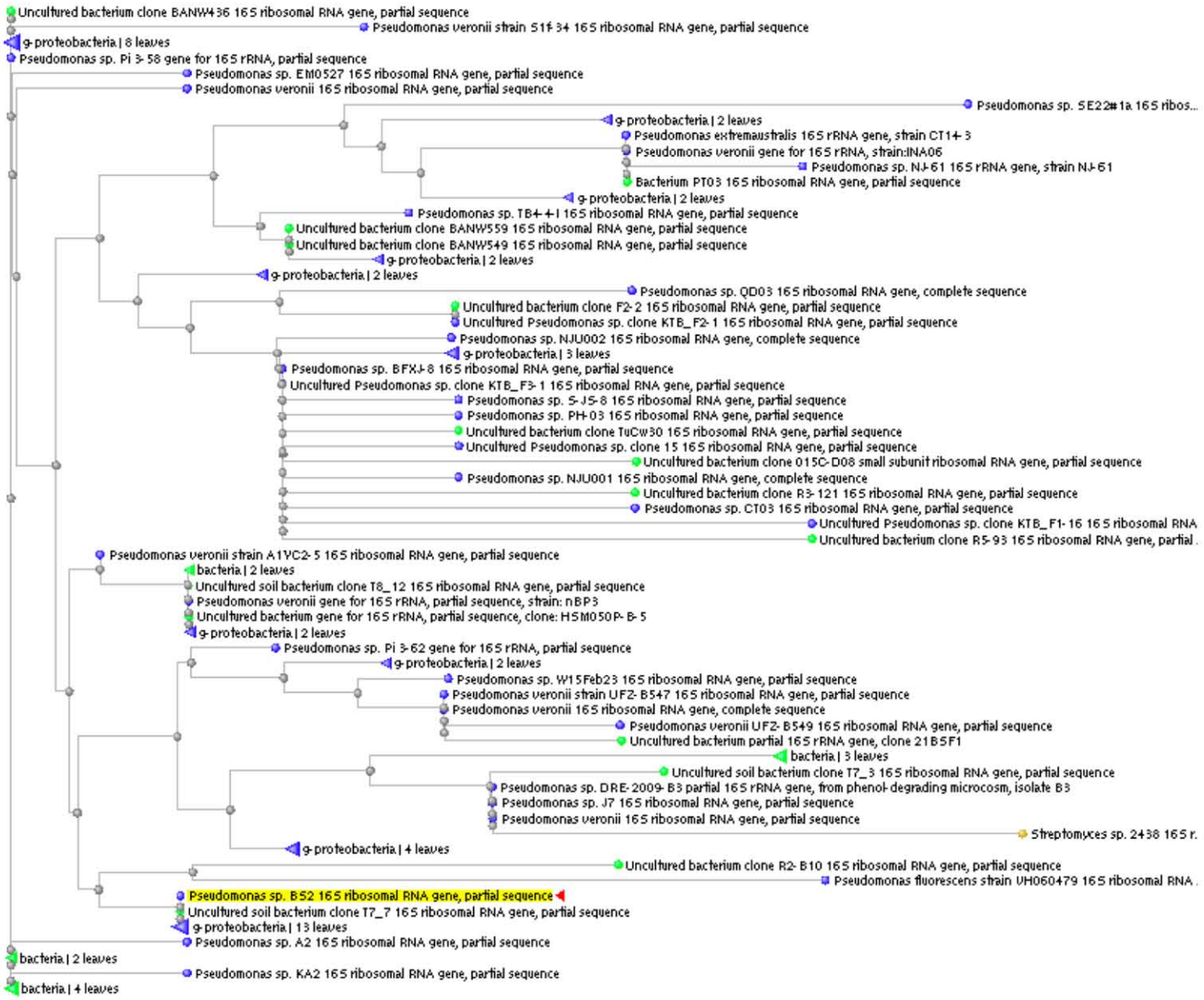


Figure 2: (a) Phylogenetic tree drawn using gene sequences obtained from strain BS2.

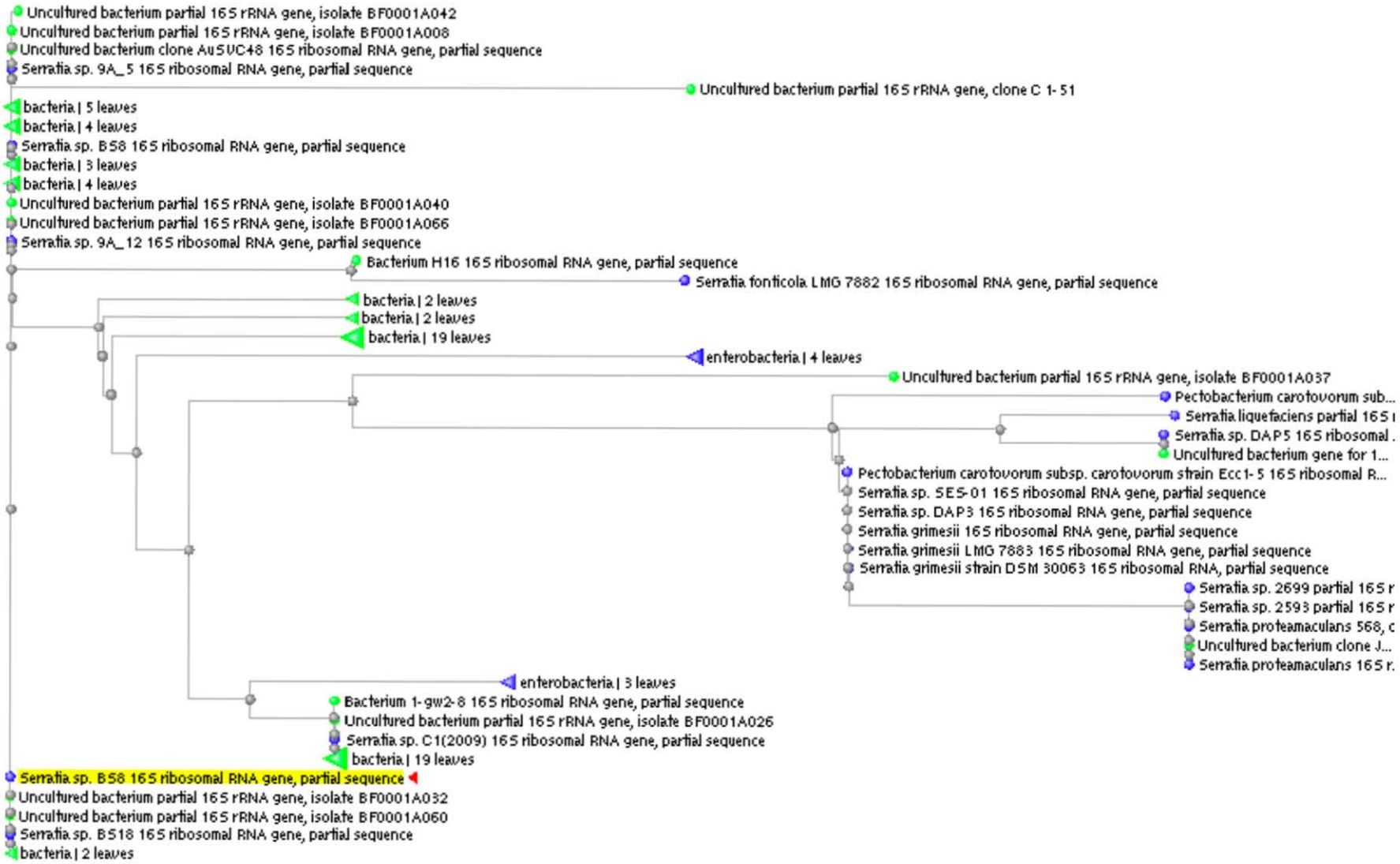


Figure 2: (b) Phylogenetic tree drawn using gene sequences obtained from strain BS8.

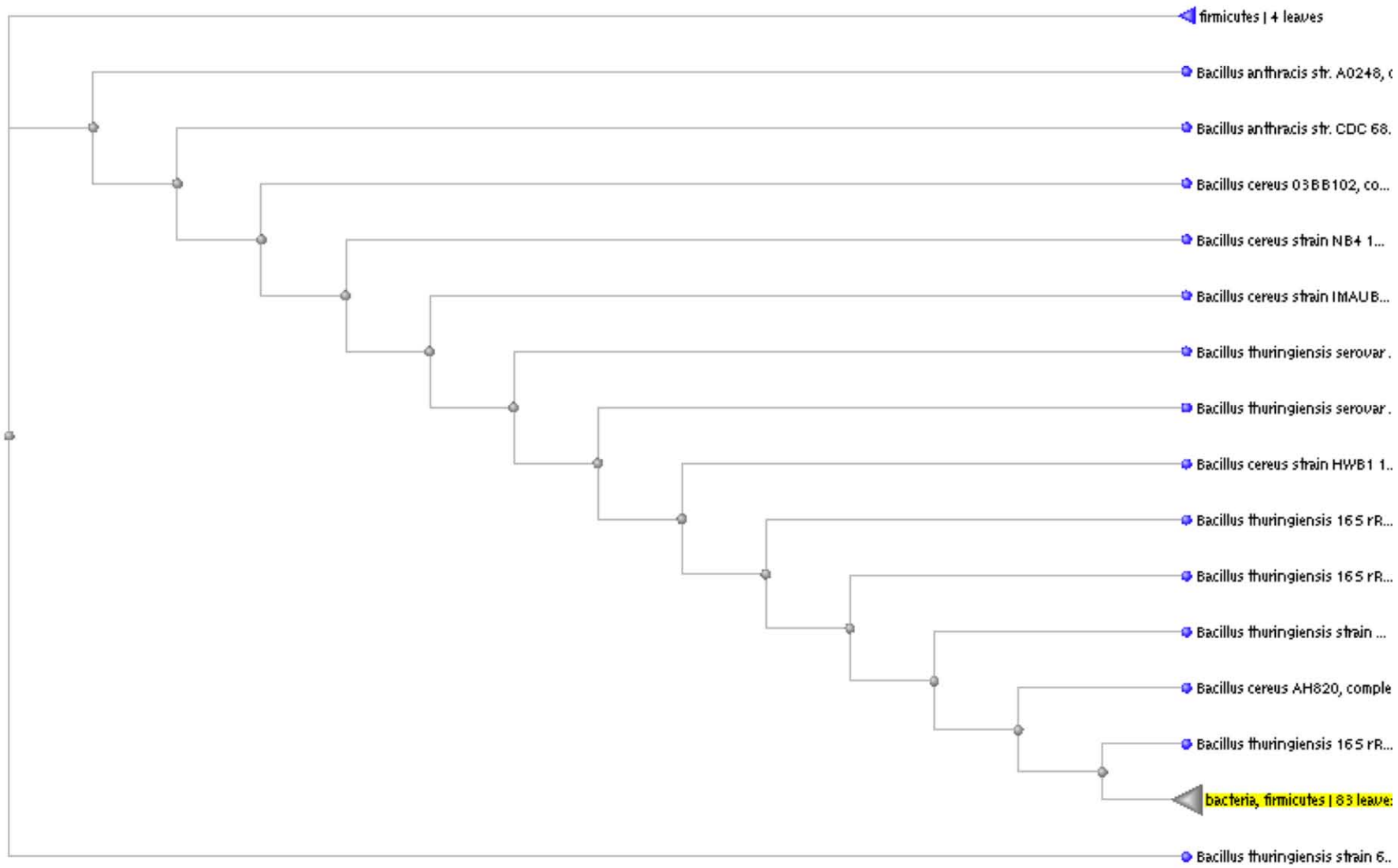


Figure 2: (c) Phylogenetic tree drawn using gene sequences obtained from strain BS9.

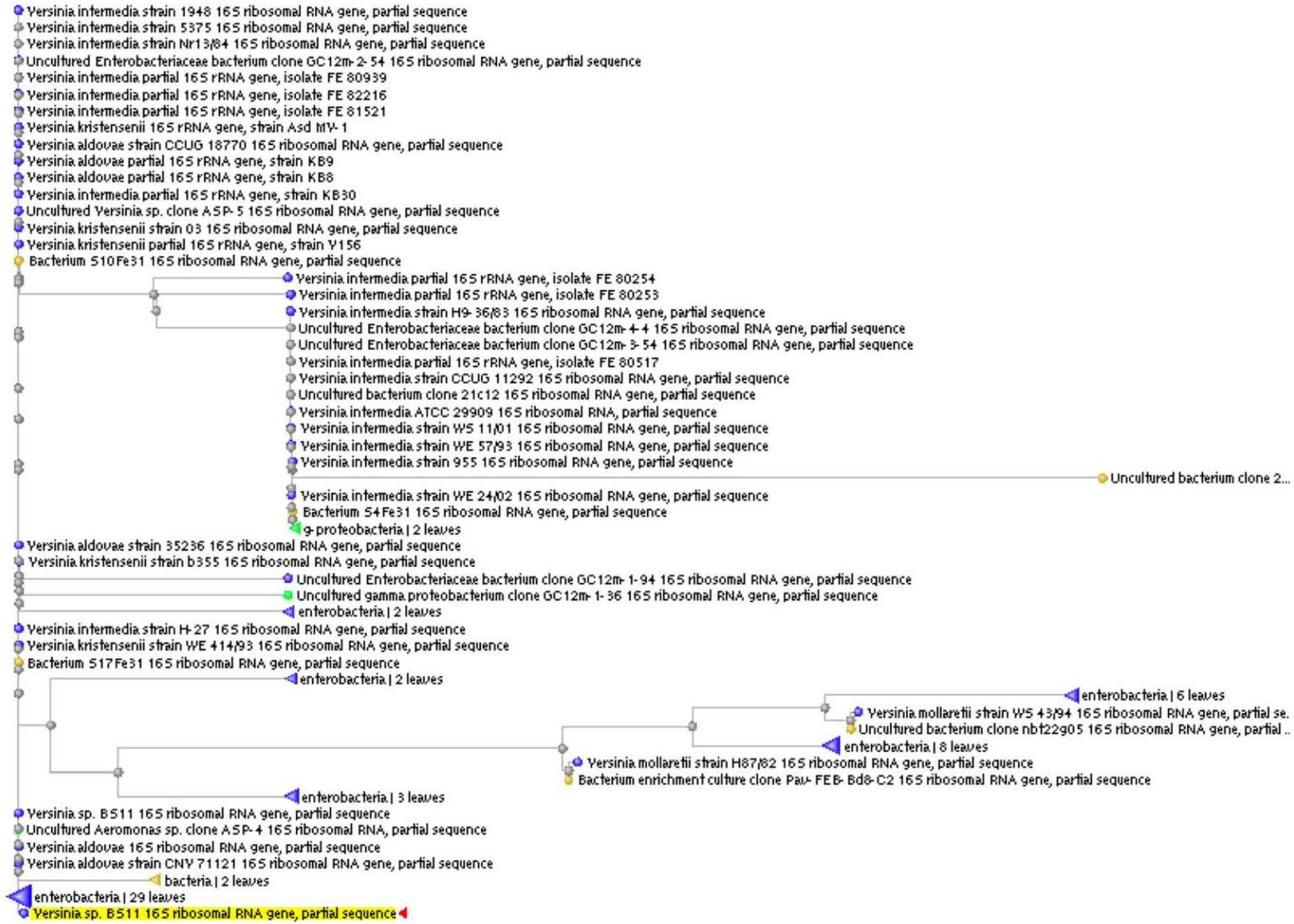


Figure 2: (d) Phylogenetic tree drawn using gene sequences obtained from strain BS11.

Figure 2: (e) Phylogenetic tree drawn using gene sequences obtained from strain BS15.

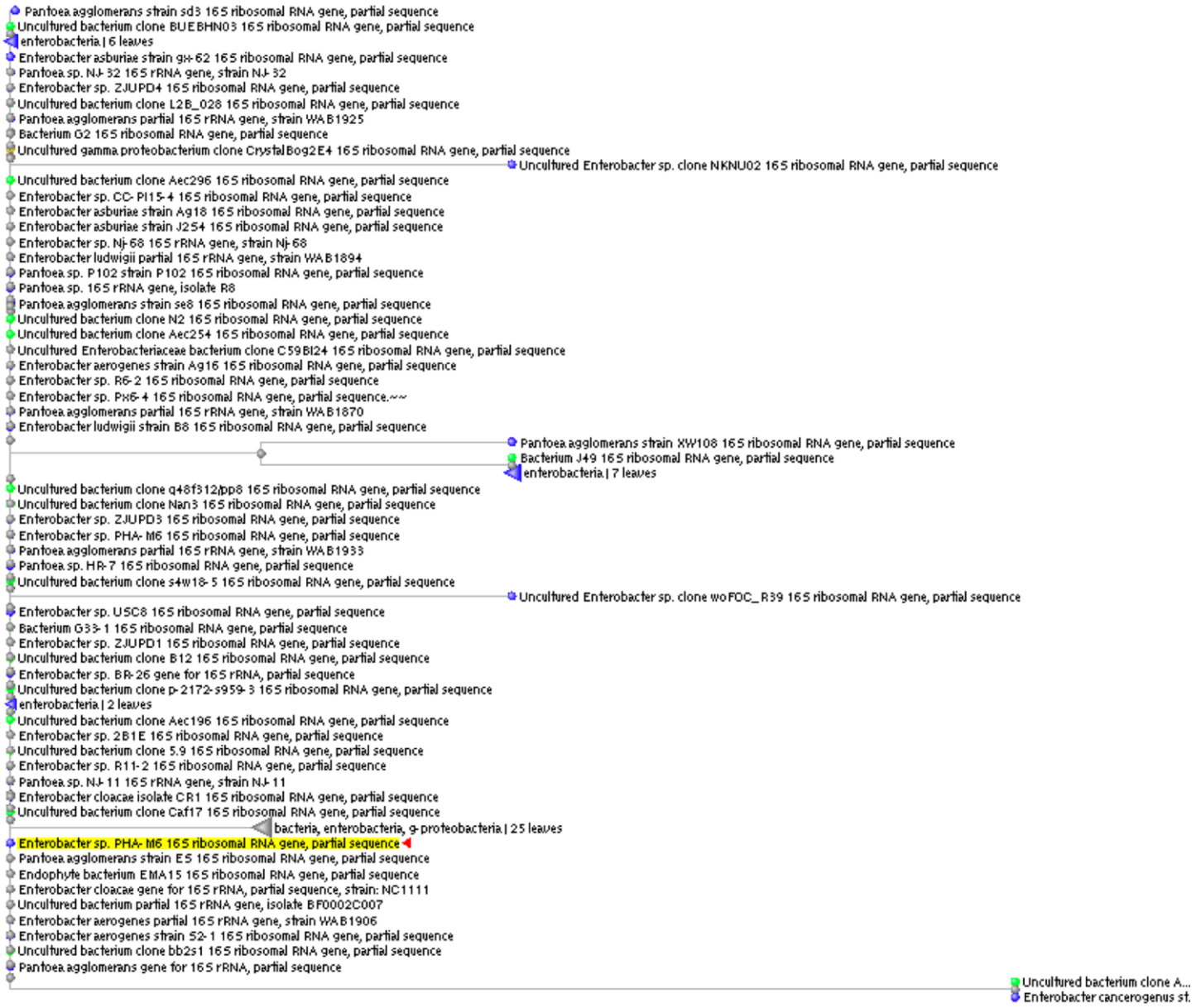


Figure 2: (f) Phylogenetic tree drawn using gene sequences obtained from strain BS25.

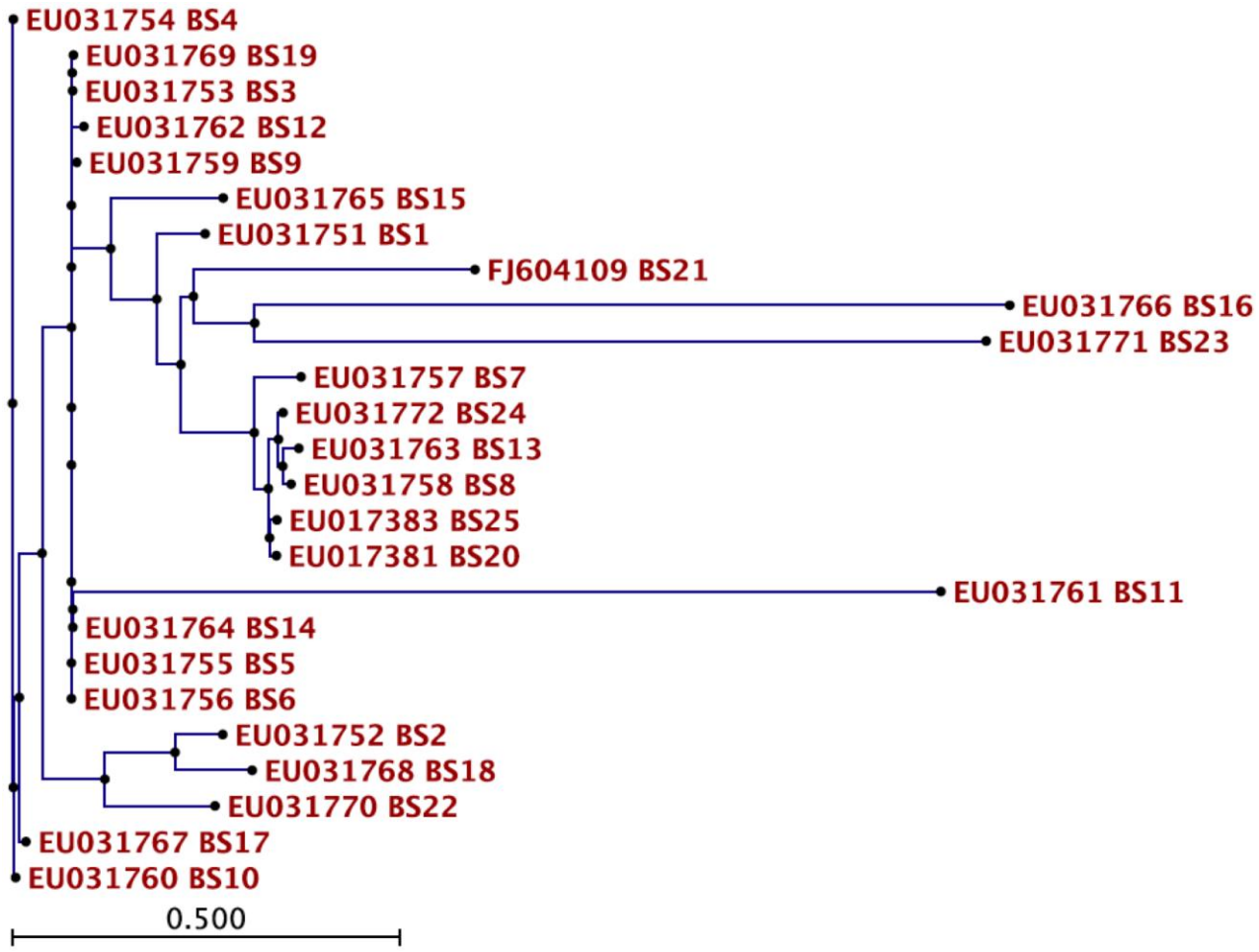


Figure 3: phylogenetic tree of all 25 EPS producing bacterial strains.

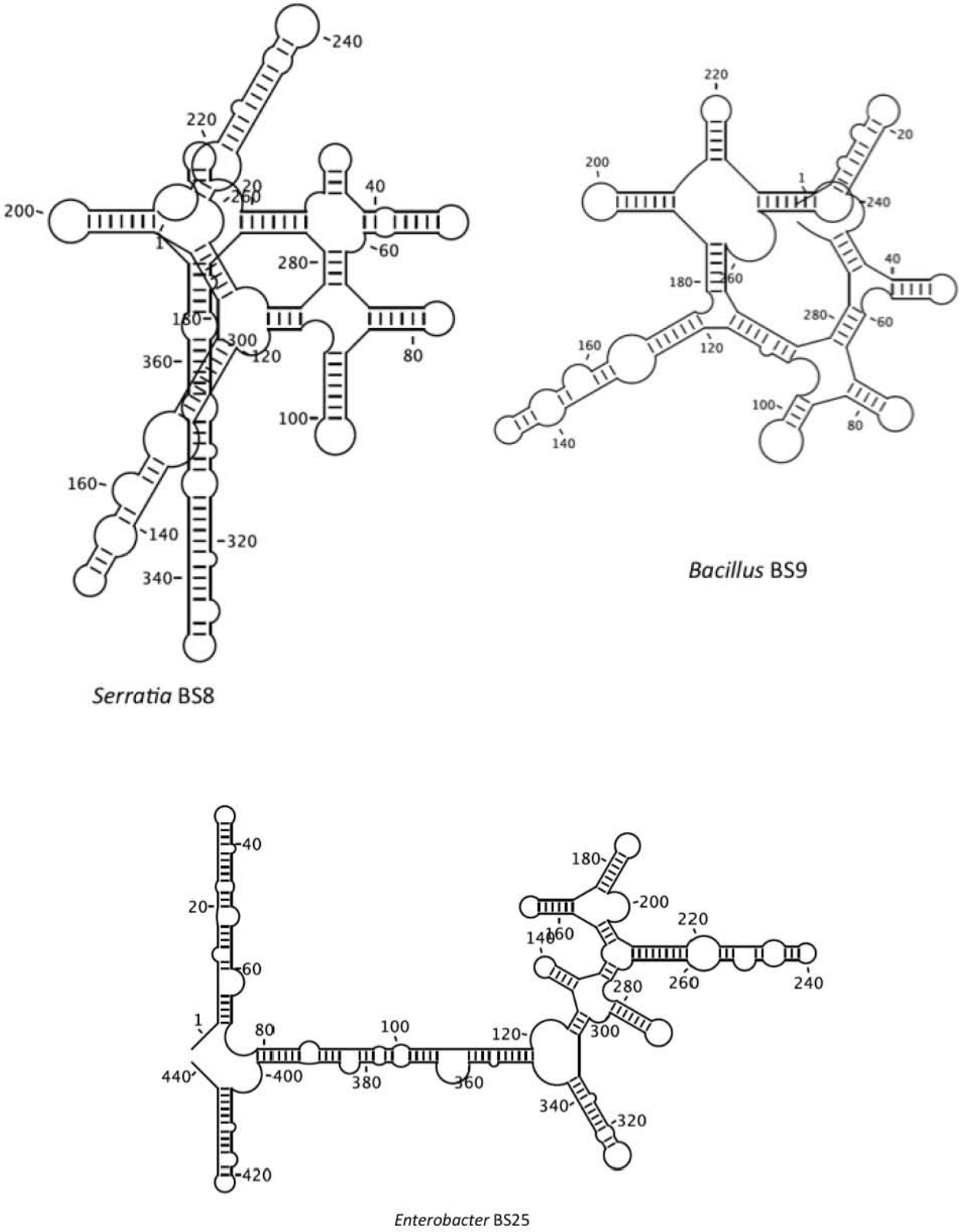
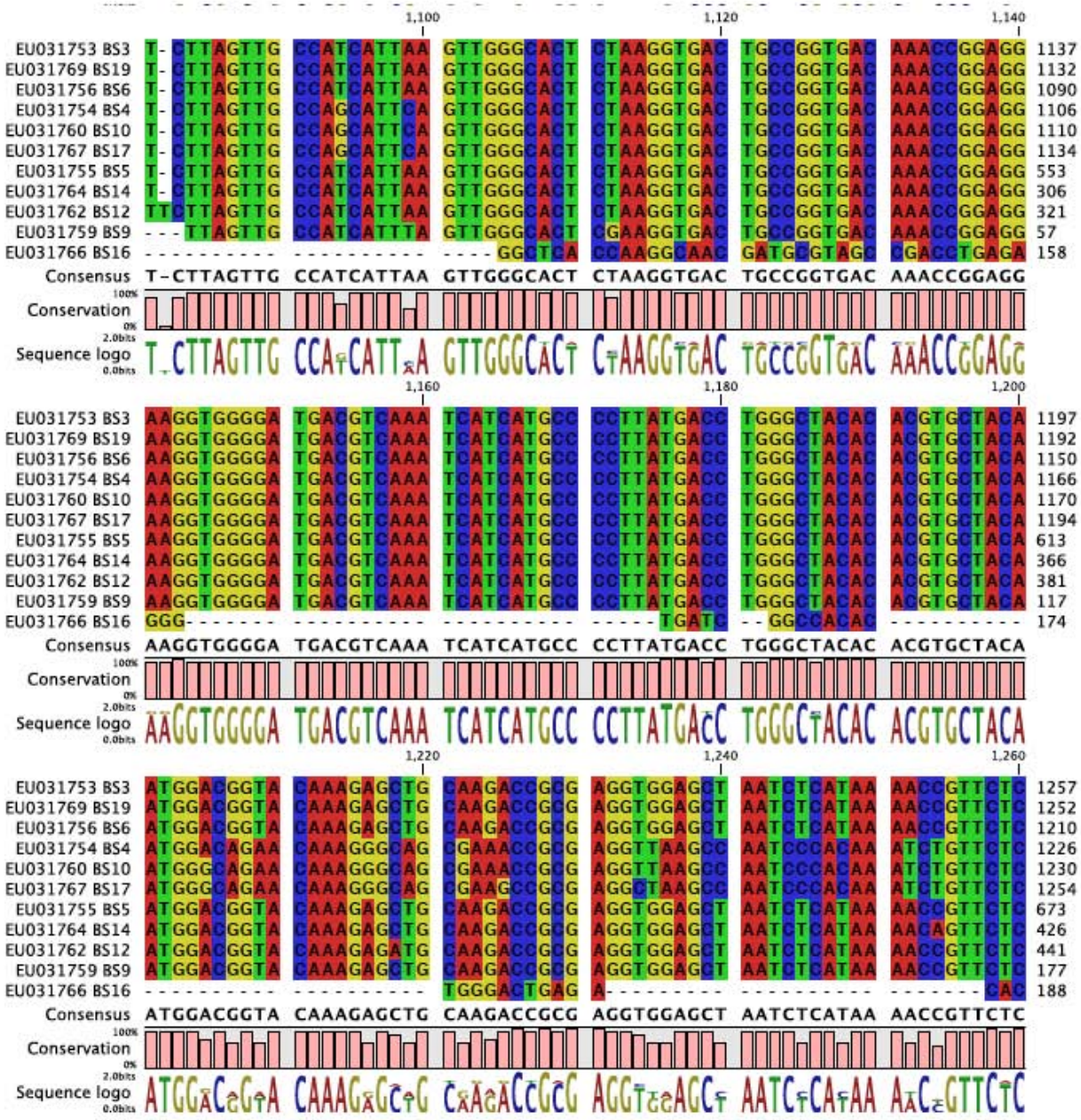


Figure 4: secondary structure of RNA gene sequences obtained from few EPS producing bacterial strain BS8, BS9 and BS25.


1,100
1,120
1,140
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EU031769 BS19 T-CTTAGTTG CCATCATTAA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG 1132
EU031756 BS6 T-CTTAGTTG CCATCATTAA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG 1090
EU031754 BS4 T-CTTAGTTG CCAGCATTCA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG 1106
EU031760 BS10 T-CTTAGTTG CCAGCATTCA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG 1110
EU031767 BS17 T-CTTAGTTG CCAGCATTCA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG 1134
EU031755 BS5 T-CTTAGTTG CCATCATTAA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG 553
EU031764 BS14 T-CTTAGTTG CCATCATTAA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG 306
EU031762 BS12 TTCTTAGTTG CCATCATTAA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG 321
EU031759 BS9 ---TTAGTTG CCATCATTTA GTTGGGCACT CGAAGGTGAC TGCCGGTGAC AAACCGGAGG 57
EU031766 BS16 ---------- ---------- ----GGCTCA CCAAGGCAAC GATGCGTAGC CGACCTGAGA 158
Consensus T-CTTAGTTG CCATCATTAA GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG
100%
Conservation
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Sequence logo
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1,200
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EU031756 BS6 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 1150
EU031754 BS4 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 1166
EU031760 BS10 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 1170
EU031767 BS17 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 1194
EU031755 BS5 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 613
EU031764 BS14 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 366
EU031762 BS12 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 381
EU031759 BS9 AAGGTGGGGA TGACGTCAAA TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA 117
EU031766 BS16 GGG---------- ---------- ---------- -----TGATC --GGCCACAC ---------- 174
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Conservation
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2.0bits
Sequence logo
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EU031756 BS6 ATGGACGGTA CAAAGAGCTG CAAGACCGCG AGGTGGAGCT AATCTCATAA AACCGTTCTC 1210
EU031754 BS4 ATGGACAGAA CAAAGGGCAG CGAAACCGCG AGGTTAAGCC AATCCCACAA ATCTGTTCTC 1226
EU031760 BS10 ATGGGCAGAA CAAAGGGCAG CGAAACCGCG AGGTTAAGCC AATCCCACAA ATCTGTTCTC 1230
EU031767 BS17 ATGGGCAGAA CAAAGGGCAG CGAAGCCGCG AGGCTAAGCC AATCCCACAA ATCTGTTCTC 1254
EU031755 BS5 ATGGACGGTA CAAAGAGCTG CAAGACCGCG AGGTGGAGCT AATCTCATAA AACCGTTCTC 673
EU031764 BS14 ATGGACGGTA CAAAGAGCTG CAAGACCGCG AGGTGGAGCT AATCTCATAA AACAGTTCTC 426
EU031762 BS12 ATGGACGGTA CAAAGAGATG CAAGACCGCG AGGTGGAGCT AATCTCATAA AACCGTTCTC 441
EU031759 BS9 ATGGACGGTA CAAAGAGCTG CAAGACCGCG AGGTGGAGCT AATCTCATAA AACCGTTCTC 177
EU031766 BS16 ---------- ---------- TGGGACTGAG A--------- ---------- -------CAC 188
Consensus ATGGACGGTA CAAAGAGCTG CAAGACCGCG AGGTGGAGCT AATCTCATAA AACCGTTCTC
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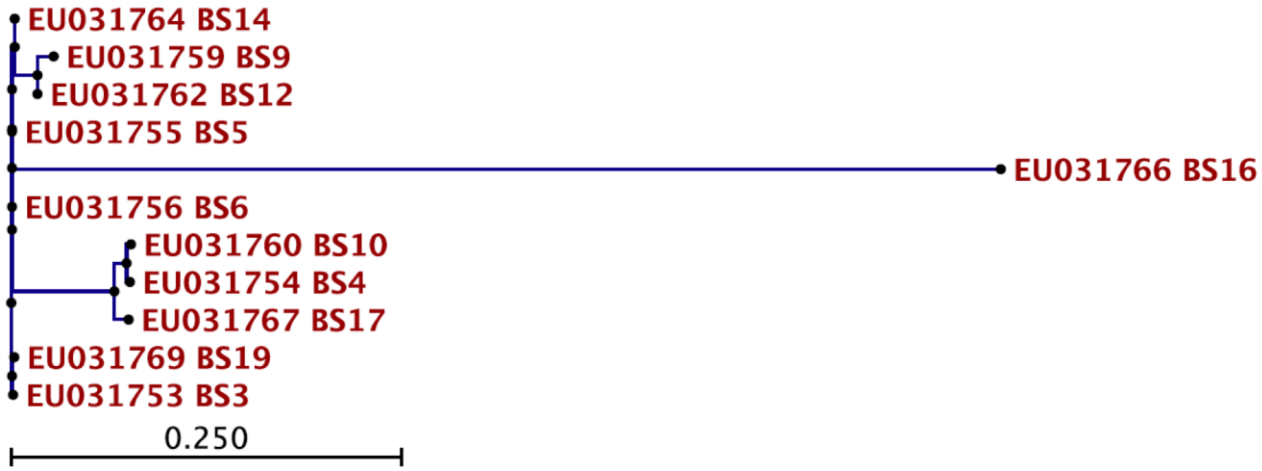
Conservation
0%
2.0bits
Sequence logo
0.0bits

(a)

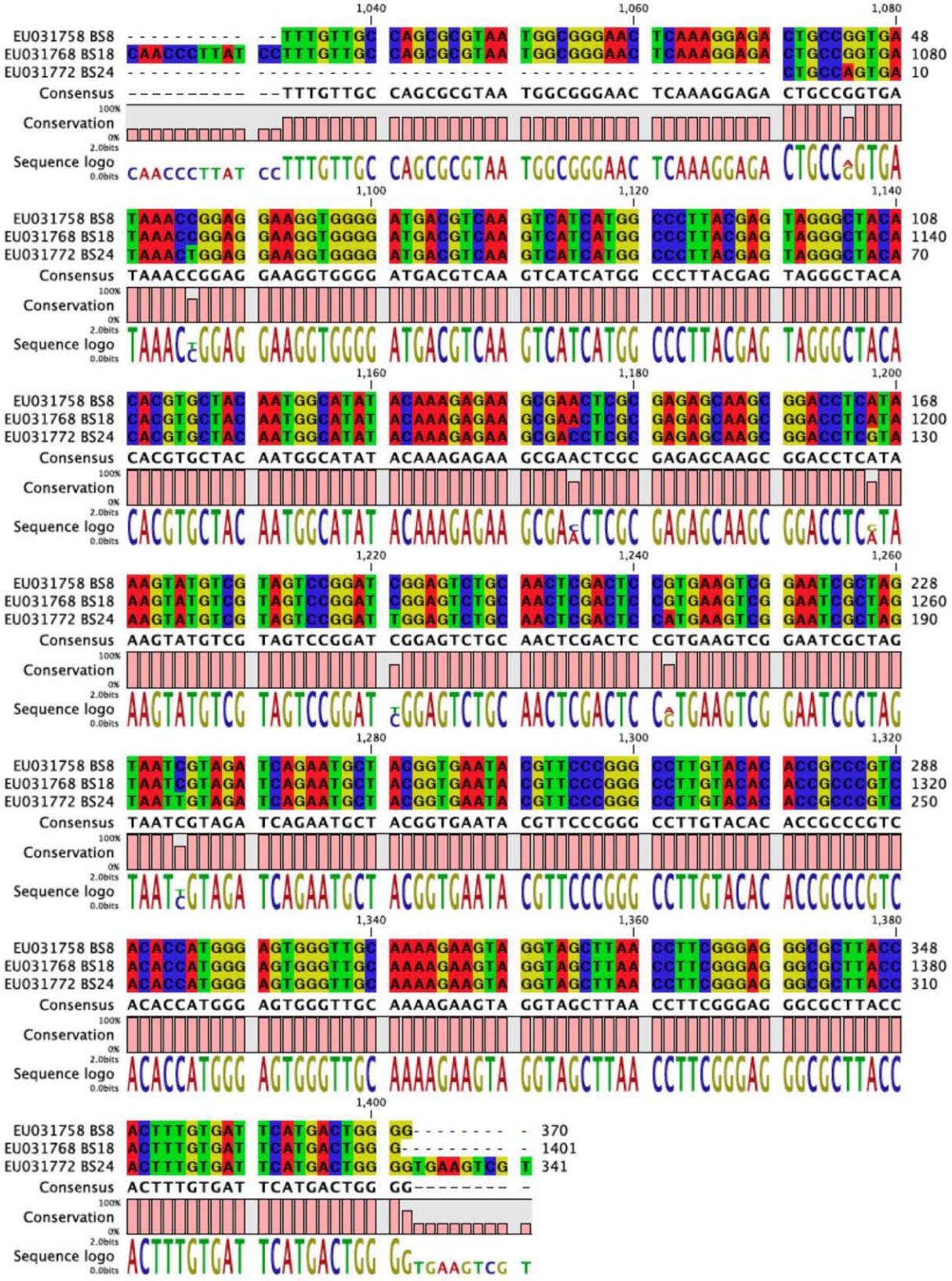


(b)

		1	2	3	4	5	6	7	8	9	10	11
Bacillus sp. BS3 (EU031753)	1		98.61	94.04	91.19	91.82	91.43	59.22	39.18	30.28	20.04	8.80
Bacillus sp. BS19 (EU031769)	2	0.01		95.09	92.21	92.03	90.40	59.04	39.65	30.65	20.28	8.91
Bacillus sp. BS6 (EU031756)	3	0.06	0.05		91.42	90.70	88.09	58.95	41.64	32.18	21.30	9.35
Bacillus sp. BS4 (EU031754)	4	0.09	0.08	0.09		98.79	94.57	56.86	38.38	29.43	19.23	8.71
Bacillus sp. BS10 (EU031760)	5	0.09	0.08	0.10	0.01		95.12	57.12	38.04	29.15	19.02	8.65
Bacillus sp. BS17 (EU031767)	6	0.09	0.10	0.13	0.06	0.05		55.71	36.96	28.27	18.31	8.46
Bacillus sp. BS5(2007) (EU031755)	7	0.59	0.59	0.59	0.64	0.64	0.67		66.16	51.11	33.84	14.85
Bacillus sp. BS14 (EU031764)	8	1.25	1.22	1.13	1.29	1.31	1.38	0.45		72.63	50.88	22.40
Bacillus sp. BS12 (EU031762)	9	1.99	1.94	1.76	2.12	2.17	2.35	0.79	0.34		32.07	23.06
Bacillus sp. BS9(2007) (EU031759)	10	∞	∞	∞	∞	∞	∞	1.60	0.80	1.77		14.53
Bacillus sp. BS16 (EU031766)	11	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	

(c)

Figure 5: (a) multiple alignment of gene sequences, (b) phylogenetic tree, and (c) pairwise comparison results of genus *Bacillus*.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1,080
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EU031768 BS18 CAACCCTTAT CCTTTGTTGC CAGCGCGTAA TGGCGGGAAC TCAAAGGAGA CTGCCGGTGA 1080
EU031772 BS24 ---------- ---------- ---------- ---------- ---------- CTGCCAGTGA 10
Consensus ---------- --TTTGTTGC CAGCGCGTAA TGGCGGGAAC TCAAAGGAGA CTGCCGGTGA
Conservation
Sequence logo
CAACCCTTAT CCTTTGTTGC CAGCGCGTAA TGGCGGGAAC TCAAAGGAGA CTGCCGGTGA
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1,120
1,140
EU031758 BS8 TAAACGGAG GAAGGTGGGG ATGACGTCAA GTCATCATGG CCCTTACGAG TAGGGCTACA 108
EU031768 BS18 TAAACGGAG GAAGGTGGGG ATGACGTCAA GTCATCATGG CCCTTACGAG TAGGGCTACA 1140
EU031772 BS24 TAAACTGGAG GAAGGTGGGG ATGACGTCAA GTCATCATGG CCCTTACGAG TAGGGCTACA 70
Consensus TAAACCGGAG GAAGGTGGGG ATGACGTCAA GTCATCATGG CCCTTACGAG TAGGGCTACA
Conservation
Sequence logo
TAAACCGGAG GAAGGTGGGG ATGACGTCAA GTCATCATGG CCCTTACGAG TAGGGCTACA
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1,180
1,200
EU031758 BS8 CACGTGCTAC AATGGCATAT ACAAAGAGAA GCGAACTCGC GAGAGCAAGC GGACCTCATA 168
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EU031772 BS24 CACGTGCTAC AATGGCATAT ACAAAGAGAA GCGACCTCGC GAGAGCAAGC GGACCTCGTA 130
Consensus CACGTGCTAC AATGGCATAT ACAAAGAGAA GCGAACTCGC GAGAGCAAGC GGACCTCATA
Conservation
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EU031758 BS8 AAGTATGTCG TAGTCCGGAT CGGAGTCTGC AACTCGACTC CGTGAAGTCG GAATCGCTAG 228
EU031768 BS18 AAGTATGTCG TAGTCCGGAT CGGAGTCTGC AACTCGACTC CGTGAAGTCG GAATCGCTAG 1260
EU031772 BS24 AAGTATGTCG TAGTCCGGAT TGGAGTCTGC AACTCGACTC CATGAAGTCG GAATCGCTAG 190
Consensus AAGTATGTCG TAGTCCGGAT CGGAGTCTGC AACTCGACTC CGTGAAGTCG GAATCGCTAG
Conservation
Sequence logo
AAGTATGTCG TAGTCCGGAT MGGAGTCTGC AACTCGACTC CRTGAAGTCG GAATCGCTAG
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1,300
1,320
EU031758 BS8 TAATCGTAGA TCAGAATGCT ACGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC 288
EU031768 BS18 TAATCGTAGA TCAGAATGCT ACGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC 1320
EU031772 BS24 TAATTGTAGA TCAGAATGCT ACGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC 250
Consensus TAATCGTAGA TCAGAATGCT ACGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC
Conservation
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1,360
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EU031758 BS8 ACACCATGGG AGTGGGTTGC AAAAGAAGTA GGTAGCTTAA CCTTCGGGAG GGCGCTTACC 348
EU031768 BS18 ACACCATGGG AGTGGGTTGC AAAAGAAGTA GGTAGCTTAA CCTTCGGGAG GGCGCTTACC 1380
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Conservation
Sequence logo
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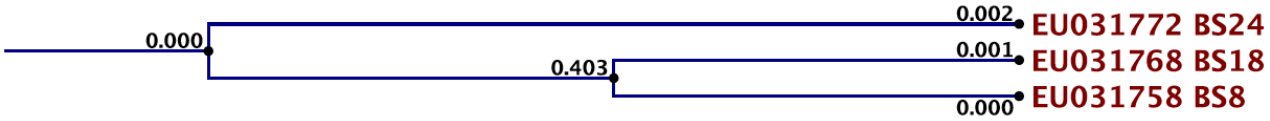
1,400
EU031758 BS8 ACTTTGTGAT TCATGACTGG GG--------- - 370
EU031768 BS18 ACTTTGTGAT TCATGACTGG G---------- - 1401
EU031772 BS24 ACTTTGTGAT TCATGACTGG GGTGAAGTCG T 341
Consensus ACTTTGTGAT TCATGACTGG GG--------- -
Conservation
Sequence logo
ACTTTGTGAT TCATGACTGG GGTGAAGTCG T

(a)

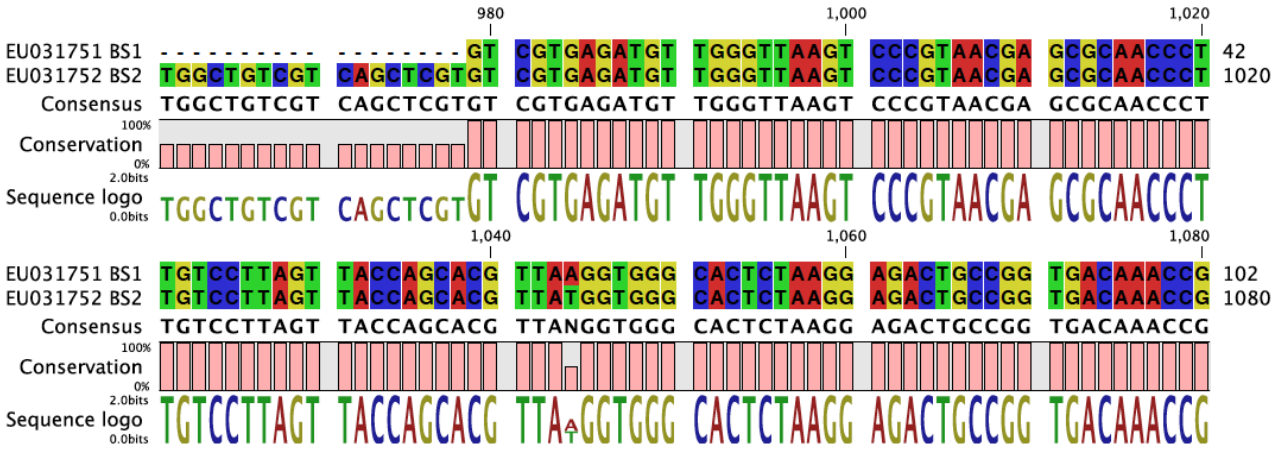


(b)

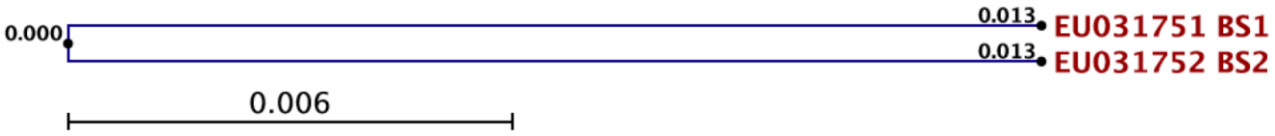
	1	2	3
Serratia sp. BS8 (EU031758) 1		26.32	85.75
Serratia sp. BS18 (EU031768) 2	3.03		22.96
Serratia sp. BS24 (EU031772) 3	0.16	∞	

(c)

Figure 6: (a) multiple alignment of gene sequences, (b) phylogenetic tree, and (c) pairwise comparison results of genus *Serratia*.



(a)



(b)

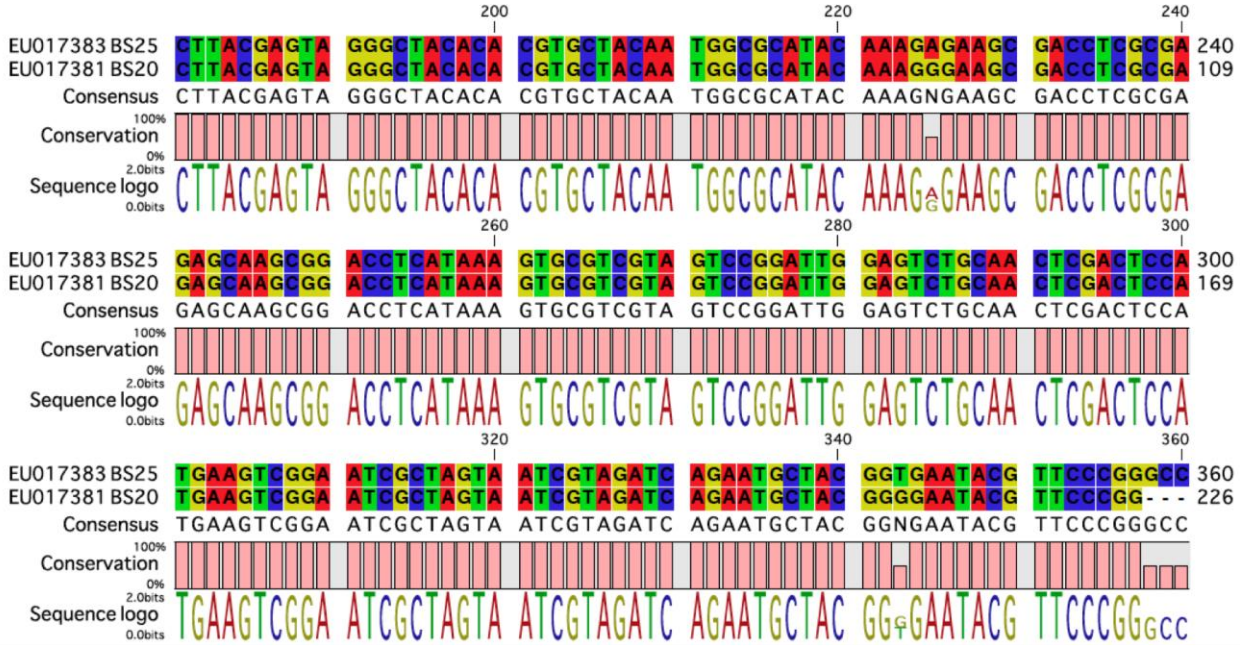
	1	2
1		26.86
2	2.77	

Pseudomonas sp. BS1(2007) (EU031751)

Pseudomonas sp. BS2 (EU031752)

(c)

Figure 7: (a) multiple alignment of gene sequences, (b) phylogenetic tree, and (c) pairwise comparison results of genus *Pseudomonas*.



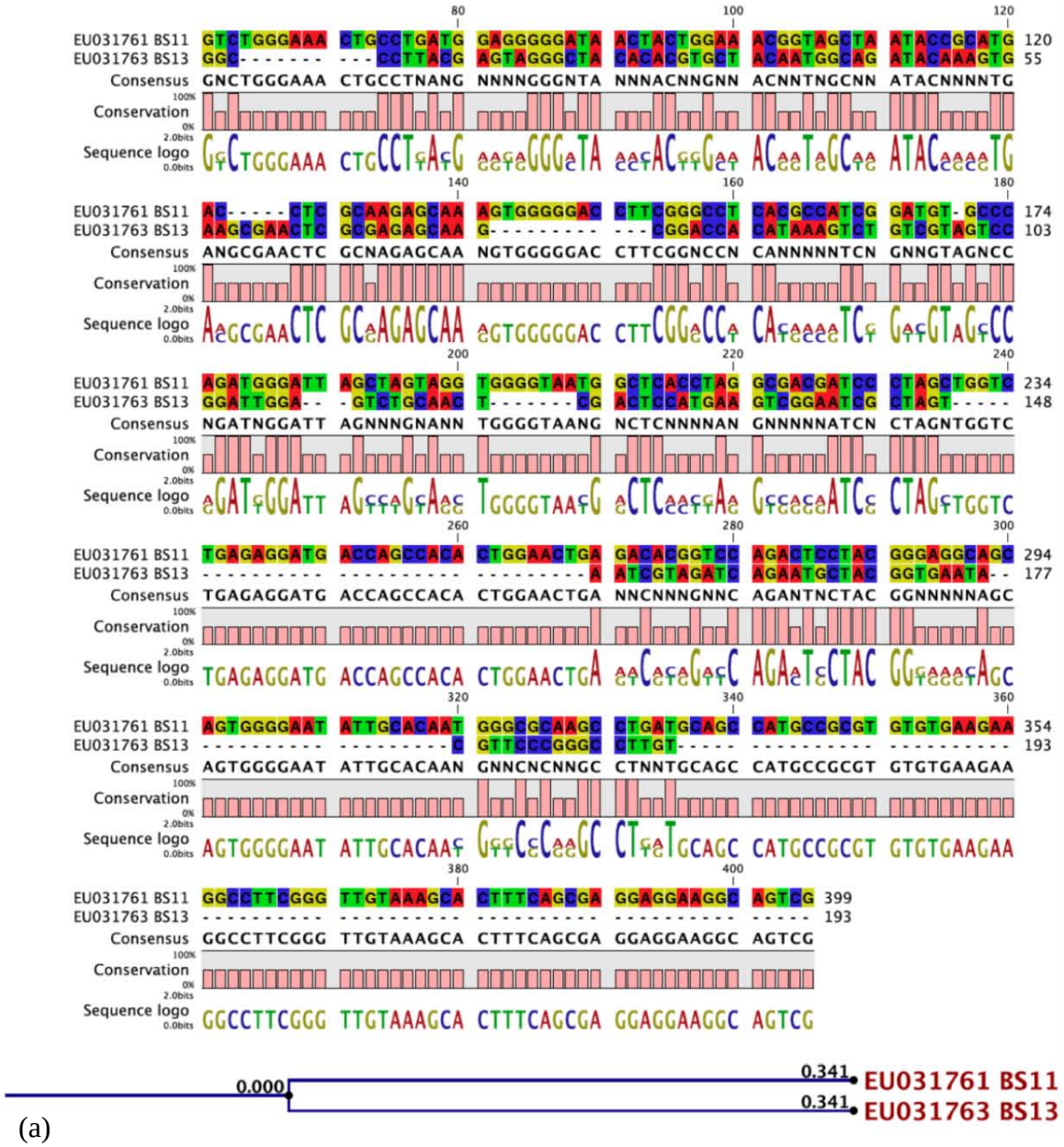
(a)

(b)

	1	2
EU017383 Enterobacter BS25	1	50.91
EU017381 Enterobacter BS20	2	0.80

(c)

Figure 8: (a) multiple alignment of gene sequences, (b) phylogenetic tree, and (c) pairwise comparison results of genus *Enterobacter*.



(a)

(b)

	1	2
1		25.43
2	3.87	

Yersinia sp. BS11 (EU031761)

Yersinia sp. BS13 (EU031763)

(c)

Figure 9: (a) multiple alignment of gene sequences, (b) phylogenetic tree, and (c) pairwise comparison results of genus *Yersinia*.

Table 1: 16S rRNA gene sequences (5′-3′ direction) obtained form 25 EPS producing bacterial strains used for bioinformatics analysis.

Strain Name	Accession number	Strain number	bp
Pseudomonas	**EU031751**	**BS1**	**386 bp**
GTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGGAGACTGCCGGTGACAAA CCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGA GGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCA CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTA GCTAGTCTAACCGCAAGGGGG			
Pseudomonas	**EU031752**	**BS2**	**1400 bp**
TTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAG AAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGG TCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATC CAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTGATTGTTTTGACGTTACCGA CAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTT AGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGC GGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGG ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGG GGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC CAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG TTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGG TGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAAT CCCAGAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGT TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGGGGACGGTTACCACGGTGTGATTCA TGACTGGGGTGAAG			
Bacillus	**EU031753**	**BS3**	**1442 bp**
ATACATGCAAGTCGAGCGAATGGATTGAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGAT AACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTC GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGA CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCT GTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA CGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTC ATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGC GACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT GTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGG CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCT TCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCC ATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA CGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTAC ATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAC ACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATG			

Bacillus	**EU031754**	**BS4**	**1399 bp**
GCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGT TTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAG TCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTA CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTT CTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCT GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAG ACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACA ATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA ACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA CCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAAC TCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTA ACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAG			
Bacillus	**EU031755**	**BS5**	**854 bp**
TGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGT GGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA GTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAAT TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAG GGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT CTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG GGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAA CTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGA GTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACA			
Bacillus	**EU031756**	**BS6**	**1357 bp**
TAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACCGCAT GGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGT AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT CTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGAC GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGC AGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCA TGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGC AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACT CCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG AACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGA GCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGT			

Pantoea	**EU031757**	**BS7**	**336 bp**
CTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATC GGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCTGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC ACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTCAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGG TAACCCGTAGGGGAGCCTGCGGCTGGATCACCTCCTTT			
Serratia	**EU031758**	**BS8**	**370 bp**
TTTGTTGCCAGCGCGTAATGGCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGT AGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATCGGAGTCTG CAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG GAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGG			
Bacillus	**EU031759**	**BS9**	**291 bp**
TTAGTTGCCATCATTTAGTTGGGCACTCGAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG GGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAA CTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC			
Bacillus	**EU031760**	**BS10**	**1409 bp**
GGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGC TTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACG GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGG TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATAC CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGC AAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTG ACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC GCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA TGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGC AACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTT GTAACACCCGAAGTCGGTGAGGTAACCTTTTTGGAGCCAGCCGCCGAAGGTGGGA			
Yersinia	**EU031761**	**BS11**	**399 bp**
TCGAGCGGCAGCGGGAAGTAGTTTACTACTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGA AACGGTAGCTAATACCGCATGACCTCGCAAGAGCAAAGTGGGGGACCTTCGGGCCTCACGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGG GTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGCA GTCG			
Bacillus	**EU031762**	**BS12**	**445 bp**
GAAGTTAACGCATTAAGCACTCCGCGTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGGCCGCACAAGCGGTGGAGCATGTGGT TTAATTCGAAGCAACGCGAAGAACCTTACCAGGTGTTCACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGCGC ATGGTTGTCGTCAGCTGGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATTCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGG TGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAG AGATGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTT			

Yersinia	**EU031763**	**BS13**	**193 bp**
ATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGTGAAGCGAACTCGCGAGAGCAAGCGGACCACATAAAGTCTGTCGTA GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGT			
Bacillus	**EU031764**	**BS14**	**566 bp**
AAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA CGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCG AGGTGGAGCTAATCTCATAAAACAGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCG CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCG			
Microbacterium	**EU031765**	**BS15**	**290 bp**
ATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGCGAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAG GTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCAAG TCATGAAAGTCGGTAACACCTGAAGCCGGTGGCCTAACCCTTGTGGAGGGAGCCGTCGAAGGTGGGATCGGTAATTAGGACTAAGTCGTAA			
Bacillus	**EU031766**	**BS16**	**223 bp**
ACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCG CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACT CCTACGGGAGGCAGCAGTAGGGAAT			
Bacillus	**EU031767**	**BS17**	**1453 bp**
ACATGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACT CCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGCA TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTG TTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA GGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTG GAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACT CTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA GAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCG CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGG GGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCA TTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG CTACAATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGA AGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCC GAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCG			
Serratia	**EU031768**	**BS18**	**1401 bp**
TCCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCTTCGGA CCAAAGTGGGGGACCTTCGGGCCTCACACCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCT GAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATG CCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGGTTCGGTGTTAATAGCACCGTTCATTGACGTTACTCGCAGAAGAAGCAC CGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGA AATCCCCGAGCTTAACTTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGG			

AGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTA AACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAGAACTTTCCAGAGATGGATTG GTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCC AGCGCGTAATGGCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACG TGCTACAATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGT CGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGG TAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGG			
Bacillus	**EU031769**	**BS19**	**1425 bp**
TGCAAGTCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTC CGGGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATT AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGT TAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG GTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG AAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTT TCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAG AGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGC ACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGG GAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCA TTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG CTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGA AGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCC GAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAG			
Enterobacter	**EU017381**	**BS20**	**226 bp**
CCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGGGAAG CGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGA TCAGAATGCTACGGGGAATACGTTCCCGG			
Photorhabdus	**FJ604109**	**BS21**	**172 bp**
TCCATGAAGTCCGAATCGAGAGTAATCGTAGATCGGAAGGCTACGGGGAATACGTTCGCTGGCCTTGTATCATGAACGGCCCGTCACACCATGGGAGTG GGTGGCAAAAGATTCCTTTACTTAACCTTCGGGAGGGCGCTGACCACTTGTGATTCAGACTGGGGGAAGTCTA			
Microbacterium	**EU031770**	**BS22**	**1394 bp**
CATGCAAGTCGAACGGTGAACACGGAGCTTGCTCTGTGGGATCAGTGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCCTGACTCTGGGATAAGCGCTGGA AACGGCGTCTAATACTGGATATGTGACGTGACCGCATGGTCTGCGTCTGGAAAGAATTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGAGGTAAT GGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT TGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGAGGGACGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACGGTACCTG CAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCG CGTCTGCTGTGAAATCCGGAGGCTCAACCTCCGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGC GCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTA GTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCT AAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACGAGAACG			

GGCCAGAAATGGTCAACTCTTTGGACACTCGTAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC TCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCT TGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGCGAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTC GACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAA CACCTGAAGCCGGTGGCCTAACCCTTGTGGAGGGAGCCGT			
Pectobacterium	**EU031771**	**BS23**	**236 bp**
TGCAAGTCGAGCGGTAGCACAGAAGAGCTTGCTCTTTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGG AAACGGTAGCTAATACCGCATAACCTCGCAAGAGCAAAGAGGGGGACCTTCGGGCCTCTCGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGAGGTAA TGGCTCACCTAGGCGACGATCCCTAGCT			
Serratia	**EU031772**	**BS24**	**341 bp**
CTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGA CCTCGCGAGAGCAAGCGGACCTCGTAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATTGTAGATCAGAATGC TACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTT TGTGATTCATGACTGGGGTGAAGTCGT			
Enterobacter	**EU017383**	**BS25**	**440 bp**
TCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCC AGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACA CACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT CCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTT GCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCAC			

Table 2: Multiple pairwise comparison of all 25 EPS producing bacterial strains. The upper columns reveals the percent of identity and the lower columns reveals the distance among them.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
EU031753	1		98.61	94.04	91.19	91.82	91.43	59.22	73.62	76.06	76.35	39.18	30.28	23.98	18.94	22.41	10.75	16.93	20.04	15.25	13.42	6.44	12.40	10.53	7.32	6.31
EU031769	2	0.01		95.09	92.21	92.03	90.40	59.04	73.32	75.77	76.85	39.65	30.65	24.27	18.52	22.67	10.87	16.51	20.28	14.76	13.00	6.52	12.55	10.66	7.40	6.37
EU031756	3	0.06	0.05		91.42	90.70	88.09	58.95	73.85	76.66	75.14	41.64	32.18	24.28	18.23	23.44	11.02	16.16	21.30	13.78	12.55	6.85	13.18	11.19	7.77	5.80
EU031754	4	0.09	0.08	0.09		98.79	94.57	56.86	75.04	77.15	76.01	38.38	29.43	24.64	19.07	23.02	11.64	16.67	19.23	14.61	13.50	6.86	12.86	10.86	6.89	6.49
EU031760	5	0.09	0.08	0.10	0.01		95.12	57.12	75.04	77.15	76.04	38.04	29.15	24.47	19.30	22.86	11.55	16.90	19.02	15.05	13.73	6.81	12.77	10.78	6.77	6.44
EU031767	6	0.09	0.10	0.13	0.06	0.05		55.71	73.59	76.53	75.39	36.96	28.27	23.75	19.49	22.58	11.92	17.79	18.31	15.49	14.65	6.67	12.38	10.45	6.58	6.33
EU031755	7	0.59	0.59	0.59	0.64	0.64	0.67		47.75	51.00	48.37	66.16	51.11	40.40	31.34	37.60	17.98	27.89	33.84	25.17	21.61	10.88	20.94	17.78	12.30	10.47
EU031768	8	0.33	0.33	0.32	0.30	0.30	0.33	0.89		84.93	70.99	33.19	25.46	29.89	26.23	22.80	11.17	22.88	16.77	13.84	17.41	6.35	15.26	13.27	6.60	8.15
EU031752	9	0.29	0.29	0.28	0.27	0.27	0.28	0.79	0.17		71.44	35.29	27.41	26.21	22.41	26.41	11.94	20.00	18.29	15.12	15.70	6.57	12.99	11.06	7.03	7.25
EU031770	10	0.28	0.28	0.30	0.29	0.29	0.30	0.87	0.37	0.36		33.84	26.07	24.18	18.70	21.94	10.99	16.20	17.50	17.76	12.83	6.52	12.77	11.05	7.21	6.45
EU031764	11	1.25	1.22	1.13	1.29	1.31	1.38	0.45	1.66	1.49	1.60		72.63	55.33	40.81	53.90	24.79	35.89	50.88	29.95	26.56	16.23	31.57	26.63	18.47	12.72
EU031762	12	1.99	1.94	1.76	2.12	2.17	2.35	0.79	3.81	2.58	3.18	0.34		35.24	22.08	34.00	15.95	17.32	32.07	11.63	9.45	11.94	20.42	13.62	9.59	0.00
EU017383	13	∞	∞	∞	∞	∞	∞	1.19	2.05	3.09	∞	0.68	1.49		72.14	70.60	34.43	64.82	53.29	39.66	45.93	20.41	50.91	42.27	20.54	25.16
EU031758	14	∞	∞	∞	∞	∞	∞	1.85	3.08	∞	∞	1.17	∞	0.35		66.59	27.49	85.75	63.34	51.30	60.09	23.99	58.11	50.54	24.34	29.28
EU031751	15	∞	∞	∞	∞	∞	∞	1.34	∞	2.98	∞	0.72	1.59	0.37	0.44		31.69	58.45	65.89	44.32	42.92	24.03	48.45	41.45	24.37	21.05
EU031761	16	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	1.56	2.55	1.81		26.01	24.47	22.72	19.27	25.79	19.46	17.76	15.64	9.93
EU031772	17	∞	∞	∞	∞	∞	∞	2.44	∞	∞	∞	1.45	∞	0.47	0.16	0.61	3.23		55.03	58.96	67.53	22.93	63.93	54.55	26.93	31.55
EU031759	18	∞	∞	∞	∞	∞	∞	1.60	∞	∞	∞	0.80	1.77	0.73	0.50	0.45	∞	0.69		44.50	38.35	26.52	61.30	52.05	31.53	17.41
EU031765	19	∞	∞	∞	∞	∞	∞	4.56	∞	∞	∞	2.04	∞	1.22	0.79	1.02	∞	0.59	1.01		61.24	16.62	39.36	47.19	24.92	28.79
EU031757	20	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	2.91	∞	0.96	0.57	1.07	∞	0.43	1.29	0.55		16.18	44.42	50.14	21.07	30.50
EU031766	21	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	3.41	∞	2.93	∞	∞		21.38	15.82	17.16	5.37
EU017381	22	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	1.83	∞	0.80	0.61	0.87	∞	0.49	0.54	1.24	1.01	∞		76.39	28.15	15.34
EU031763	23	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	2.87	∞	1.10	0.81	1.14	∞	0.70	0.76	0.91	0.82	∞	0.28		28.35	19.40
EU031771	24	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	2.74	1.83	∞	∞	∞	2.38	2.33		12.50
FJ604109	25	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	∞	4.62	2.15	∞	∞	1.83	∞	2.24	1.96	∞	∞	∞	∞	

CHAPITRE 4.

BIOFLOCCULATION ET DE DÉSHYDRATATION UTILISANT LES SOUCHES DE MYCÈTES FILAMENTEUX

PART I

A NEW, PELLET-FORMING FUNGAL STRAIN: ITS ISOLATION, MOLECULAR IDENTIFICATION, AND PERFORMANCE FOR SIMULTANEOUS SLUDGE-SOLIDS REDUCTION, FLOCCULATION, AND DEWATERING

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PART II

SIMULTANEOUS SOLIDS AND PATHOGENS REDUCTION, SETTLING AND DEWATERING (SSPRSD) USING A FILAMENTOUS FUNGAL STRAIN *PENICILLIUM EXPANSUM BS30* ISOLATED FROM WASTEWATER SLUDGE

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RÉSUMÉ

Dans cette étude, un champignon filamenteux (*Penicillium expansum* BS30) isolé de boues activées d'usine de traitement des eaux a été utilisé afin de réduire les solides dans les boues, de réduire la prolifération de pathogènes, d'améliorer la floculation des boues ainsi que la déshydratation (SSPRSD). Ces essais ont été réalisés en erlenmeyers et en fermenteur de 10L. Le rôle de la souche fongique dans le processus de SSPRSD a été évalué à différentes températures et à différentes concentrations d'inoculum (spores). Les conditions optimales pour le procédé sont une température d'incubation de 25°C et une concentration d'inoculum de 10^6 spores/mL. Dans ces conditions, les solides totaux et les solides volatiles totaux ont été dégradés à plus de 50% et à plus de 53% respectivement. Le temps capillaire de succion (CST) (moins de 13s) est plus bas que le temps requis pour l'assèchement des boues (CST <20s). Les populations de coliformes et de Salmonelle (indicateur de pathogènes) ont été réduites de 2 logs et de 4 logs respectivement. Des études génétiques ont également été réalisées sur la biosynthèse des pénicillines, sur la ségrégation des gènes et sur la machinerie de dégradation des matières organiques toxiques (MOT). Le champignon produit effectivement des gènes de production de pénicillines, mais ne contient pas de gènes MOT, ce qui rend cette souche incapable de dégrader ce genre de substances.

ABSTRACT

A filamentous fungal strain (*Penicillium expansum* BS30) isolated from a municipal wastewater treatment plant was used in this study to simultaneously reduce sludge solids, pathogens, and improve the sludge settling and dewaterability (SSPRSD) in shake-flask and 10 L bioreactor experiments. Fungal strain role in SSPRSD process was evaluated at different temperatures and inoculum (spores) concentrations. The best performance of the process was achieved at incubation temperature 25°C and inoculum concentration of 10^6 spores/mL. At these

optimal conditions, suspended solids (SS) and volatile suspended solids (VSS) were degraded >50 and >53 %, respectively. The capillary suction time (CST) value recorded (<13 s) was lower than that required for sludge dewaterability (<20 s). The populations of total coliforms and *Salmonella* (pathogen indicators) were reduced by 2 and 4 log cycles, respectively. A study on molecular screening of penicillin biosynthesis gene cluster and toxic organic compounds degrading machinery of the fungal strain was also conducted. It was found that the fungal strain, possessed the penicillin-producing gene, however, it did not contain toxic organic compounds degrading genes, and therefore may not be helpful in degrading these compounds.

Key words: Filamentous fungi; Activated sludge; Zeta potential; Sludge settling; dewatering.

INTRODUCTION

Biological wastewater treatment results in the generation of a considerable amount of sludge as a byproduct that should be safely disposed or recycled. Cost of sludge treatment has been estimated to be 50–60% of the total expense of a wastewater treatment plant (Egemen *et al.* 2001). Conventional disposal method of land filling is becoming more and more difficult due to limited landfill spaces and secondary pollution problems. Therefore, efforts have been made to reduce volume and mass of the wastewater sludge by aerobic/anaerobic digestion. One of the most important steps in sludge disposal is the solid-liquid separation (flocculation, settling and dewatering). In general, sludge settling is enhanced by the addition of synthetic polymers, but they are known to be expensive and may further pollute the environment (Chang *et al.* 2002; Deng *et al.* 2003). To minimize the use of synthetic flocculants, an alternative and novel approach is to use ecofriendly biocoagulants/bioflocculants.

Role of extracellular polymeric substances (EPS) produced by sludge microorganisms (during wastewater treatment or sludge digestion) on sludge flocculation has been extensively studied in recent years (Urbain *et al.* 1993; Higgins and Novak 1997; Jorand *et al.* 1998; Houghton *et al.* 2001; Liao *et al.* 2001; Sobeck and Higgins 2002; Vogelaar *et al.* 2005). In general, EPS facilitates bioflocs formation. Bioflocs are formed together with microbial aggregates, filamentous bacterial strains, and organic and inorganic sludge particles, which are held together by EPS (Novak *et al.* 2003). Several EPS producing bacterial strains have been isolated from various environmental sources (Kurane *et al.* 1994; Nakata and Kurane 1999; Fujita *et al.* 2001; Zang *et al.* 2002; Deng *et al.* 2003; Gao *et al.* 2006). Further, filamentous fungal (FF) strains play an important role in bioflocculation of solids. They act as a backbone to form larger and stronger bioflocs than bacterial biopolymers (Alam *et al.* 2001). It is also widely known that the presence of excessive filamentous microbial growth in activated sludge may lead to operational problems of

sludge bulking (poor sludge settling and thickening) which hamper the solid-liquid separation process (Jenkins *et al.* 2004; Jenkins *et al.* 1993; Wanner 1994; Davenport *et al.* 2000).

In previous study, a filamentous fungal (FF) strain (*Penicillium expansum* BS30) isolated from wastewater sludge (Genbank accession number EF491160) was used to degrade sludge solids (sludge decomposition) and to improve sludge dewaterability at different suspended solids concentration with and without heat treatment (Bala Subramanian *et al.* 2008). The sludge dewaterability was improved up to 10 g/L of initial SS concentration used. The raw activated sludge, inoculated with the fungal spores, revealed floc formation during growth, better dewaterability than that inoculated with the fungal beads. This process was named as SSRSD (Simultaneous Sludge-Solids Reduction, Settling and Dewatering) (Bala Subramanian *et al.* 2008).

Due to extreme seasonal variations, the operating temperature, pH, dissolved oxygen and sludge characteristics (excess nutrient/substrate in feed, feed rate and C/N ratio) in a municipal wastewater treatment plant may vary to a large extent. Apparently, this variation may affect the fungal growth (biomass concentration), which in turn may change the performance of sludge solids reduction, biofloc formation and sludge dewaterability. Further, the amount of fungal spores added (inoculated) to sludge can severely affect the solids reduction and biofloc formation. High concentration of spores may cause excessive growth of fungal biomass that could deteriorate the biofloc formation (sludge bulking), whereas lower concentration of spores may result in lower growth which may decrease the solids degradation rate. Therefore, the objective of this research was to optimize the SSRSD process at different temperatures and inoculum (spores) concentrations, to achieve maximum sludge degradation and sludge settling/dewatering. Impact of fungal growth on pathogen reduction was also investigated. Many fungal strains are known to possess enzymatic system to degrade toxic pollutants (González *et al.* 2003; Liu *et al.* 2004; Quintero *et al.* 2008). Therefore, besides the sludge solids reduction and improvement in sludge

dewatering by fungal mediated aerobic digestion process, in this study we also examined whether toxic organic compounds degrading genes were present or expressed during growth of this fungal strain.

MATERIALS AND METHODS

Activated sludge

Activated sludge was collected (before adding chemical polymers) from the Victoriaville wastewater treatment plant in Québec, Canada. The WWTP treats domestic wastewater employing a sequential batch reactor. The activated sludge had total solids (TS) concentration of 4.6±0.6 g/L, suspended solids (SS) concentration of 3.2±0.4 g/L and a pH of 6.38±0.08. The wastewater sludge was collected directly from the thickener. Collected sludge samples were stored at 4ºC for less than 24 h before use and there was no sludge degradation during storage. The Victoriaville WWTP employs 10-15 g/L SS for aerobic sludge digestion, therefore, in this study lower range of 10 g/L of SS were used. For the shake flask experimental studies, samples were collected in May 2008. The reproducibility studies were conducted from the activated sludge samples collected in August 2008 and November 2008, for shake flask and 10 L reactor experiments, respectively.

Microorganisms used

A filamentous fungal strain *Penicillium expansum* BS30 isolated from activated sludge (collected from Communauté Urbaine du Québec, Quebec, QC, Canada) was used in this study (Bala Subramanian *et al.* 2008).

Experimental plan

Four sets of experiments were performed, to assess the optimal conditions for the fungal mediated SSPRSD process, by varying two parameters [temperature and inoculum concentration

(spores/mL)]. Temperature range evaluated in this study was 15, 25, 35 and 45° C, and the initial (beginning of the experiments) spore concentration was varied in the order of 10^4, 10^5, 10^6 and 10^7 spores/mL. Each experimental setup was carried out at a single temperature (temperature variations of ±0.5ºC as per the manufacturer, New Brunswick G25 shaker incubator, GMI, Inc. Minnesota, USA) in five experimental flasks, which consisted of four different concentrations of inoculum (fungal spores) and a control without inoculum. Fungal spores were obtained as described by Bala Subramanian *et al.* (2008).

Each set of five experimental flasks (described above), with different inoculum concentrations (10^4, 10^5, 10^6 and 10^7 spores/mL), and a control was designated as: (i) study temperature at 15°C with control and four different inoculum concentrations were named as 15D-Con, 15D-4, 15D-5, 15D-6 and 15D-7; (ii) temperature at 25°C with control and four different inoculum concentrations were named as 25D-Con, 25D-4, 25D-5, 25D-6 and 25D-7; (iii) temperature at 35°C with control and four different inoculum concentrations were named as 35D-Con, 35D-4, 35D-5, 35D-6 and 35D-7; and (iv) temperature at 45°C with control and four different inoculum concentrations were named as 45D-Con, 45D-4, 45D-5, 45D-6 and 45D-7. The experiments were conducted in 500 mL shake flasks, each containing 200 mL of fresh sludge with suspended solids concentrations of 10 g/L and incubated for 5 days at 200 rpm in an orbital shaker.

The samples were drawn at regular intervals to measure changes in suspended solids. SS concentration was measured during five days of experiments (APHA, 1995). Initial and final pH of the growth medium were also measured. Zeta potential of fungal grown sludge was measured at the end of incubation time (5^{th} day). The sludge settling and dewatering characteristics were measured in terms of sludge volume index (SVI) and capillary suction time (CST), respectively, at the end of growth.

Sludge volume index

SVI was measured to determine the sludge settling efficiency of the fungal biomass grown in sludge after 5 days of incubation. Each experimental sample was transferred into a 100 mL graduated measuring cylinder for $SVI_{0.1}$ measurement as described by Bala Subramanian *et al.* (2008). The effect of cations (Ca^{++} ions) on sludge settling was measured by adding 100 mg/L of $CaCl_2$ solution to the sludge prior to performing the SVI test. Effect of cationic synthetic polymers (CSP) on SVI was studied by adding 0.1 g/L of CSP to fresh sludge.

Capillary suction time

Samples were prepared similar to the SVI experiments. CST experiments were carried out using standard protocol as mentioned by manufacturer (Triton electronics, Model 304M CST, Dunmow, Essex, UK). All four different sets of experiments along with control at different inoculum concentrations were subjected to CST test. Also, the effect of cations (Ca^{++} ions) and CSP on dewatering were measured.

Pathogens removal and competitive fungal growth in sludge

Pathogens removal was determined by measuring concentration of *Salmonella* and fecal coliforms in the beginning and at the end of 5 days experiments. All sludge samples were serially diluted and plated on suitable agar media, to enumerate the microbial growth in colony forming units (CFU/mL). Two different media, endo agar (EA) (fecal coliforms) and Lactose selenite and tetrathionate agar [(LSTA) selenite and tetrathionate were externally supplemented with lactose broth] (fecal *Salmonella*) were used to enumerate the initial and final concentrations of pathogen in the sludge (Kramer and Liu, 2002; Quinlan 1990). After pathogens enumeration, this SSRSD process was renamed as SSPRSD process (Simultaneous Solids and Pathogens Reduction, Settling and Dewatering). Also, competitive growth of the filamentous fungi with natural sludge

microorganisms was examined by plating initial and final sludge samples on potato dextrose agar (PDA) plates.

Molecular corroboration of antibiotic producing and pollutant degradative genes

The mechanism of pathogens reductions in SSPRSD process, mediated by *Penicillium expansum* BS30 strain, was evaluated using molecular biology techniques (White *et al.* 1990; Vanittanakon *et al.* 2002). Fungal genomic DNA isolation and PCR amplification followed by gene sequencing to confirm the presence of penicillin biosynthesis gene cluster in the *P. expansum* BS30 was conducted as described in the previous study (Bala Subramanian *et al.* 2008). Specific PCR primers for the penicillin biosynthesis gene cluster amplification were utilized as reported by Kosalková *et al.* (2007). The forward primer <5′-agaccaatgcagcaggcc-3′> and reverse primer <5′-gttgacgcactccttcca-3′> sequences were custom synthesized (Centre Hospitalier Université Laval (CHUL), Quebec, QC, Canada). The amplified PCR products were purified using Qiagen gel extraction kit and sequenced (ABI 3730xl DNA analyzer, Applied Biosystems, Foster City, CA). The obtained penicillin biosynthetic gene sequences were submitted to Genbank [National Center for Biotechnology Information (NCBI), Bethesda, MD, USA] under the accession number FJ604110. The 18S-28S rRNA gene sequences of *P. expansum* BS30 strain accession number EF491160.

Molecular screening of the laccase gene (which degrades toxic compounds like lignin and endocrine disrupters), aromatic (phenol, xlyene, toluene, napthalene) and aliphatic (alkanes) hydrocarbon degrading genes was examined using specific PCR primers. PCR amplification and direct sequencing of degradative genes were conducted as mentioned (Smits *et al.* 1999; Ringelberg *et al.* 2001; D'souza *et al.* 1996; Hoshida *et al.* 2001; Gonzalez *et al.* 2003; Collins and Dobson 1997; O'Leary *et al.* 2001).

Zeta potential

Surface charge [zeta potential (ζ)] of fresh sludge and fungal spores grown on sludge from all four different experiments were measured using zetaphoremeter (Zetaphoremeter IV, Zetacompact Z8000, CAD Instrumentation, France) with the application of the Smoluckowski equation (Liao *et al.* 2001). Also, the effect of cations (Ca^{++}) on zeta potential value was measured from SVI and CST tests samples. In order to check the reproducibility of the zeta potential results, a minimum of 12 samples were measured (at the same time) from the same experimental setup.

Reproducibility studies in shake flask and 10 L reactor

In order to obtain reproducible results, at 25°C with spore concentration of 10^6 spores/mL, the experiments were conducted in shake flasks using triplicates including a control as well. The initial and final variations of pH, SS, VSS, SVI, CST, zeta potential, pathogen reduction and fungal adaptation were studied. Presence of laccase enzyme producing genetic machinery was verified (repeated) using PCR.

Further, the obtained optimized conditions (at 25ºC with 10^6 spores/mL) of SSPRSD processes from the repeated studies in shake flasks were verified using 10 L (working volume) reactor experiments. Sludge samples were collected as mentioned above and 10 L of sludge at SS concentration of 10 g/L was used in two 20 L (total volume) reactors operated simultaneously (one control and the other inoculated with fungi). The reactor experiments were carried out at 25ºC with 10^6 spores/mL, with aeration using an aquarium type of sparger fixed at the bottom of the reactor and agitation speed of 30 to 50 rpm. The reactor was operated and monitored for 5 days. The pH, SS, VSS, SVI, CST, zeta potential, pathogen reduction and fungal adaptation were analyzed as described above.

RESULTS AND DISCUSSION

Microbial adaptation (competitive growth)

Competition and adaptation of the filamentous fungal strain in the SSPRSD process was examined with the natural microbial community that exists in sludge by plating the samples collected at the end of growth (5^{th} day). The results revealed that fungal strain exhibited adaptation in sludge and grew competitively over natural microbial flora. This was confirmed by final cell count of the fungal colonies on PDA plates (Table 1) in all four sets of different experiments. The highest fungal colony count was observed in samples incubated at 25 and 35ºC (optimum for growth), and at initial spore concentration of 10^6 spores/mL. Therefore, 25-35ºC was the optimal range of temperature for FF growth. The lower temperature at 25ºC with 10^6 spores/mL was chosen for further studies on reproducibility. The reason for the decreased fungal concentration in experiments with inoculum concentration 10^7 (spores/mL) irrespective of operating temperatures, could be due to substrate limitation.

Variation of pH

Initial (before fungal inoculation) pH of the sludge was 6.43. The final pH (after fungal growth) of the fungal grown sludge ranged from 4.74 to 8.36 (Figure 1). The final pH of the sludge incubated at 15 and 25ºC decreased from 6.43 to 4.80, irrespective of inoculum concentrations including the control, this could be due to production of organic acids during fungal growth. The final (5^{th} day) pH of sludge samples incubated at 35°C changed to a lesser extent (differences of 0.52 – 0.63 from initial pH), whereas the final pH of sludge samples incubated at 45°C increased up to 8.40 (Figure 1).

The trend of pH changes for all four different incubation temperatures (15, 25, 35 and 45°C) did not exhibit a direct relation with the inoculum (spores) concentration. The observed

variation was mainly due to the effect of temperature on FF strain and natural sludge microbial growth. Higher incubation temperature (45ºC) probably led to cell lysis and caused pH increase. The pH increase could be due to ammonia release from degradation of sludge proteins (and/or proteins released by cell lysis).

SS reduction during fungal growth

The profiles of SS reduction and/or utilization (as nutrients) by fungal biomass, during 5 days of incubation at different temperatures and initial spore concentrations, are presented in Figure 2. There was an increased SS reduction between days 4 and 5 (Figure 2). This was due to the fact that fungal spores were inoculated in the sludge might have taken some time to germinate. After germination fungal biomass growth was slow in the initial period. Once sufficient fungal biomass was produced, the fungi degraded the sludge solids at a comparatively faster rate and thus SS reduction rate was increased (after day 4) (Figure 2). Profiles of SS reduction in all experiments displayed significant decrease in SS concentration than control, which revealed that fungal biomass utilized (degraded) sludge solids irrespective of temperatures and initial spore concentrations. In control (without inoculation of fungal biomass), 18 to 21% SS degradation was observed. The SS degradations in control sludge (15D-Con, 25D-Con, 35D-Con and 45D-Con) were due to metabolic activity of the natural microbial community present in the sludge (Figure 2). SS reduction of 30 to 50% was observed under different experimental conditions (Figure 2) and was of the same order as demonstrated by other researchers (Mason *et al.* 1987; Novak *et al.* 2003). The degradability of sludge solids mainly depends on sludge retention time (SRT) in the treatment process, solids concentration, microbial community, and operating conditions [dissolved oxygen (DO), temperature and pH] (Metcalf and Eddy 1991). In order to pronounce that the sludge is digested, a minimum of 39% VSS must be degraded, and 400 degree-days value is required to achieve sludge stabilization (Metcalf and Eddy 1991). In this study, more than 53% VSS reduction

was achieved in 125 degree-days (Table 2). Therefore, this process was comparable/better than a conventional sludge digestion process and those reported in the literature (Table 2).

The data on SS degradation, obtained at different temperatures and initial spore concentrations, were analyzed by estimating the first-order degradation rate constant (K_d). The values of K_d obtained are presented in Figure 3. The coefficient of determination value (R^2) varied between 0.78 and 0.98. The maximum value of K_d (1.02 d^{-1}) observed at 25ºC and at 10^6 initial spores/mL, was 2.3 times higher than the control. The K_d values, at 25ºC and at initial spore concentration of 10^5 spores/mL (0.93 d^{-1}) and at 10^7 spores/mL (0.94 d^{-1}), were similar and insignificantly different from the maximum value (1.02 d^{-1}). Increase or decrease of temperature from 25ºC, substantially reduced the K_d value, indicating temperature sensitivity of the fungi to grow in sludge and its capability to degrade sludge solids. However, irrespective of temperature and initial spore concentration, K_d value was significantly higher than control (1.7 times higher at 35 and 45ºC and 2.2 times higher at 15ºC). K_d value in control, increased with increase in temperature from 15 to 25ºC, however, it remained almost unchanged at a temperature higher than 25ºC demonstrating higher resilience of sludge dwelling microorganisms (than the inoculated fungi) to an increase of temperature.

The K_d value could vary depending on characteristics of feed sludge (sludge type), digester-operating conditions, pH, temperature, TSS (solids concentration) and oxygen concentration (Metcalf and Eddy 1991). The first-order degradation rate constant (K_d) values of 0.05 d^{-1} at 15ºC and 0.14 d^{-1} at 25ºC, for activated sludge, were reported by Metcalf and Eddy (1991), and WEF (1998). Similarly, K_d values of 0.32 – 0.64 d^{-1} at 18ºC, 0.40 d^{-1} at 20ºC and 0.20 d^{-1} at 10ºC have been reported for activated sludge (Salem *et al.* 2003; Henze *et al.* 1999). Arunachalam *et al.* (2004) reported K_d value of 0.096 d^{-1} for mixed mode of operation, whereas, in batch digestion K_d was 0.264 to 0.696 d^{-1}. Thus, the maximum value of K_d reported in this work was 2.5 to 13.2 times

higher than those reported in the literature, under various operating conditions. This confirms that this process is much more efficient for sludge solids degradation than a conventional aerobic sludge digestion process.

Pathogen removal

Fecal coliform bacteria are considered as the principal indicator for the presence of potentially dangerous pathogens. Fecal coliforms decreased by 2 log cycles in all experiments, whereas 1 log cycle was observed in the control, except at 45ºC (2 log cycles reduction). The reason for 2 log cycles reduction at 45ºC in control was probably due to the heat effect on coliforms. Further, a different degree of pathogen reduction was observed at different temperatures (Table 1) and at different inoculum (spores) concentrations. This was possibly due to difference in FF growth and its competition for sludge nutrients against pathogens and sludge microorganisms at different cultivation conditions.

Salmonella reduction in all experiments was 4-5 log cycles, and 2 log cycles in control. The reason for higher *Salmonella* reduction (4-5 log cycles) than coliforms reduction (2 log cycles) could be due to its susceptibility to growth of FF in sludge. Higher pathogens reductions in experiments than control could be also due to production of antimicrobial compounds by FF and/or competition with other microorganisms. Likewise, the reason for pathogen reduction in the control was due to competition among pathogens and the natural sludge microorganisms.

The penicillin biosynthetic gene sequences similarity search was carried out using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/). Similarity search results revealed more than 97% homology with the existing gene sequences of penicillin producers (*Penicillium chrysogenum* Wisconsin 54-1255, GenBank Acession number AM920428), which confirmed that FF used in this study possessed penicillin biosynthetic gene cluster necessary to produce penicillin during its growth. Though, the penicillin biosynthetic gene was present in the genome of

P. expansum BS30, the antibiotic (penicillin) production/expression in the sludge was not known. This fact must be studied, and relation to antibiotic resistance development of natural microbial community should be verified.

Toxic organic compound degrading genes

PCR amplification and direct sequencing of *P. expansum* BS30 genome using specific primers for various degradative genes (Table 3) revealed no PCR amplification and sequencing signals. The toxic organic compounds degradative genes were not present in the isolated FF strain.

Sludge volume index

The sludge with good settling characteristics should have $SVI_{0.1}$<30 mL (Tarre and Green, 1994). $SVI_{0.1}$ for all four different experiments with or without addition of Ca^{++} ions were >30 mL (Figure 4). Addition of Ca^{++} ions slightly improved or did not have any significant impact on sludge settling (Figure 4). The reason for high $SVI_{0.1}$ value >30 mL was due to higher SS concentration used in this study, which hindered the solids settling. Effect of cationic synthetic polymers (CSP) on SVI was also studied by adding 0.1 g/L of CSP to fresh sludge. The $SVI_{0.1}$ value was 30 mL for fresh sludge (with CSP) and 90 mL for control sludge (5 days incubated without CSP). The lowest $SVI_{0.1}$ (≤40 mL) was found for sludge sample incubated at 25°C with inoculum (spore) concentration of 10^6 spores/mL with cations.

The reason for increased SVI value (irrespective of the operating temperature) at initial higher inoculum concentration (10^7 spores/mL) could be due to the fact that fungal biomass was decreased from the initial value of 10^7 to ≈10^5 CFU/mL (Table 1). Since there was no growth of fungi and hence there was no floc formation, which rendered increased SVI values.

Bioflocs were formed containing fungal mycelium and sludge solids (comprising natural microbial flora, EPS, non-degraded solids and/or inert solids), which rendered lower SVI values

(SVI ≈ 40 mL). Fungal mycelium interacted with sludge solids and other natural microbes which aided in the formation of strong and larger flocs. The fungal hyphae served as a backbone to form bioflocs.

Capillary suction time

Capillary suction time test is widely used to measure the sludge dewaterability. Generally, the lower CST value is better for sludge filterability and dewaterability. For sludge dewatering, using a filter press, CST values between 15 and 20 s and for a centrifuge, CST values of 15 s or less are acceptable (CST Equipment manual, Triton Electronics Ltd, Dunmow, Essex, UK). In control (without fungal spore inoculation), CST values were found to be higher than treated samples with and without fortification of Ca^{++} (Figure 5). Sludge samples incubated at 15, 25 and 35°C exhibited better dewatering (CST values <15 s) than the sludge incubated at 45°C (CST values >60 s), irrespective of the inoculum concentrations. These results demonstrated that the isolated fungi when cultivated in sludge performed well at wide range of temperatures from 15 to 35°C to dewater the sludge. In most cases, addition of calcium ions slightly deteriorated or did not have any impact on the dewaterability of the fungal grown sludge at all experimental conditions (Figure 5).

CST value of 13.2 s was observed when CSP (0.1 g/L) was added to fresh sludge (with and without addition of Ca^{++}). FF strain grown sludge exhibited better CST values (<13.2 s) at 25 and 35ºC. Poor dewaterability at higher temperature (45°C) could be due to increased pH (Figure 1), which increased hydroxyl ions (OH^-) in the FF grown sludge. As described earlier, sludge solids and fungal biomass were negatively charged; therefore, further increase in negatively charged ions (direct proportional to pH) had a detrimental effect on sludge dewatering.

One important point is that, the SVI values were found higher than the required values in all experiments, but the sludge dewatering capacity (CST values) was improved/better than the

control and CSP fortified sludge. This confirmed that SVI measurement was not a direct measurement of dewatering and could be used to measure only sludge flocculation and settling.

Zeta potential

Zeta potential (ZP) values of -77.49±7.03 and -49.42±3.67 mV were recorded for fresh sludge and pure fungal biomass (grown in synthetic media), respectively. Furthermore, the biofloc containing sludge solids and fungal biomass (inoculated spores after 5 days of incubation) also revealed negative charge (Figure 6), in all four different sets of experiments. Although fungal mycelium carried a negative charge (anionic in nature), the interactions with negatively charged sludge solids most likely occurred due to physical entrapment.

The ZP values of samples inoculated at 15 to 35°C ranged between –67.34±3.7 and –52.91±4.2 mV. Samples inoculated at 45°C, irrespective of inoculum concentrations, exhibited higher negative charge (between –89.68±3.2 and –85.07±2.1 mV). Higher incubation temperature (45ºC) might have enhanced the release of hindered ions from sludge solids, which increased ZP values. This was also one of the probable reasons for higher CST values at 45ºC than other 3 sets of experiments (Figure 5), as discussed before.

In order to improve the biofloc formation, Ca^{++} ions were used as a linker or bridging agent between the two negatively charged sludge particles (Bala Subramanian *et al.*, 2008). However, addition of calcium ions in sludge samples only rendered less negative charge (ZP values, –44.29±6.3 to –24.66±3.2 mV), but did not show any significant reduction in SVI and CST values. This revealed that ZP was not directly related to SVI and/or CST values. There are many other parameters that are responsible for sludge settling and dewatering characteristics (such as concentration, surface charge and hydrophobicity of EPS present in sludge and sludge characteristics) as mentioned elsewhere (Eriksson *et al.* 1992; Houghton *et al.* 2001; Mikkelsen and Keiding 2002).

Fungal pellet formation

In the previous study (Bala Subramanian *et al.* 2008), fungal pellets were produced in the synthetic media and this was used as inoculum to degrade SS and to improve sludge dewaterability. In the present study, instead of fungal pellets, spores were used as inoculum to conduct SSPRSD process. Though, use of spores as inoculum, the fungal pellets did not form effectively (monitored by visual observation), however, dewaterability was substantially improved with concomitant degradation of substantial amount of sludge solids and pathogen reduction (Figure 2, Tables 1 and 4). Thus, this study established that there was no need of production of beads/pellets in synthetic medium to achieve simultaneous dewaterability and solids degradation as reported in earlier study (Bala Subramanian *et al.*, 2008). This could lead to significant reduction in process operation cost.

On the other hand, it is well known that filamentous organisms cause major problems in sludge settling (sludge bulking) (Richard 2003); however, in this study it has been demonstrated that the floc forming fungal strain was helpful in improving sludge solids degradation and sludge dewaterability. Thus, it is suggested that a large-scale study needs to be carried out in order to strengthen the concept for eventual practical applications.

Reproducibility

Reproducibility results are presented in Table 4. In shake flask reproducible studies, SS and VSS decreases of 23.96±0.13 and 27.09±0.32%, respectively, were observed in the control whereas 48±0.27 and 53.96±0.45%, respectively, decreases were observed in FF inoculated flasks. In 10 L reactor, the SS and VSS decreases were found higher than shake flask results (Table 4). The reason for improved SS degradation in 10 L reactor could be due to availability of more oxygen. A decrease of pH was found at the end of experiments (5^{th} day) in shake flasks as well as 10 L reactor.

The sludge settling and dewatering capacities of FF grown sludge were improved substantially in both shake flask and 10 L reactor studies (Table 4). This could be due to adaptation of fungal biomass in sludge and formation of flocs. Coliforms (2 log cycle) and *Salmonella* (4 log cycle) reduction were observed in FF grown sludge. Toxic organic compounds degrading genes were not present in the genome of the FF strain as per the results of molecular biology studies. Simultaneous degradation of toxic organic compounds, during growth of the FF may not occur. The reproducibility study confirmed that isolated FF strain could be used to achieve SSPRSD processes in the 10 L reactor.

CONCLUSIONS

A new process for simultaneous sludge solids degradation, pathogen reduction, and sludge settling and dewatering improvement employing a sludge isolated filamentous fungi was demonstrated. A maximum suspended solids degradation of 50% was achieved at a temperature 25°C and initial fungal spore concentration of 10^6 spores/mL. The first-order solids degradation rate constant (K_d) value (1.02 d^{-1}) at 25ºC with initial spore concentration of 10^6 spores/mL was 2.3 times higher than the control. A decrease in temperature to 15ºC resulted in decrease of K_d value, however, it was still 2.2 times higher than the control. In this study, a 2.5 to 13.2 fold higher K_d value was observed than those reported in the literatures on aerobic sludge digestion. In fungal treated sludge, indicator pathogen removal of 4-5 log cycles was achieved. At the end of the process, sludge could be dewatered easily with CST value less than 13 s, which was less than the required CST value (20 s). Moreover, CST value was below 20 s at temperatures 15 to 35ºC, but deteriorated at 45ºC. Fungal strain possessed the penicillin biosynthesis gene but did not contain toxic organic compound degrading genes. The reproducibility study, conducted in 10 L bioreactor, revealed enhanced SS reduction (54%), 2-4 log cycles pathogen reduction and better sludge dewaterability (<20 s).

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REFERENCES

Alam, M. Z., Fakhru'l-Razi, A., Molla, A. H., and Roychoudhury, P. K. (2001). "Treatment of wastewater sludge by liquid state bioconversion process." *J Environ Sci Health.*, A36(7), 1237–1243.

American Public Health Association (APHA)/American Water Works Association/Water Environment Federation, (1995). Standard Methods for the Examination of Water and Wastewater, 19th eds, Washington DC, USA.

Arunachalam,R., Shah, H.K., and Ju, L.K. (2004). "Aerobic Sludge Digestion under Low Dissolved Oxygen Concentrations." *Water Environ Res.*, 76 (5), 453 – 462.

Bala Subramanian, S., Yan, S., Tyagi, R. D., and Surampalli, R. Y. (2008). "A New, Pellet Forming Fungal Strain: Its Isolation, Molecular Identification, and Performance for Simultaneous Sludge Solids Reduction, Flocculation, and Dewatering." *Water Environmental Research.*, 80 (9), 840-852.

Chang, L. L., Bruchb, D. M., Griskowitzc, N. J., and Dentel, S. K. (2002). "NMR spectroscopy for determination of cationic polymer concentrations." *Water Research*., 36(9), 2255–2264.

Collins, P.J., and Dobson, A.D.W. (1997). "Regulation of laccase gene transcription in Trametes versicolor." *Appl. Environ. Microbiol.*, 63, 3444-3450.

D'souza, T. M., Boominathan, K., and Reddy, C. A. (1996). "Isolation of Laccase Gene-Specific Sequences from White Rot and Brown Rot Fungi by PCR." *Appl. Environ. Microbiol.*, 62(10), 3739-3744.

Davenport, R. J., Curtis, T. P., Goodfellow, M., Stainsby, F. M., and Bingley, M. (2000). "Quantitative use of fluorescent in situ hybridization to examine relationships between mycolic acid-containing actinomycetes and foaming in activated sludge plants." *Appl. Environ. Microbiol*., 66, 1158–1166.

Deng S. B., Bai, R. B., Hu, X. M., and Luo, Q. (2003). "Characteristics of a bioflocculant produced by Bacillus mucilaginosus and its use in starch wastewater treatment." *Appl Microbiol Biotechnol.*, 60, 588–593.

Egemen, E., Corpening, J., and Nirmalakhandan, N. (2001). "Evaluation of an ozonation system for reduced waste sludge generation." *Water Sci. Technol.*, 44 (2–3), 445–452.

Eriksson L., Steen, I., and Tendaj, M. (1992). "Evaluation of sludge properties at an activated sludge plant." *Water science and technology.*, 25, 251-265.

Fujita, M., Ike, M., Jang, J. H., Kim, S. M., and Hirao, T. (2001). "Bioflocculation production from lower-molecular fatty acids as a novel strategy for utilization of sludge digestion liquor." *Water Science & Technology,*, 44, 237-243.

Gao, J., Ying, B.H., Xiu, X.M., Xia, L.Y., Qian, L., and Fen, Z.Y. (2006). "Characterization of a bioflocculant from a newly isolated Vagococcus sp. W31." *J. Zhejiang Univ. Science B.*, *7*(3), 186–192.

González, T., Terrón, M. C., Zapico, E. J., Yagüe, S., Téllez, A., Junca, H., and González, A. E. (2003). "Identification of a new laccase gene and confirmation of genomic predictions by cDNA sequences of Trametes sp. I-62 laccase family." *Mycol. Res.*, 107(6), 727-735.

Henze, M., Gujer, W., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.V.R., and Van Loosdrecht, M.C.M. (1999). "Activated Sludge Model No.2d, ASM2D." *Water Science and Technology.*, 39 (1), 165–182.

Higgins, M. J., and Novak, J. T. (1997). "Characterization of Exocellular Protein and Its Role in Bioflocculation." *J. Environ. Eng*., 123, 479-485.

Hoshida, H., Mitsuhide, N., Hidenobu, K., Kanako, K., Toru, H., Koji, M., Rinji, A., and Yoshinor, N. (2001). "Isolation of five laccase gene sequences from the white-rot fungus *Trametes sanguinea* by PCR, and cloning, characterization and expression of the laccase cDNA in yeasts." *Journal of bioscience and bioengineering*., 92 (4), 372-380.

Houghton, J. I., Quarmby, J., and Stephenson, T. (2001). "Municipal wastewater sludge dewaterability and the presence of microbial extracellular polymer." *Water Sci Technol.*, 44(2-3), 373-379.

Jenkins, D., Richard, M. G., and Daigger, G. T. (1993). "Manual on the causes and control of activated sludge bulking and foaming." 2nd ed. Lewis Publishers, Chelsea, Mich.,

Jenkins, D., Richard, M. G., and Daigger, G. T. (2004). "Manual on the causes and control of activated sludge bulking and foaming and other solids separation problems." Lewis Publishers, Washington DC, p. 190.

Jorand, F., Bigne, F. B., Block, J. C., and Urbain, V. (1998). "Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances." *Water Sci Technol.*, 37(4-5), 307-315.

Kosalková, K., Sáiz, M. R., Barredo, J. L., and Martín, J.F. (2007). "Binding of the PTA1 transcriptional activator to the divergent promoter region of the first two genes of the penicillin pathway in different *Penicillium* species." *Curr Genet.*, 52, 229–237.

Kramer, T.A., and Liu, J. (2002). "Enumeration of Coliform Bacteria in Wastewater Solids Using Defined Substrate Technology." *Water Environment Research.*, 74 (6), 526-530.

Kurane, R., Ahatamochi, K., Kakuno, T., Kiyohira, M., and Kawaguchi, K. (1994). "Characterization of liquid bioflocculant produced by *Rhodococcus erythropolis*." *Biosci. Biotechnol. Biochem.*, 58, 1977-1982.

Liao, B. Q., Allen, D. G., Droppo, I. G., Leppard, G. G., and Liss, S. N. (2001). "Surface properties of sludge and their role in bioflocculation and biosettleability." *Water Res.*, 35(2), 339-350.

Liu, Y.H., Liu, Y., Chen, Z.S., Lian, J., Huang, X. and Chung, Y.C. (2004). "Purification and characterization of a novel organophosphorus pesticide hydrolase from *Penicillium lilacinum BP303*." *Enzyme and Microbial Technology.*, 34 (3-4), 297-303.

Mason, C. A., Hamer, G., Fleischmann, T. H., and Lang, C. (1987). "Bioparticulate solubilization and biodegradation in semi-continuous aerobic thermophilic digestion." *Water, Air, and Soil Pollution*., 34, 399-407.

Metcalf and Eddy Inc., (1991). "Wastewater engineering: Treatment, disposal, reuse." (3rd Edn ed.), McGraw-Hill, Inc., New York, NY.

Mikkelsen, L. H., and Keiding, K. (2002). "Physico-chemical characteristics of full scale sewage sludges with implications to dewatering." *Water research.*, 36, 2451-2462.

Nakata, K., and Kurane, R. (1999). "Production of an extracellular polysaccharide bioflocculant by *Klebseilla pneumoniae*." *Biosci. Biotechnol. Biochem.*, 63, 2064-2068.

Novak, J. T., Sadler, M. E., and Murthy, S. N. (2003). "Mechanisms of floc destruction during anaerobic and aerobic digestion and the effect on conditioning and dewatering of biosolids." *Water Res*., 37 (13), 3136–3144.

O'Leary, N. D., O'Connor, K. E., Duetz, W., and Dobson A. D. W. (2001). "Transcriptional regulation of styrene degradation in *Pseudomonas putida* CA-3. Microbiology." 147, 973-979.

Quinlan, R. J. (1990). "Registration requirements and safety considerations for microbial pest control agents in the European Economic Community" In: Laird M, Lacey LA, & Davidson EW ed. Safety of microbial pesticides. Boca Raton, Florida, CRC Press, pp 11-18.

Quintero, J.C., Moreira, M.T., Feijoo, G., and Lema, J.M. (2008). "Screening of white rot fungal species for their capacity to degrade lindane and other isomers of hexachlorocyclohexane (HCH)." *Cien. Inv. Agr., 35(2), 159-167.*

Richard, M. (2003). "Activated sludge microbiology problems and their control." *Presented at the 20th annual USEPA national operator trainers conference.* June 08. Buffalo, NY.

Ringelberg, D. B., Talley, J. W., Perkins, E. J., Tucker, S. G., Luthy, R. G., Bouwer, E. J., and Fredrickson, H. L. (2001). "Succession of phenotypic, genotypic and metabolic community characteristics during in vitro bioslurry treatment of polycyclic aromatic hydrocarbon-contaminated sediments." *Appl. Environ. Microbiol.*, 67(4), 1542-1550.

Salem, S., Berends, D. H. J. G., Heijnen, J. J., and Van Loosdrecht, M. C. M. (2003). "Bio-augmentation by nitrification with return sludge." *Water Research.*, 37 (8), 1794-1804.

Smits, T. H. M., Rothlisberger, M., Witholt, B., and Van Beilen, J. B. (1999). "Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains." *Environ. Microbiol*., 1(4), 307-317.

Sobeck, D. C., and Higgins, M. J. (2002). "Examination of Three Theories for Mechanisms of Cation-Induced Bioflocculation." *Water Res*., 36, 527-538.

Tarre, S., and Green, M. (1994). "Precipitation potential as a major factor in the formation of granular sludge in an upflow sludge-blanket reactor for denitrification of drinking water." *Applied Microbiology and Biotechnology*., 42(2-3), 482-486.

Urbain, V., Block, J. C., and Manem, J. (1993). "Bioflocculation in activated sludge: an analytical approach." *Water Res.*, 27(5), 829-838.

Vanittanakom, N., Vanittanakom P., and Hay, R. J. (2002). "Rapid Identification of *Penicillium marneffei* by PCR-Based Detection of Specific Sequences on the rRNA Gene." *J of Clinical Microbiol*., 40(5), 1739–1742.

Vogelaar, J. C. T., Keizer, A. D., Spijker, S., and Lettinga, G. (2005). "Bioflocculation of mesophilic and thermophilic activated sludge." *Water Res.*, 39(1), 37-46.

Wanner, J. (1994). "Activated sludge bulking and foaming control." Technomic Publishing, Basel, Switzerland.

WEF. (1998). "Design of wastewater treatment plants" 4th ed., Manual of practice No.8, vol.3, Chapters 17-24, Water Environment Federation, Alexandria, VA.

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics." *In* M. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. *Academic Press, Inc.*, San Diego, Calif., p. 315–322.

Zang, J., Wang, R, Ziang, P., and Liu, Z. (2002). "Production of an exopolysaccharides bioflocculant by So*rangium cellulosum." Lett in Appl. Microbiol.,* 34, 178-181.

Table 1: Enumeration of pathogens and filamentous fungi growth during SSPRSD processes.

Agar media	Total fecal coliforms (CFU/mL)		*Salmonella* (CFU/mL)		Fungi (CFU/mL)	
Time	Day "0"	Day "5"	Day "0"	Day "5"	Day "0"	Day "5"
Enumeration of pathogenic & fungal strains at 15°C with different inoculum concentration						
15D-Con	$2.4\pm0.20\times10^{7}$	$7.0\pm0.34\times10^{6}$	$1.25\pm0.5\times10^{7}$	$6.5\pm0.20\times10^{5}$	No fungi	No fungi
15D-4		$2.0\pm0.24\times10^{5}$		$1.5\pm0.18\times10^{3}$	10^{4}	$2.0\pm0.35\times10^{5}$
15D-5		$1.5\pm0.12\times10^{5}$		$0.5\pm0.26\times10^{3}$	10^{5}	$5.0\pm0.23\times10^{5}$
15D-6		$6.3\pm0.11\times10^{5}$		$1.2\pm0.14\times10^{3}$	10^{6} 10^{7}	$1.3\pm0.15\times10^{6}$
15D-7		$3.3\pm0.39\times10^{5}$		$1.3\pm0.32\times10^{3}$		$9.0\pm0.24\times10^{5}$
Enumeration of pathogenic & fungal strains at 25°C with different inoculum concentration						
25D-Con	$2.4\pm0.20\times10^{7}$	$9.3\pm0.49\times10^{6}$	$1.25\pm0.5\times10^{7}$	$7.0\pm0.21\times10^{5}$	No fungi	No fungi
25D-4		$1.1\pm0.21\times10^{5}$		$4.0\pm0.61\times10^{3}$	10^{4}	$1.0\pm0.56\times10^{5}$
25D-5		$3.5\pm0.31\times10^{5}$		$0.1\pm0.48\times10^{3}$	10^{5}	$6.3\pm0.27\times10^{6}$
25D-6		$2.9\pm0.42\times10^{5}$		$1.0\pm0.30\times10^{3}$	10^{6} 10^{7}	$1.3\pm0.35\times10^{7}$
25D-7		$2.6\pm0.39\times10^{5}$		$1.5\pm0.29\times10^{3}$		$2.3\pm0.45\times10^{6}$
Enumeration of pathogenic & fungal strains at 35°C with different inoculum concentration						
35D-Con	$2.4\pm0.20\times10^{7}$	$2.1\pm0.30\times10^{6}$	$1.25\pm0.5\times10^{7}$	$7.5\pm0.34\times10^{5}$	No fungi	No fungi
35D-4		$1.5\pm0.46\times10^{5}$		$1.0\pm0.29\times10^{3}$	10^{4}	$6.0\pm0.10\times10^{5}$
35D-5		$2.2\pm0.50\times10^{5}$		$0.5\pm0.21\times10^{3}$	10^{5}	$1.0\pm0.05\times10^{6}$
35D-6		$1.1\pm0.34\times10^{5}$		$0.5\pm0.24\times10^{3}$	10^{6} 10^{7}	$2.2\pm0.38\times10^{7}$
35D-7		$2.0\pm0.23\times10^{5}$		$0.5\pm0.42\times10^{3}$		$2.0\pm0.23\times10^{6}$
Enumeration of pathogenic & fungal strains at 45°C with different inoculum concentration						
45D-Con	$2.4\pm0.20\times10^{7}$	$9.0\pm0.33\times10^{5}$	$1.25\pm0.5\times10^{7}$	$6.0\pm0.12\times10^{5}$	No fungi	No fungi
45D-4		$6.0\pm0.14\times10^{5}$		$1.0\pm0.22\times10^{3}$	10^{4}	$3.0\pm0.13\times10^{5}$
45D-5		$4.0\pm0.49\times10^{5}$		$0.1\pm0.19\times10^{3}$	10^{5}	$6.0\pm0.32\times10^{5}$
45D-6		$2.4\pm0.09\times10^{5}$		$0.2\pm0.40\times10^{3}$	10^{6} 10^{7}	$4.0\pm0.12\times10^{6}$
45D-7		$3.3\pm0.60\times10^{5}$		$0.1\pm0.33\times10^{3}$		$5.0\pm0.21\times10^{5}$

Table 2: Comparison of Degree-days and sludge solids degradation.

System used	Conditions	Degree days (d)	Solids degradation (%)	References
1.5 L reactor	Thermophilic digestion at 60°C for 3 d	180	59% of TSS	Mason *et al.* 1987
3 L reactor	Aerobic digestion at 20° C for 50 d	1000	50% of VSS	Novak *et al.* (2003)
Shake flask	15ºC for 5 days without FF strain*	75	19% of SS	This study
	15ºC for 5 days with FF strain		30 to 39% of SS	This study
	25ºC for 5 days without FF strain	125	21 to 24% of SS	This study
	25ºC for 5 days with FF strain		41 to 51% of SS	This study
	35ºC for 5 days without FF strain	175	20% of SS	This study
	35ºC for 5 days with FF strain		36 to 43% of SS	This study
	45ºC for 5 days without FF strain	225	21% of SS	This study
	45ºC for 5 days with FF strain		35 to 44% of SS	This study
	25ºC for 5 days without FF strain	125	27% of VSS	This study
	25ºC for 5 days with FF strain		54% of VSS	This study
10 L reactor	25ºC for 5 days without FF strain	125	26% of SS	This study
	25ºC for 5 days with FF strain		55% of SS	This study
	25ºC for 5 days without FF strain		32% of VSS	This study
	25ºC for 5 days with FF strain		60% of VSS	This study

*FF strain – Filamentous Fungal strain

Table 3: Various degradative gene primers used in this study for PCR amplification and direct sequencing.

Gene	Primer Direction	Primer Sequence 5′ - 3′	References
Toluene di-oxygenase *(TodC1)*	Forward	GCG AGA TAG AAG CGC TCT TG	Smits *et al.* 1999
	Reverse	GTA TTG ATA CCT GGG AGG AAG	
Toluene mono-oxygenase *(tmoA)*	Forward	GCT ATG TTA CCG AAG AGC AGC	
	Reverse	GGA ATA GAT CCC AGT ACC AGG	
Naphthalene di-oxygenase *(Ndo)*	Forward	TTT GTG TGC GGY TAC CAC GGN TGG GG	
	Reverse	TCT CAC CTA CAA AAG TTT TCC GCA AAA ARS CTT CCA GT	
Catechol 2,3, oxygenase *(xylE)*	Forward	CAA GGC CCA CGA CGT GGC NTT	
	Reverse	CGG TTA CCG GAC GGG TCG AAG AAG T	
Alkane monooxygenase (alk B)	TS2S F	AAY AGA GCT CAY GAR YTR GGT CAY AAG	Ringelberg *et al.* 2001
	TS2S mod F	AAY AGA GCT CAY GAR ITI GGI CAY AAR	
	TS2S mod2 F	AAY AGA GCT CAY GAR ITI TCI CAY AAR	
	Deg1RE	GTR AGI CTR GTR GTR CGC TTA AGG TG	
	Deg1RE2	GTR TCR CTR GTR GTR CGC TTA AGG TG	
Laccase gene	lac1F	CAC TGG CAC GGN TTC TTC CA	D'souza *et al.* 1996
	lac1R	GTG ACT ATG ATA CCA GAA NGT	
	lac2 F	CAY TGG CAY GGN TTY TTY CA	Hoshida *et al.* 2001.
	lac2 R1	TGR AAR TCD ATR TGR CAR TG	
	lac2 R2	GTR ACR GTR TAD CAR AAR GT	
	lcc1	ATG GCG AAG CTG CAG TTC	Gonzalez *et al.* 2003
	CD13	GCA CGA GAG CGA AAC TAA	
	LCC2	ATG TCG AGG TTC CAC TC	
	CD23	AAC GCG GAC CGA CGC TTC GC	
	LCC3	ATG TCG GGC TTC CGT CTC CTT	
	CD33	ACA GTA CTG CAA CAA	
	Forward	ATT GGC ACG GCT TCT TCC	Collins and Dobson, 1997.
	Reverse	GAT CTG GAT GGA GTC GAC	
styS gene	Forward	TGCGGGCAGCTCTACTTGGAAAAT	O'Leary *et al.* 2001
	Reverse	CTGGCGGAAGGGCGGAACATC	
styR gene	Forward	CGCCCCTTTCAAACGATTCAT	
	Reverse	ATGACCACAAAGCCCACAGTA	
styA gene	Forward	GGCCGCGATAGTCGGTGCGTA	
	Reverse	AGAAAAAGCGTATCGGTATT	
styD gene	Forward	GTAGGCGATAACCAACGAGCG	
	Reverse	ATGACAAGGAGCCTAACCATGAAC	

Table 4: Reproducibility results from shake flask and 10L reactor studies conducted at temperature 25°C with inoculum concentrations of 10^6 spores/mL.

Parameter	***Shake flask***		***10 L reactor***	
	Day "0"	Day "5"	Day "0"	Day "5"
		pH variations		
Control	6.82±0.02	5.43±0.03	6.53±0.05	5.76±0.06
Experiment		5.48±0.02		5.89±0.08
		% of SS reduction		
Control		23.96±0.13		25.76±0.78
Experiment		48.91±0.27		54.77±0.76
		% of VSS reduction		
Control		27.09±0.32		31.65±0.46
Experiment		53.96±0.45		59.55±0.95
		Zeta potential (mV)		
Control		-61.67±6.9		-60.12±5.3
Experiment		-59.83±7.3		-58.96±4.5
		SVI (mL)		
Control	100	90	100	60
Experiment		55		35
		CST (seconds)		
Control	80	38.6±0.15	245	124±0.57
Experiment		12.6±0.52		16±0.74
		Pathogens reduction (CFU/mL)		
Control (Fecal *coliforms*)	$1.9\pm0.76\times10^7$	$1.0\pm0.60\times10^6$	$2.1\pm0.43\times10^7$	$1.7\pm0.67\times10^6$
Experiment (Fecal *coliforms*)		$0.8\pm0.86\times10^5$		$1.6\pm0.78\times10^5$
Control (*salmonella*)	$1.7\pm0.76\times10^7$	$2.8\pm0.65\times10^5$	$3.6\pm0.45\times10^7$	$0.5\pm0.43\times10^6$
Experiment (*salmonella*)		$3.7\pm0.56\times10^3$		$1.8\pm0.59\times10^3$
		Fungal adaptation (CFU/mL)		
Control	No fungi	No fungi	No fungi	No fungi
Experiment	10^6	$8.0\pm0.93\times10^6$	10^6	$4.5\pm0.76\times10^6$

FIGURE LEGENDS

Figure 1: Measurement of pH changes during 5 days of fungal growth in sludge samples at four different temperatures with various inoculum concentrations (10^4, 10^5, 10^6 and 10^7 spores/mL) as follows; (a) incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

Figure 2: Profile of SS concentration measured during 5 days of fungal growth in sludge samples at four different temperatures with various inoculum concentrations (10^4, 10^5, 10^6 and 10^7 spores/mL) as follows; (a) incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

Figure 3: Profiles of K_d at different temperatures and initial spore concentrations.

Figure 4: Measurement of sludge settling by SVI method after 5 days of fungal growth in sludge samples at four different temperatures with various inoculum concentrations (10^4, 10^5, 10^6 and 10^7 spores/mL) without ( ) and with cations ( ); (a) incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

Figure 5: Measurement of sludge dewatering by CST test after 5 days of fungal growth in sludge samples at four different temperatures with various inoculum concentrations (10^4, 10^5, 10^6 and 10^7 spores/mL) without ( ) and with cations ( ); (a) incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

Figure 6: Zeta potential value of fungal grown sludge solids after 5 days of incubation at four different temperatures with various inoculum concentrations (10^4, 10^5, 10^6 and 10^7 spores/mL) without ( ) and with cations ( ); (a) incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

Figure 1

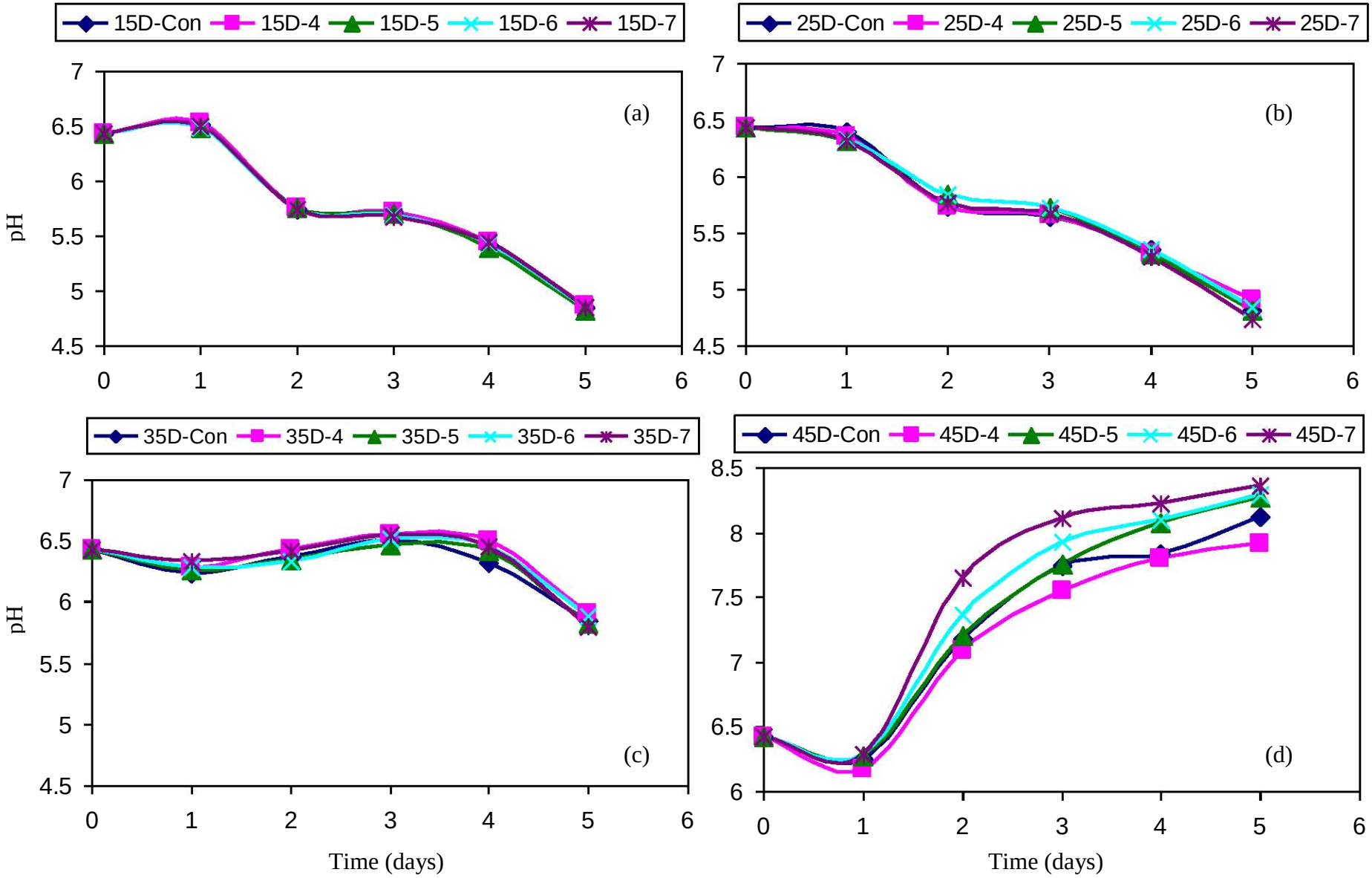

15D-Con
15D-4
15D-5
15D-6
15D-7
(a)
25D-Con
25D-4
25D-5
25D-6
25D-7
(b)
35D-Con
35D-4
35D-5
35D-6
35D-7
(c)
45D-Con
45D-4
45D-5
45D-6
45D-7
(d)
pH
Time (days)

Figure 2

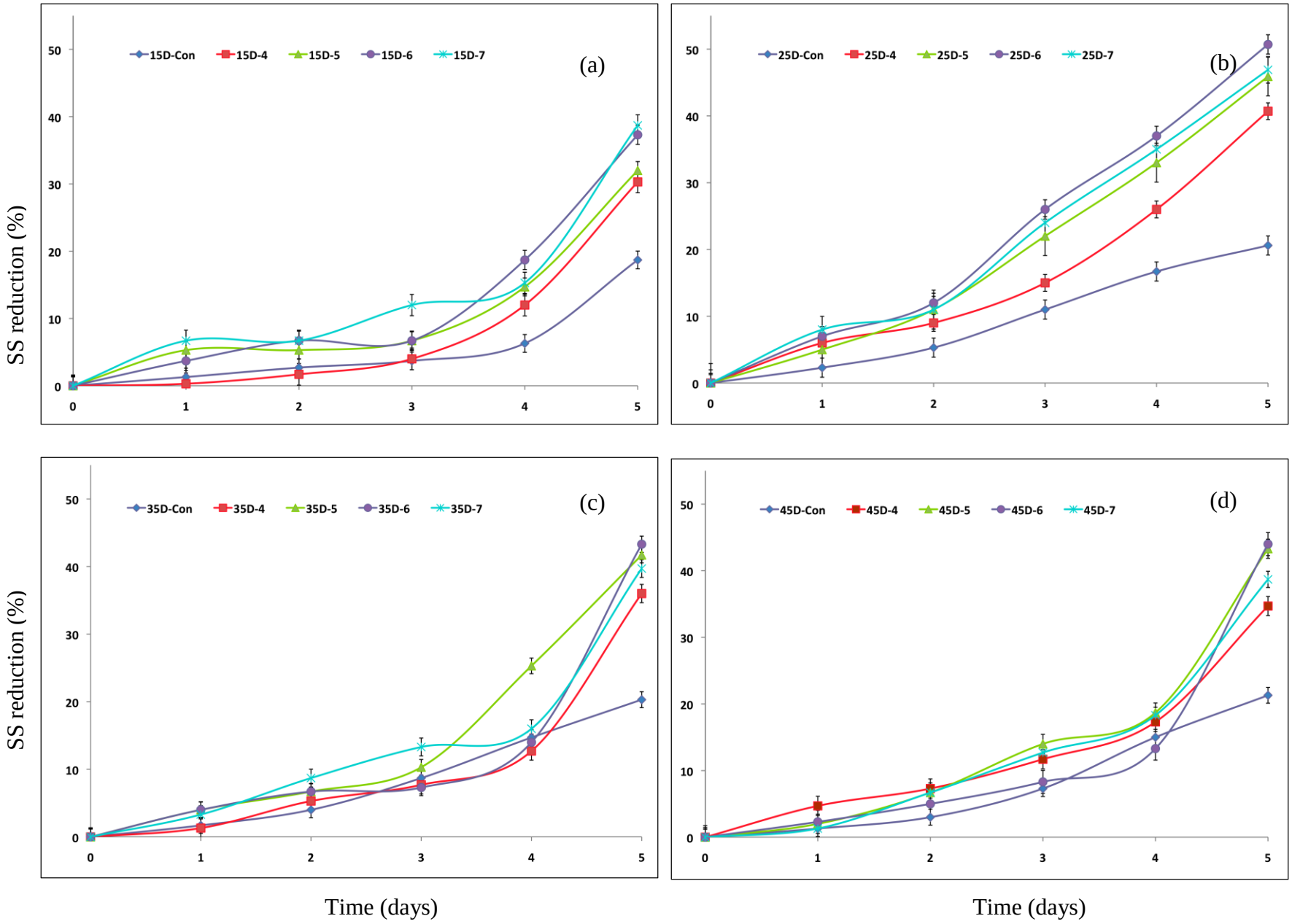

15D-Con
15D-4
15D-5
15D-6
15D-7
(a)
25D-Con
25D-4
25D-5
25D-6
25D-7
(b)
35D-Con
35D-4
35D-5
35D-6
35D-7
(c)
45D-Con
45D-4
45D-5
45D-6
45D-7
(d)
SS reduction (%)
SS reduction (%)
Time (days)
Time (days)

Figure 3: Profiles of K_d at different temperature and initial spore concentration.

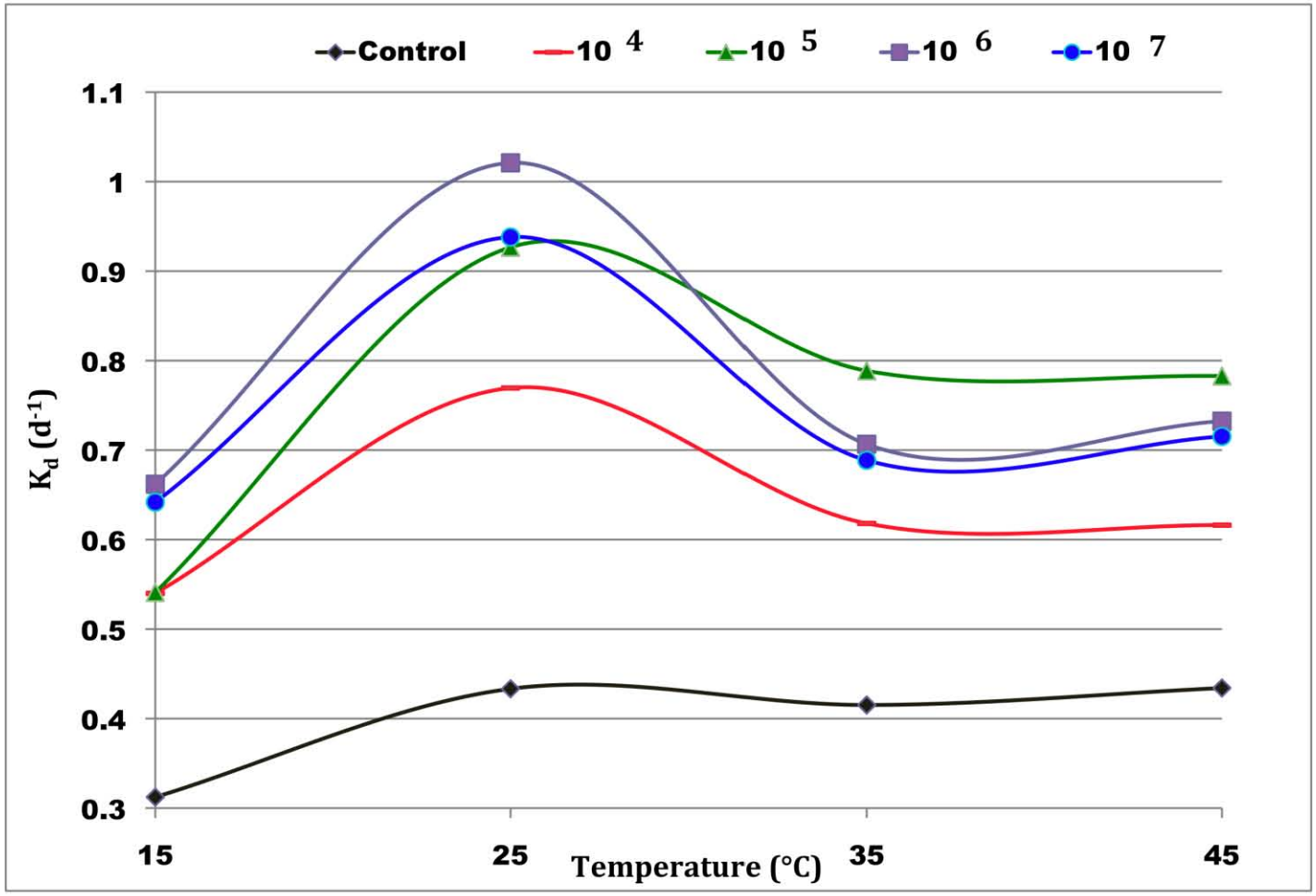


Figure 4

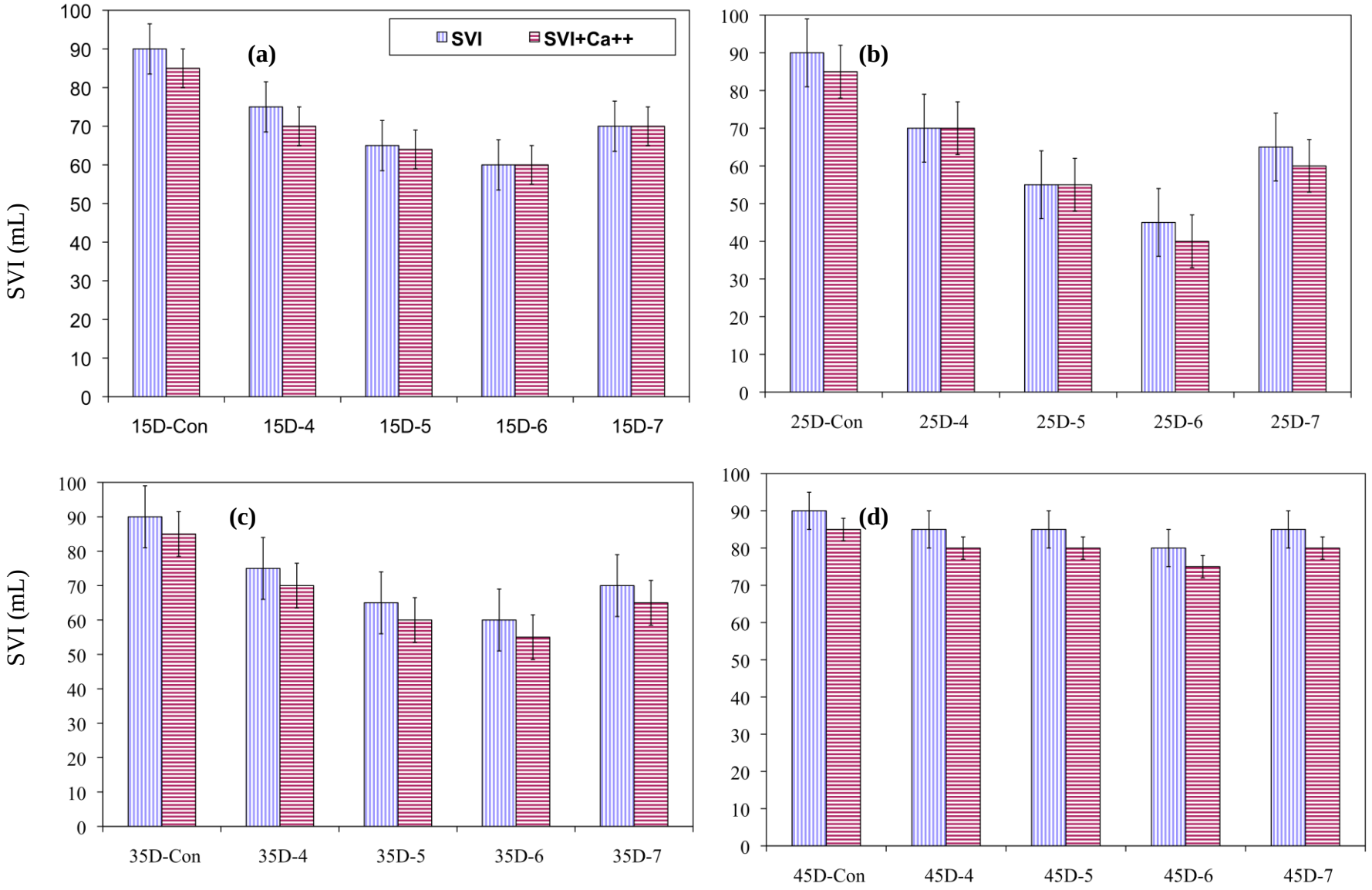

SVI
SVI+Ca++
(a)
(b)
(c)
(d)
SVI (mL)
SVI (mL)
100
90
80
70
60
50
40
30
20
10
0
15D-Con
15D-4
15D-5
15D-6
15D-7
25D-Con
25D-4
25D-5
25D-6
25D-7
35D-Con
35D-4
35D-5
35D-6
35D-7
45D-Con
45D-4
45D-5
45D-6
45D-7

Figure 5

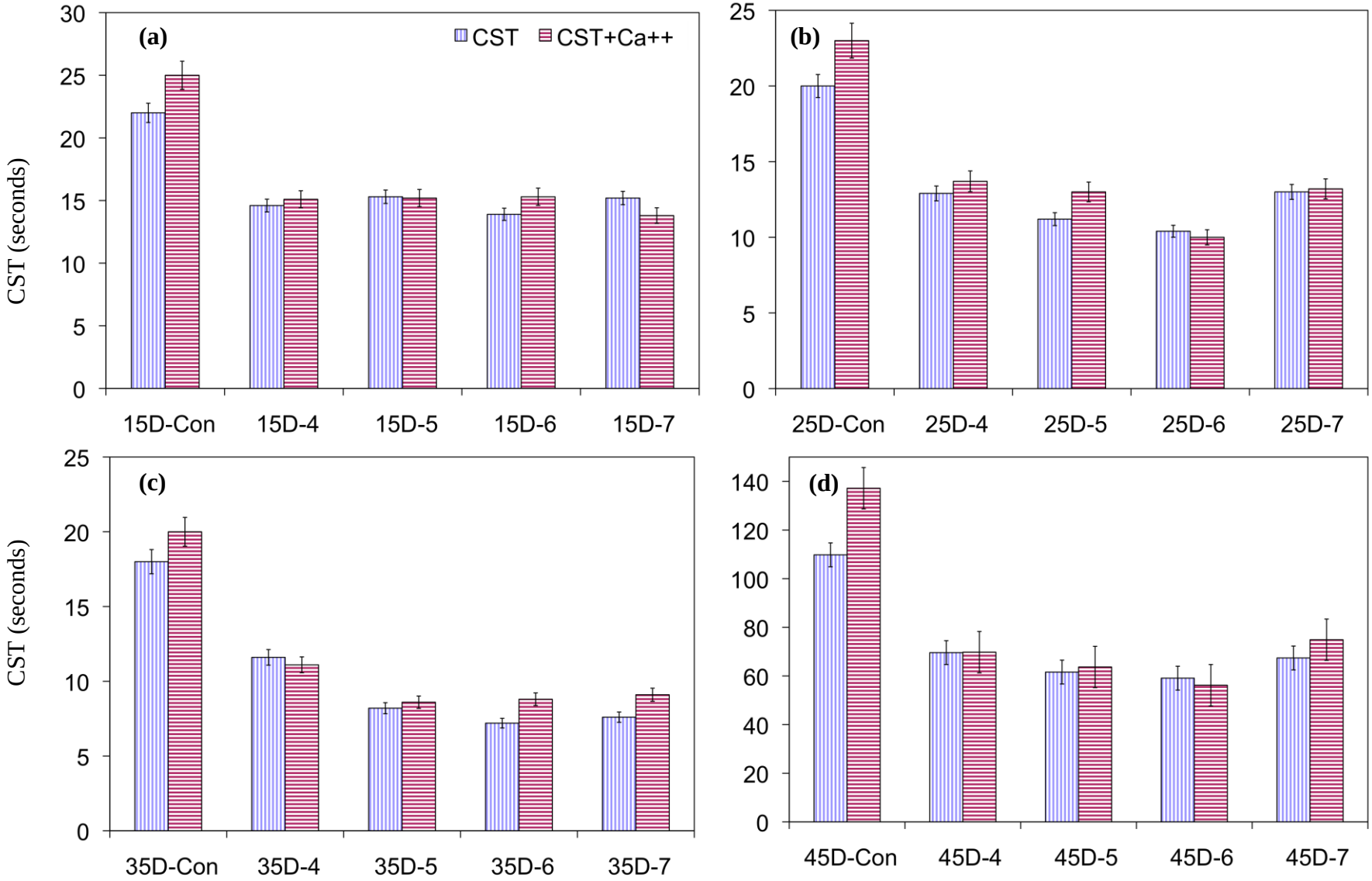
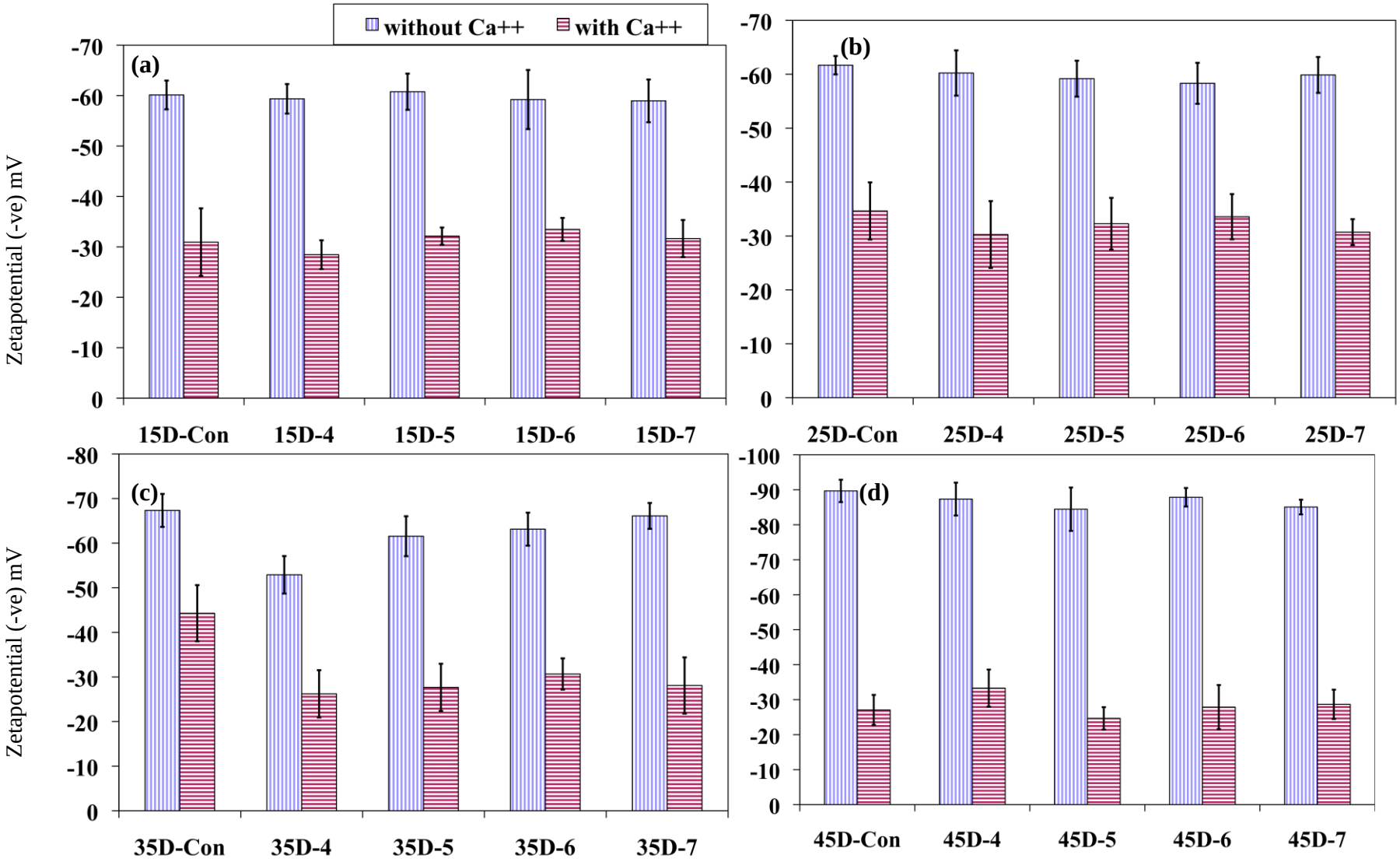

(a)
CST
CST+Ca++
CST (seconds)
30
25
20
15
10
5
0
15D-Con
15D-4
15D-5
15D-6
15D-7
(b)
25
20
15
10
5
0
25D-Con
25D-4
25D-5
25D-6
25D-7
(c)
CST (seconds)
25
20
15
10
5
0
35D-Con
35D-4
35D-5
35D-6
35D-7
(d)
140
120
100
80
60
40
20
0
45D-Con
45D-4
45D-5
45D-6
45D-7

Figure 6


without Ca++
with Ca++
(a)
(b)
(c)
(d)
Zetapotential (-ve) mV
Zetapotential (-ve) mV
15D-Con
15D-4
15D-5
15D-6
15D-7
25D-Con
25D-4
25D-5
25D-6
25D-7
35D-Con
35D-4
35D-5
35D-6
35D-7
45D-Con
45D-4
45D-5
45D-6
45D-7

CHAPITRE 5.

CONCLUSIONS ET RECOMMANDATIONS

5.1 CONCLUSIONS

Cette étude a permis de montrer que les deux souches bactériennes "non filamenteuses productrices de substances polymériques extracellulaires (SPE) et les "champignons filamenteux" peuvent être utilisées dans les procédés de décantation et de déshydratation des boues. Les principales conclusions obtenues à l'issu de ce travail sont résumées ci-dessous.

5.1.1 Identification moléculaire des bactéries productrices de SPE isolées à partir de boues d'épuration des eaux usées municipales et utilisées pour la bioflocculation

Parmi les vingt-cinq souches bactériennes isolées à partir de boues d'eaux usées municipales, 15 d'entre-elles ont été capables de produire des EPS capsulaires. Elles appartenaient, pour la plupart, au genre *Bacillus*. La quantité de SPE (dépôt gluant) (3.6-35.8 g / L) et de bouillon bactérien (5,0 - 36,4 g / L), produite par les souches isolées a été plus élevée que la quantité de SPE produite par le consortium de micro-organismes dans les boues. Une augmentation notable de la viscosité (105-179 mPas) des bouillons bactériens après 3 jours de fermentation confirme que les souches isolées produisent bien des EPS. La caractérisation des suspensions d'EPS par un zêtamètre a montré qu'ils portent une charge négative. Cependant, leur charge est moins négative que celle des SPE présents dans les boues fraîches. De plus, les SPE (dépôt gluant) ont révélé une meilleure activité de floculation du kaolin des boues de décantation que les EPS capsulaires et les bouillons bactériens, avec et sans addition de Ca^{++}. Les SPE produits par la souche bactérienne BS8 (genre *Serratia*) ont montré la meilleure activité de décantation des boues d'épuration. Les valeurs de CST ont sensiblement augmenté avec l'ajout d'extraits de SPE provenant de souches isolées. L'addition de cations a permis d'améliorer le procédé de déshydratation et de décantation des boues. Les SPE produits par six souches bactériennes sélectionnées et par un consortium de micro-organismes provenant de boues activées ont été caractérisés en termes de protéines (0.7-4%)

et d'hydrates de carbone (4-7%). Les extraits de SPE sont très riches (89-94%) en éléments autres que les glucides totaux et les protéines totales (lipides, ADN, ARN).

5.1.2 Une nouvelle souche de champignon formant des flocs : isolement, identification moléculaire et mesure de sa capacité à réduire, floculer et déshydrater simultanément les boues solides.

Une nouvelle souche de champignon formant des flocs provenant des boues d'épuration municipales a été isolée et caractérisée. Une identification moléculaire a montré que le champignon appartenait au genre *Penicillium expansum* BS30. La souche isolée montre une bonne adaptation à la boue. La biomasse fongique est chargée négativement (charge globale anionique).

Il a été démontré que le champignon filamenteux isolé est valorisable pour les procédés de réduction et de décantation des boues. Nous avons enregistré un taux de dégradation maximal des matières solides par la biomasse fongique de 68%, cultivée sur la boue fraîche (sans stérilisation). Ce taux est supérieur à la fois à celui du contrôle (dégradation de 22%) et celui des procédés classiques de digestion aérobie. Toutefois, le pourcentage de déshydratation de ces boues était faible lorsque la concentration en matières en suspension (MES) dépassait 2,5 g/L. Nous avons obtenu une dégradation maximale des MES (64%) et une meilleure déshydratation des boues fongiques brutes (non stérilisées), inoculées avec le champignon cultivé, avec un temps de succion capillaire CST <20 s et à une concentration initiale en MES de 15 g/L. Les boues brutes inoculées avec les spores du champignon ont montré une réduction substantielle de la MES tandis que le taux de déshydratation a diminué pour une concentration initiale en MES de 15 g/L. L'ajout de Ca^{++} a permis d'améliorer le taux de déshydratation des boues fongiques stérilisées mais non pas celui des boues brutes.

5.1.3 Utilisation de la souche filamenteuse *Penicillium expansum* BS30, isolée à partir de boues d'épuration des eaux usées municipales, pour la réduction des agents pathogènes, la déshydratation et la décantation simultanées des boues solides (SSPRSD).

Un nouveau procédé utilisant un champignon filamenteux isolé des boues a été développé pour la dégradation des boues solides, la réduction des agents pathogènes et l'amélioration de la décantation et la déshydratation des boues. Nous avons pu atteindre un taux maximal de dégradation de 50% de la MES à une température de 25°C et à une concentration de spores fongiques de 10^6 spores/mL. Dans ces conditions de température et de concentration de spores, la constante de dégradation de premier ordre (K_d) a atteint 1,02 d^{-1}. Cette valeur est 2,3 fois plus élevée que celle du contrôle. Une diminution de la température à 15 ° C entraîne une diminution de la valeur de K_d, qui reste toutefois 2,2 fois plus élevée que celle du contrôle. Dans cette étude, nous avons atteint des valeurs de K_d de 2,5 à 13,2 fois supérieures à celles rapportées par la littérature sur les procédés de digestion aérobie des boues. Le procédé a aussi permis une réduction de l'indicateur de suppression de pathogènes de 4-5 unités log dans les boues traitées. A la fin du cycle de dégradation, il est possible de déshydrater les boues avec une valeur de CST inférieure à 13s (inférieure à la valeur de CST de 20s requise). Par ailleurs, la valeur de CST était inférieure à 20s à des températures allant de 15 à 35ºC, mais elle s'est détériorée en augmentant la température à 45ºC. La souche de champignon isolée possède des gènes de biosynthèse de la pénicilline, mais ne contient pas de composés organiques toxiques dégradant les gènes. L'étude de la reproductibilité du procédé, menée dans un bioréacteur de 10L, a conduit à un taux de réduction des solides en suspension important de 54%, à une réduction de 2-4 log et à un meilleur taux de déshydratation des boues (CST <20s par rapport au contrôle).

5.2 RECOMMANDATIONS

La présente étude a clairement démontré l'amélioration des opérations de décantation et de déshydratation au cours des procédés de traitement des boues en utilisant à la fois les SPE produits par des souches microbiennes et les champignons filamenteux. Toutefois, les recommandations suivantes sont proposées pour optimiser et améliorer le procédé afin de pouvoir le mettre en œuvre dans des applications à grandes échelles.

1. Les biopolymères (SPE) et les polymères cationiques synthétiques (CSP) peuvent être combinés (le ratio optimal doit néanmoins être déterminé) pour procéder à la décantation et la déshydratation des boues; cela permettrait une meilleure floculation des boues et, à terme, contribuerait à réduire la consommation de CSP, la toxicité (approche écologique) et le coût global du procédé.
2. Les composants des SPE (glucides, protéines et lipides) doivent être séparés et purifiés afin d'étudier l'aptitude de chacun d'eux à décanter et à déshydrater les boues. Cela permettra de comprendre la raison pour laquelle les SPE bactériens ont montré de meilleurs résultats que ceux obtenus avec le consortium de SPE et les SPE extraits des boues. De même, la caractérisation moléculaire des biopolymères en termes de poids moléculaire et de groupements chimiques actifs doit être entreprise. Ceci permettrait d'ouvrir une nouvelle voie pour la mise en œuvre et l'amélioration des procédés de bioflocculation utilisant les polymères purifiés.
3. L'identification moléculaire de gènes *eps* (gènes responsables de la synthèse et la production de SPE bactériens) en utilisant des sondes ADN et des amorces PCR spécifiques nous permettra d'identifier les organismes porteurs de ces gènes. Leur clonage permettrait de produire des EPS en grandes quantités pour le traitement des boues.

4. La composition du milieu de culture peut également modifier la charge et la composition des SPE. L'ajout de cations bivalents ou trivalents dans la composition des milieux de culture pourrait stimuler et améliorer la production de protéines extracellulaires (SPE). De même, en modifiant le ratio protéines/glucides dans le milieu de culture, il serait possible d'obtenir des SPE ayant la composition chimique désirée avec moins de charge négative. Puisque l'ajout de Ca^{++} aux préparations de SPE a permis une amélioration de la déshydratation des boues, l'emploi d'autres cations, comme l'alun et $FeCl_3$ devrait être étudié pour leurs effets sur le procédé de déshydratation.
5. L'efficacité du processus de réduction des agents pathogènes, de déshydratation et de décantation simultanées des boues solides (SSPRSD) devra être vérifiée à grande échelle dans un bioréacteur de 1000L.
6. Il serait intéressant de mettre en place un procédé utilisant les bactériophages dans le but de contrôler la croissance excessive des champignons filamenteux et la surproduction de SPE bactériennes. En effet, l'utilisation des bactériophages pourrait constituer une nouvelle approche pour l'amélioration des procédés de digestion, de déshydratation et de réduction des pathogènes. De même, il serait judicieux d'étudier l'action des bactériophages combinée à celle des EPS et/ou à celle des champignons filamenteux dans l'amélioration des phénomènes de réduction de la MES, des pathogènes, de décantation et de déshydratation des boues.

ANNEXE I

Données

EXTRACELLULAR POLYMERIC SUBSTANCES (EPS) PRODUCING BACTERIAL STRAINS OF MUNICIPAL WASTEWATER SLUDGE: ISOLATION, MOLECULAR IDENTIFICATION, EPS CHARACTERIZATION AND PERFORMANCE FOR SLUDGE SETTLING AND DEWATERING

EPS concentrations (g/L)		
Bacterial strains	Slime EPS	Bacterial broth
BS1	28.0	28.4
BS2	05.4	05.6
BS3	18.0	20.6
BS4	05.0	05.5
BS5	07.0	07.6
BS6	06.4	06.6
BS7	26.8	27.4
BS8	25.0	25.2
BS9	03.6	06.6
BS10	26.0	26.4
BS11	27.7	29.6
BS12	07.4	07.8
BS13	27.0	28.4
BS14	26.4	28.0
BS15	16.8	18.8
BS16	09.8	10.4
BS17	23.4	24.6
BS18	24.6	25.8
BS19	34.0	34.8
BS20	24.6	25.2
BS21	26.8	27.1
BS22	18.2	18.8
BS23	20.4	21.0
BS24	35.8	36.4
BS25	31.4	32.2
Consortium	04.6	07.2

Figure 2: EPS concentrations produced by 25 bacterial strains and consortium at the end of 3 days of fermentation.

Bacterial strains	Slime EPS		Capsular EPS		Bacterial broth	
	Zetapotential (mV)	Stdev	Zetapotential (mV)	stdev	Zetapotential (mV)	stdev
BS1	-35.955000	3.385398	-37.943333	1.096543	-35.216667	1.494205
BS2	-20.145000	1.163931	-51.745556	1.731048	-35.702222	2.201362
BS3	-24.067778	7.712585	-37.863333	12.546786	-13.630000	6.100162
BS4	-40.916667	0.837326	-60.684444	4.499900	-40.179167	1.943869
BS5	-18.387500	3.364324	-15.687778	2.621711	-11.354167	1.057032
BS6	-11.218333	2.607468	-13.477778	0.530113	-6.755000	0.893639
BS7	-27.819167	3.879078	-39.570000	2.140707	-32.350000	0.509313
BS8	-26.357500	4.433163	-28.573333	12.188710	-25.728889	3.910455
BS9	-24.163333	1.542403	-36.940000	1.218113	-26.360000	1.837479
BS10	-24.306667	4.224548	-60.234444	4.560623	-40.764167	1.691011
BS11	-9.283333	5.155184	-35.675556	1.669806	-15.272222	1.340735
BS12	-30.636667	4.431071	-43.333333	3.039235	-30.788333	3.441949
BS13	-23.523333	6.090619	-20.381111	5.950285	-29.064167	17.709187
BS14	-36.382222	4.720871	-50.928889	3.443085	-9.477778	3.930171
BS15	-18.196667	5.393563	-46.817500	2.682364	-26.767500	3.355289
BS16	-47.400000	4.222200	-49.700000	2.999560	-55.360000	3.255550
BS17	-32.513333	3.501385	-25.611111	6.775785	-42.397500	2.132839
BS18	-24.918333	16.662588	-33.557778	2.890985	-27.848889	2.233900
BS19	-31.961111	3.915474	-33.730000	2.306648	-26.455000	3.522304
BS20	-23.945000	4.780404	-31.681111	2.283853	-30.993333	1.705858
BS21	-41.344444	1.214641	-66.812222	2.122957	-43.952500	1.395839
BS22	-25.183333	2.193502	-43.280000	2.334470	-26.908333	0.753981
BS23	-34.995000	0.536021	-39.571429	1.117474	-34.674444	0.919553
BS24	-33.085556	7.125902	-36.887778	5.159672	-34.330000	2.289303
BS25	-22.747778	3.706055	-29.240000	14.059195	-22.660833	5.108089

Stdev – Standard deviation

Figure 3: Zeta potential value of slime EPS, capsular EPS and bacterial broth produced from 25 EPS producing bacterial strains.

	Zetapotential (mV)		
Bacterial strains	pH 4	pH 10	pH 7
BS1	-18.355000	-48.290000	-35.216667
BS2	-31.500000	-49.700000	-35.702222
BS3	-26.280000	-46.935000	-13.630000
BS4	-34.600000	-35.520000	-40.179167
BS5	-38.750000	-67.030000	-11.354167
BS6	-45.800000	-68.670000	-6.755000
BS7	-48.800000	-73.400000	-32.350000
BS8	-39.070000	-70.660000	-25.728889
BS9	-42.780000	-69.620000	-26.360000
BS10	-53.000000	-65.640000	-40.764167
BS11	-50.830000	-58.100000	-15.272222
BS12	-42.200000	-78.690000	-30.788333
BS13	-39.870000	-78.280000	-29.064167
BS14	-50.340000	-75.320000	-9.477778
BS15	-36.430000	-63.680000	-26.767500
BS16	-39.610000	-66.690000	-55.360000
BS17	-45.900000	-79.400000	-42.397500
BS18	-45.000000	-78.890000	-27.848889
BS19	-37.990000	-69.320000	-26.455000
BS20	-37.530000	-66.870000	-30.993333
BS21	-50.070000	-73.370000	-43.952500
BS22	-41.610000	-78.630000	-26.908333
BS23	-43.850000	-75.870000	-34.674444
BS24	-46.370000	-73.590000	-34.330000
BS25	-46.350000	-77.710000	-22.660833
Consortium	-42.791818	-73.092727	-37.970000
Sludge			-77.270000

Figure 4: Zeta potential value of EPS in bacterial broth from 25 EPS producing bacterial strains and consortium produced at different pH (4, 7 & 10).

(a)

Sludge Volume Index (mL/g)		
Bacterial strains	without Ca++	with Ca++
BS2	70	64
BS8	60	54
BS9	70	62
BS11	64	62
BS15	74	66
BS25	74	64
Consortium	120	94
Control	160	104
Cationic polymer	56	56

(b)

CST (seconds)		
Bacterial strains	without Ca++	with Ca++
BS2	66.8	41.9
BS8	48.4	36.4
BS9	67.1	54.1
BS11	59.1	38.4
BS15	45	42.3
BS25	77.1	29.4
Consortium	136	110
Control	158	127
Cationic polymer	13.2	13.2

Figure 5: Sludge settling and dewatering efficiency of slime EPS [10 % V/V - (0.23, 0.30, 0.24, 0.25, 0.21 and 0.32 mg/mL)] from six selected bacterial strains (BS2, BS8, BS9, BS11, BS15 and BS25) and consortium with and without cations. (a) Sludge volume index, and (b) Capillary suction time.

	Slime EPS	
Bacterial strains	TC (g/L)	TP (g/L)
BS2	0.132	0.017
BS8	0.158	0.084
BS9	0.168	0.050
BS11	0.150	0.102
BS15	0.094	0.027
BS25	0.142	0.063
Consortium	0.111	0.138
Sludge EPS	0.228	0.142

Figure 6: Extracted slime EPS total carbohydrates (TC) and total protein (TP) concentration from six selected pure bacterial strains, consortium and fresh sludge.

ANNEXE II

Données

A NEW, PELLET-FORMING FUNGAL STRAIN: ITS ISOLATION, MOLECULAR IDENTIFICATION, AND PERFORMANCE FOR SIMULTANEOUS SLUDGE-SOLIDS REDUCTION, FLOCCULATION, AND DEWATERING

(a) SS decrease (g/L) in NSTNT

	Days					
Experiments	0	1	2	3	4	5
NSTNT1	1	1	0.9	0.9	0.89	0.85
NSTNT2.5	2.5	2.5	2.3	2.2	2	1.95
NSTNT5	5	4.9	4.8	4.7	4.6	4.6
NSTNT10	10	10	9.6	9.3	9.2	9.05
NSTNT15	15	15	14.6	14	13.95	13.6

(b) SS decrease (g/L) in STNT

	Days					
Experiments	0	1	2	3	4	5
STNT1	1	1	0.98	0.95	0.9	0.85
STNT2.5	2.5	2.5	2.5	2.5	2.35	2.35
STNT5	5	5	4.8	4.65	4.55	4.1
STNT10	10	10	9.95	9.5	9	8.95
STNT15	15	15	14.5	14.2	14	13.5

(c) SS decrease (g/L) in NSTS

	Days					
Experiments	0	1	2	3	4	5
NSTS1	1	0.9	0.9	0.9	0.7	0.7
NSTS2.5	2.5	2	1.4	1.4	1.2	1.1
NSTS5	5	4.2	3.9	3.7	3.5	3
NSTS10	10	10	8.1	6	5.5	5
NSTS15	15	13.1	10.7	9	8.3	7.8

(d) SS decrease (g/L) in STS

	Days					
Experiments	0	1	2	3	4	5
STS1	1	0.9	0.8	0.8	0.7	0.6
STS2.5	2.5	2.4	2	1.9	1.1	1
STS5	5	4.5	4.1	3.1	2.7	2.2
STS10	10	7.2	6.7	6	5.3	4.5
STS15	15	11.4	10.4	9.2	8.6	7.8

(e) SS decrease in NSTB

	Days					
Experiments	0	1	2	3	4	5
NSTB1	1	0.9	0.7	0.5	0.5	0.4
NSTB2.5	2.5	2.4	2.1	1.9	1.5	0.9
NSTB5	5	4.4	3.7	3.3	3	2.7
NSTB10	10	8.5	7.5	6.8	5.9	4.9
NSTB15	15	13.6	12	9.9	8.5	7.5

(f) SS decrease in STB

	Days					
Experiments	0	1	2	3	4	5
STB1	1	0.9	0.7	0.7	0.6	0.5
STB2.5	2.5	2.1	1.8	1.4	1.1	0.8
STB5	5	3.2	3	2.9	2.7	2.6
STB10	10	7.3	6.1	5.5	5.3	5
STB15	15	10.9	9.3	8.3	7.9	7.5

Figure 4: Profile of suspended-solids concentration (g/L) measured 5 d, under conditions as follows: (a) nonsterilized sludge, not treated with fungi (NSTNT) used as control; (b) sterilized sludge, not treated with fungi (STNT) used as control; (c) nonsterilized sludge inoculated with fungal spores (NSTS); (d) sterilized sludge inoculated with fungal spores (STS); (e) nonsterilized sludge inoculated with fungal beads (NSTB); and (f) sterilized sludge inoculated with fungal beads (STB).

(a) Sludge volume index (mL)

SS (g/L)	NSTS	STS	NSTB	STB	NSTNT	STNT
1	4	15	8	26	5	3
2.5	9	35	25	28	10	6
5	20	90	60	30	20	12
10	75	93	90	32	48	22
15	95	96	95	48	85	55

(b) SS concentration in SVI supernatant (g/L)

SS (g/L)	NSTS	STS	NSTB	STB	NSTNT	STNT
1	0	0.1	0	0.2	0.4	0.22
2.5	0	0.1	0	0.2	0.3	0.23
5	0.1	0	0.2	1.1	1.4	1.2
10	0.3	0.1	1.1	1.5	2.0	1.6
15	1.8	0.3	2.0	1.6	3.1	2.3

Figure 5: (a) Sludge-volume index (SVI) of fungal-grown sludge solids after 5 d of incubation; and (b) suspended solids (g/L) concentration measured in the supernatant of SVI. STNT = sterilized sludge non-treated (control); NSTNT = nonsterilized sludge non-treated (control); STS = sterilized sludge inoculated with spores; NSTS = nonsterilized sludge inoculated with spores; STB = sterilized sludge inoculated with beads; and NSTB = nonsterilized sludge inoculated with beads.

(a) NSTNT

CST (seconds)		
Experiments	Without Ca^{++}	With Ca^{++}
NSTNT1	7	6
NSTNT2.5	11.1	7.6
NSTNT5	23.8	10.8
NSTNT10	204.2	73.8
NSTNT15	469.6	295

(b) STNT

CST (seconds)		
Experiments	Without Ca^{++}	With Ca^{++}
STNT1	8.3	6.9
STNT2.5	9.6	6.85
STNT5	32.4	7
STNT10	83.7	52.9
STNT15	184.3	160

(c) NSTS

Experiments	Without Ca^{++}	With Ca^{++}
NSTS1	5.3	4.6
NSTS2.5	5.7	7.3
NSTS5	7.5	9
NSTS10	13	14.7
NSTS15	31.3	41.8

(d) STS

Experiments	Without Ca^{++}	With Ca^{++}
STS1	5.4	6.2
STS2.5	20.8	8.9
STS5	25.2	18.7
STS10	13.3	9.3
STS15	59.5	34.1

(e) NSTB

Experiments	Without Ca^{++}	With Ca^{++}
NSTB1	7.4	8
NSTB2.5	8.2	7.2
NSTB5	9.5	11.6
NSTB10	18	18.5
NSTB15	19.4	38.4

(f) STB

Experiments	Without Ca^{++}	With Ca^{++}
STB1	9	8.4
STB2.5	10.2	9.5
STB5	55	30.3
STB10	77	62.3
STB15	302	282.1

Figure 6: Capillary suction time (CST) measurements of control and experimental results carried out without cations and with cations (Ca^{++}) as follows: (a) nonsterilized sludge, not treated with fungi (NSTNT) used as control; (b) sterilized sludge, not treated with fungi (STNT) used as control; (c) nonsterilized sludge inoculated with fungal spores (NSTS); (d) sterilized sludge inoculated with fungal spore (STS); (e) nonsterilized sludge inoculated with fungal beads (NSTB); and (f) sterilized sludge inoculated with fungal beads (STB).

(a) STS

Experiments	Zetapotential (mV)	Stdev
STS1	-29.258333	2.013347
STS2.5	-32.123889	1.887928
STS5	-24.235000	2.058804
STS10	-31.745556	0.754401
STS15	-36.454762	4.670621

(b) STB

Experiments	Zetapotential (mV)	Stdev
STB1	-19.418333	0.895625
STB2.5	-22.533333	0.870188
STB5	-30.357222	1.185624
STB10	-31.341667	1.317262
STB15	-34.152778	1.589291

(c) NSTB

Experiments	Zetapotential (mV)	Stdev
NSTB1	-43.347778	1.932289
NSTB2.5	-33.015556	1.067836
NSTB5	-34.088889	1.234008
NSTB10	-31.679333	1.357913
NSTB15	-39.012778	1.366472

(d) NSTS

Experiments	Zetapotential (mV)	Stdev
NSTS1	-36.187500	1.827289
NSTS2.5	-39.234444	1.448031
NSTS5	-33.304286	1.460577
NSTS10	-34.706667	2.022868
NSTS15	-35.956111	1.551705

Stdev – Standard deviation

Figure 7: Zeta potential value of fungal-grown sludge solids after 5 d incubation. STS = sterilized sludge inoculated with spores; NSTS = nonsterilized sludge inoculated with spores; STB = sterilized sludge inoculated with beads; and NSTB = nonsterilized sludge inoculated with beads.

ANNEXE III

Données

SIMULTANEOUS SLUDGE SOLIDS AND PATHOGENS REDUCTION, SETTLING AND DEWATERING (SSPRSD) USING A NEW FILAMENTOUS FUNGAL STRAIN *PENICILLIUM EXPANSUM BS30* ISOLATED FROM WASTEWATER SLUDGE

(a)

pH						
	Days					
Experiments	0	1	2	3	4	5
15D-Con	6.43	6.52	5.75	5.72	5.42	4.85
15D-4	6.43	6.53	5.76	5.72	5.44	4.87
15D-5	6.43	6.49	5.76	5.70	5.39	4.83
15D-6	6.43	6.48	5.74	5.69	5.43	4.85
15D-7	6.43	6.50	5.75	5.68	5.44	4.85

(b)

	Days					
Experiments	0	1	2	3	4	5
25D-Con	6.43	6.40	5.74	5.65	5.36	4.81
25D-4	6.43	6.35	5.74	5.66	5.31	4.91
25D-5	6.43	6.32	5.84	5.73	5.32	4.81
25D-6	6.43	6.33	5.84	5.73	5.36	4.84
25D-7	6.43	6.32	5.76	5.67	5.29	4.74

(c)

	Days					
Experiments	0	1	2	3	4	5
35D-Con	6.43	6.24	6.37	6.50	6.32	5.85
35D-4	6.43	6.29	6.43	6.55	6.50	5.91
35D-5	6.43	6.26	6.34	6.47	6.42	5.82
35D-6	6.43	6.29	6.33	6.51	6.45	5.88
35D-7	6.43	6.33	6.42	6.55	6.45	5.80

(d)

	Days					
Experiments	0	1	2	3	4	5
45D-Con	6.43	6.25	7.18	7.74	7.83	8.12
45D-4	6.43	6.17	7.09	7.55	7.80	7.91
45D-5	6.43	6.27	7.20	7.76	8.08	8.27
45D-6	6.43	6.29	7.36	7.93	8.10	8.29
45D-7	6.43	6.29	7.65	8.11	8.22	8.36

Figure 1: Measurement of pH changes during 5 days of fungal growth in sludge samples at four different temperatures with varying inoculum (10^4, 10^5, 10^6 and 10^7) concentration (a) samples incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

(a)

SS decrease (g/L)						
	Days					
Experiments	0	1	2	3	4	5
15D-Con	10	9.87	9.73	9.63	9.37	8.13
15D-4	10	9.97	9.83	9.60	8.80	6.97
15D-5	10	9.47	9.47	9.33	8.53	6.80
15D-6	10	9.63	9.33	9.33	8.13	6.27
15D-7	10	9.33	9.33	8.80	8.47	6.13

(b)

	Days					
Experiments	0	1	2	3	4	5
25D-Con	10	9.77	9.47	8.90	8.33	7.94
25D-4	10	9.40	9.10	8.50	7.40	5.93
25D-5	10	9.50	8.90	7.80	6.70	5.41
25D-6	10	9.30	8.80	7.40	6.30	4.93
25D-7	10	9.20	8.90	7.60	6.50	5.31

(c)

	Days					
Experiments	0	1	2	3	4	5
35D-Con	10	9.83	9.60	9.13	8.53	7.97
35D-4	10	9.87	9.47	9.23	8.73	6.40
35D-5	10	9.60	9.33	8.97	7.47	5.83
35D-6	10	9.60	9.33	9.27	8.60	5.67
35D-7	10	9.67	9.13	8.67	8.40	6.03

(d)

	Days					
Experiments	0	1	2	3	4	5
45D-Con	10	9.87	9.70	9.27	8.50	7.87
45D-4	10	9.53	9.27	8.83	8.27	6.53
45D-5	10	9.80	9.33	8.60	8.13	5.67
45D-6	10	9.77	9.50	9.17	8.67	5.60
45D-7	10	9.87	9.33	8.73	8.17	6.13

Figure 2: Profile of SS concentration measured during 5 days of fungal growth in sludge samples at four different temperatures with varying inoculum (10^4, 10^5, 10^6 and 10^7) concentration (a) samples incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

Temperature	Initial inoculum concentration (spores/mL)				
	Control	10^4	10^5	10^6	10^7
15	0.3123	0.54	0.541	0.6619	0.6418
25	0.4333	0.7694	0.9267	1.021	0.9381
35	0.4152	0.6181	0.7886	0.7066	0.6886
45	0.4342	0.6162	0.7828	0.7324	0.7153

Equation obtained in this study.		
15D-Con	y = -0.3123x + 10.236	R^2 = 0.7329
15D-4	y = -0.54x + 10.544	R^2 = 0.7357
15D-5	y = -0.541x + 10.286	R^2 = 0.77853
15D-6	y = -0.6619x + 10.438	R^2 = 0.80169
15D-7	y = -0.6418x + 10.282	R^2 = 0.78885
25D-Con	y = -0.4333x + 10.151	R^2 = 0.97916
25D-4	y = -0.7694x + 10.313	R^2 = 0.92998
25D-5	y = -0.9267x + 10.369	R^2 = 0.97173
25D-6	y = -1.021x + 10.341	R^2 = 0.97456
25D-7	y = -0.9381x + 10.264	R^2 = 0.97232
35D-Con	y = -0.4152x + 10.216	R^2 = 0.95244
35D-4	y = -0.6181x + 10.495	R^2 = 0.75635
35D-5	y = -0.7886x + 10.505	R^2 = 0.86748
35D-6	y = -0.7066x + 10.511	R^2 = 0.70357
35D-7	y = -0.6886x + 10.371	R^2 = 0.82974
45D-Con	y = -0.4342x + 10.286	R^2 = 0.91516
45D-4	y = -0.6162x + 10.279	R^2 = 0.87466
45D-5	y = -0.7828x + 10.546	R^2 = 0.8406
45D-6	y = -0.7324x + 10.614	R^2 = 0.70816
45D-7	y = -0.7153x + 10.494	R^2 = 0.867

Figure 3: Profiles of K_d at different temperatures and initial spore concentration.

(a)

	SVI (mL)	
Experiments	Without Ca^{++}	With Ca^{++}
15D-Con	90	85
15D-4	75	70
15D-5	65	64
15D-6	60	60
15D-7	70	70

(b)

Experiments	Without Ca^{++}	With Ca^{++}
25D-Con	90	85
25D-4	70	70
25D-5	55	55
25D-6	45	40
25D-7	65	60

(c)

Experiments	Without Ca^{++}	With Ca^{++}
35D-Con	90	85
35D-4	75	70
35D-5	65	60
35D-6	60	55
35D-7	70	65

(d)

Experiments	Without Ca^{++}	With Ca^{++}
45D-Con	90	85
45D-4	85	80
45D-5	85	80
45D-6	80	75
45D-7	85	80

Figure 4: Measurement of sludge settling by SVI method after 5 days of fungal growth in sludge samples at four different temperatures with varying inoculum (10^4, 10^5, 10^6 and 10^7) concentration without cations and with cations (Ca^{++}) (a) samples incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

(a)

	CST (s)	
Experiments	Without Ca^{++}	With Ca^{++}
15D-Con	22.0	25.0
15D-4	14.6	15.1
15D-5	15.3	15.2
15D-6	13.9	15.3
15D-7	15.2	13.8

(b)

	CST (s)	
Experiments	Without Ca^{++}	With Ca^{++}
25D-Con	20.0	23.0
25D-4	12.9	13.7
25D-5	11.2	13.0
25D-6	10.4	10.0
25D-7	13.0	13.2

(c)

	CST (s)	
Experiments	Without Ca^{++}	With Ca^{++}
35D-Con	18.0	20.0
35D-4	11.6	11.1
35D-5	08.2	08.6
35D-6	07.2	08.8
35D-7	07.6	09.1

(d)

	CST (s)	
Experiments	Without Ca^{++}	With Ca^{++}
45D-Con	109.8	137.2
45D-4	69.6	69.8
45D-5	61.6	63.7
45D-6	59.1	56.2
45D-7	67.4	74.9

Figure 5: Measurement of sludge dewatering by CST test after 5 days of fungal growth in sludge samples at four different temperatures with varying inoculum (10^4, 10^5, 10^6 and 10^7) concentration without cations and with cations (Ca^{++}) (a) samples incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.

(a)

Zetapotential (mV)				
Experiments	Without Ca^{++}	Stdev	With Ca^{++}	Stdev
15D-Con	-60.126667	2.853248	-30.940000	6.699666
15D-4	-59.358333	2.931692	-28.448333	2.854249
15D-5	-60.765000	3.587337	-32.131667	1.702767
15D-6	-59.216667	5.860523	-33.463333	2.276354
15D-7	-58.960000	4.236667	-31.650000	3.677700

(b)

Experiments	Without Ca^{++}	Stdev	With Ca^{++}	Stdev
25D-Con	-61.677700	5.300000	-34.640000	5.300000
25D-4	-60.230000	6.200000	-30.270000	6.200000
25D-5	-59.178889	4.800000	-32.270000	4.800000
25D-6	-58.320000	4.200000	-33.570000	4.200000
25D-7	-59.870000	2.400000	-30.720000	2.400000

(c)

Experiments	Without Ca^{++}	Stdev	With Ca^{++}	Stdev
35D-Con	-67.340000	3.700000	-44.290000	6.300000
35D-4	-52.910000	4.200000	-26.220000	5.300000
35D-5	-61.557778	4.477032	-27.660000	5.300000
35D-6	-63.140000	3.700000	-30.670000	3.500000
35D-7	-66.110000	2.900000	-28.090000	6.300000

(d)

Days				
Experiments	Without Ca^{++}	Stdev	With Ca^{++}	Stdev
45D-Con	-89.680000	3.200000	-27.060000	4.300000
45D-4	-87.350000	4.700000	-33.310000	5.300000
45D-5	-84.450000	6.200000	-24.660000	3.200000
45D-6	-87.863333	2.647215	-27.880000	6.300000
45D-7	-85.070000	2.100000	-28.660000	4.200000

Stdev – Standard deviation

Figure 6: Zeta potential value of fungal grown sludge solids after 5 days of incubation at four different temperatures with varying inoculum (10^4, 10^5, 10^6 and 10^7) concentration without cations and with cations (Ca^{++}) (a) samples incubated at 15°C, (b) 25°C, (c) 35°C and (d) 45°C.