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Biosurfactant production from shrimp shell waste by *Pseudomonas stutzeri*

Deepa Kadam & Devayani Savant*
SIES Indian Institute of Environment Management, Sri Chandrasekarendra Saraswati Vidyapurum, Nerul, Navi Mumbai India
*[E-mail: devayanis@sies.edu.in]

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Biosurfactant producing *Pseudomonas stutzeri* strain L1 was isolated from a marine fishing port in Mumbai. Biosurfactant production by the strain was tested using crude substrates like de-oiled cakes of soybean, sunflower and coconut; fish waste, shrimp shell waste, sugarcane and mosambi waste. The isolate exhibited emulsification activity in most of the substrates with the highest in shrimp shell waste. Nutritional and environmental parameters for maximum biosurfactant production were optimized by changing one variable at a time. Biosurfactant was recovered by acid precipitation. About 4 to 6 g/l biosurfactant could be obtained in the optimized medium which showed emulsification index of 65 % and surface tension reduction upto 40 dynes/cm.

[Keywords: Shrimp shell waste; Biosurfactant; *P. stutzeri*; Emulsification; Oil pollution; Waste management]

Introduction

Oil pollution has become a persistent problem in the oceans world over. Offshore oil wells, under- water leakage of oil pipelines, accidents of ships, ballast water release, discharge of industrial and municipal wastewaters, diesel pump and ship cleaning activities, loading and unloading activities at port and natural seeps are the various causes of marine oil pollution[1]. The oil forms a thin film or slick on the water surface and affects the marine flora, fauna and human beings[2,3,4]. It also damages boats, fishing gears, port installations and greatly diminishes the value of shores and heritage sites as recreational resources. If not treated, crude oil spills would require a very long period of time to naturally biodegrade; it nearly takes about 22 years for complete biodegradation of 1 kg of crude oil by natural processes[5]. Many methods are being used to remove oil from water including physical removal by booms, skimmers and sorbents; chemical methods like use of gelling agents and dispersants and biological methods like fertilization, seeding with different bacteria and applications of biosurfactants[6]. All these methods have their own advantages and disadvantages. Dispersants contain chemical surfactants which are highly toxic to aquatic flora and fauna. Biosurfactants are structurally diverse group of surface active amphiphilic compounds produced by different microorganisms and have wide applications in control of oil pollution. They beat chemical surfactants in specificity, low toxicity, high biodegradability, effectiveness at extremes of temperature, pH, salinity and widespread applicability[7]. They assist in emulsification and degradation of oily waste and can be used in control of oil pollution. (Fig.1) But their high cost of production, which is 3-10 times more than the chemical surfactants; low yield and difficulties in downstream processing limit their commercial production[8,9]. Many low cost substrates have been recently reviewed by different researchers for biosurfactant production[8,9,10,11,12]. Animal fat, molasses, starch industry waste, olive oil mill effluent, agro based products like wheat bran, rice bran and soap stock,

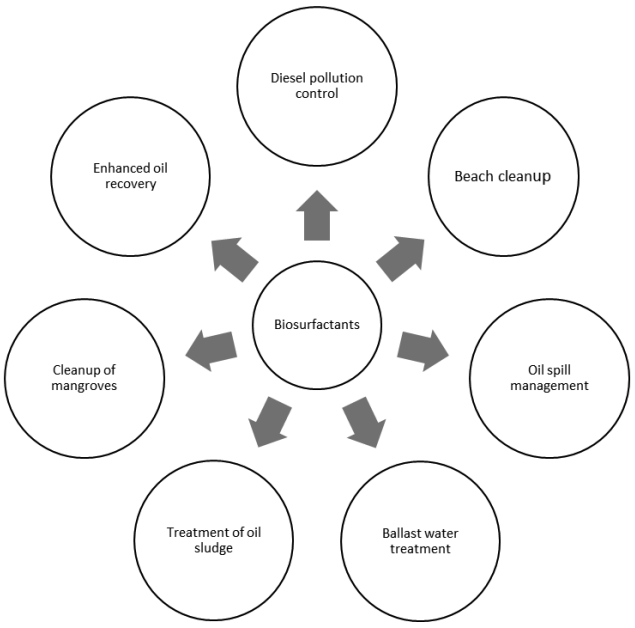


Fig. 1 — Environmental applications of biosurfactants

dairy industry waste are some of the crude substrates that have been used to reduce the production cost.

In this work, various deoiled cakes, fruit juice wastes, fresh fish waste and shrimp shell waste were tested for biosurfactant production.

To compete with synthetic surfactants, it is also necessary to have an effective microorganism for biosurfactant production. Since majority of applications are in marine ecosystem, it is thought that biosurfactants from marine bacteria would be more effective. Moreover, marine microbes have some novel structural and functional properties. Several high molecular weight polymer and glycolipid type biosurfactant and bioemulsifiers are produced by marine microbes and have important potential application in different industries[13]. Many marine bacteria have been explored for production of surface active molecules of biosurfactant and bioemulsifier e.g. *Acinetobacter, Arthrobacter, Pseudomonas, Myroides. Halomonas, Alcanivorax, Rhodococcus* and *Halomonas.* Marine ecosystems therefore provide an excellent opportunity to select potent microorganisms. In this study, biosurfactant producing marine bacteria were isolated from various sites in and around Mumbai harbour and the selected strain was further studied for utilization of various crude substrates for biosurfactant production.

Materials and Methods

Marine water samples were obtained from 12 different sites in and around Mumbai including salt pans, oil refineries, oil spill areas, mangroves and shipping harbour. Samples were collected aseptically and were inoculated in 100 ml Artificial Sea Water (ASW) medium[14] with 2% (v/v) engine oil as hydrocarbon substrate at pH 7.2 + 0.2. The samples were incubated at 30 °C temperature with agitation of 90-100 rpm. Two to three sequential transfers were given for each sample within a period of 30 days. After enrichment, the isolates were obtained on nutrient agar plates.

Various qualitative tests like oil displacement test[15], drop collapse assay[16], emulsification index (E- 24)[17,18], hemolytic activity[19,20], Blue agar plate method[21] were used for detection of biosurfactant producing isolates. Un-inoculated medium was used as negative control and 1% SDS as the positive control.

Surface tension was determined by ring method using Du Nouy ring tensiometer (K6, Komal Scientific, India). The test was carried out at room temperature on cell free supernatant of the culture obtained after centrifuging the culture broth at 8000 rpm for 20 minutes. Distilled water and 0.1% (w/v) SDS solutions were used as negative and positive controls respectively.

The isolate L1 was subjected to Gram staining and biochemical tests such as catalase, oxidase, indole, methyl red test, Voges Prauskeur test and citrate utilization as described in Bergey's Manual of Determinative Bacteriology[22]. Further identification was carried out by 16s rDNA sequencing. DNA was isolated by using InstaGene TM Matrix Genomic DNA isolation kit. PCR was performed using MJ Research TC -225 Peltier Thermal Cycler. 1 µl DNA extract was added in a total volume of 20 µl PCR reaction solution. The PCR was conducted using primers 27F and 1492R. PCR program consisted thirty five cycles of amplification including denaturation at 94 °C for 45 sec, annealing at 55 °C for 60 sec and extension at 72 °C for 60 sec. The PCR product was purified by using Montage PCR clean up kit (Millipore). The sample was sequenced using 518F/800R primers. Sequencing reactions were performed using an ABI PRISM BigDyeTM Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase. The sequences were analyzed using Sequence Scanner software. BLAST was used to find the sequence similarity. The programme MUSCLE 3.7 was used for multiple sequence alignment. Phylogenetic analysis was carried out using hyML 3.0 aLRT.

Different crude substrates were tested for biosurfactant production by the isolate. De-oiled cakes of soybean, sunflower, coconut (kopra), ground nut and fresh fish waste, mosambi (*Citrus limetta)* waste, sugarcane waste and shrimp shell waste were used at 2% w/v concentration. All the sources were sun dried and powdered in a domestic grinder except fresh fish waste. Shrimp shell waste was further tested at concentrations ranging 1% - 8% w /v. Biosurfactant production was measured in terms of emulsification index.

For optimization of biosurfactant production, a series of experiments were conducted by changing one variable at a time, keeping the other factors fixed at specific set of conditions. 2 % v/v inoculum of 24 hrs old freshly grown culture was used in all the experiments. Biosurfactant production was measured in terms of emulsification index. Whereever necessary, uninculated medium was used as the negative control and 1% w/v SDS was used as the positive control.

Sugars like lactose, sucrose, fructose, xylose were used at 2 % w/v to evaluate the capacity of *P. stutzeri* to

produce biosurfactant. Inorganic nitrogen sources tested were urea, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, KNO_3, while keeping nitrogen concentration same as that of original concentration of $NaNO_3$ in ASW medium. Organic sources such as yeast extract, beef extract, peptone, tryptone were tested at 0.1% w/v concentration.

Effect of salinity on biosurfactant production was determined by adding NaCl at different concentrations ranging 1 to 10% w/v. The effect of pH was checked by changing the pH using 1N HCl and 1N NaOH in range of 5 to 8. Effect of temperature was evaluated by incubating the culture media at temperatures 25 °C, 30 °C, 37 °C and 45 °C.

Amino acids, surfactants and some hydrocarbons are known for stimulation of biosurfactant production. Amino acids like leucine, isoleucine, arginine, glutamine, lysine and surfactants like SDS, Tween 80 and Triton X-100; hydrocarbons like N-hexadecane and kerosene and chemicals like magnesium sulfate and dipotassium hydrogen phosphate were tested at 1g/l concentration for enhancement of biosurfactant production.

Bacterial cells were removed by centrifugation at 12,000 rpm at 4 °C for 15 min. Culture supernatant was acidified with 6 N HCl to obtain a pH of 2.0 and kept overnight in a refrigerator at 4 °C. The precipitated biosurfactant was extracted three times with two volumes of chloroform:methanol (2:1 v/v) mixture. Pooled solvent extracts were concentrated by evaporation in a rota-vacuum evaporator. The yield of biosurfactant was estimated gravimetrically[23].

Results and Discussion

Twenty seven isolates were obtained from artificial sea water medium supplemented with 2% engine oil. The isolates were subjected to screening tests for biosurfactant production. The isolate L1 gave positive results for qualitative tests like oil displacement, drop collapse, emulsification index, surface tension reduction and Blue agar plate as shown in Table 1. Hence, it was selected for further studies. The isolate L1 was Gram negative rod, motile, non-spore forming, non-fluorescent, without any pigment and showed growth under aerobic conditions. It was identified as *Pseudomonas stutzeri* on the basis of morphological tests, biochemical tests as shown in Table 2 and further confirmed by 16 S rRNA analysis (Fig. 2) (GenBank accession no. KR080473).

As shown in Fig. 3, the isolate L1 showed utilization of various crude substrates and biosurfactant production in terms of emulsification index. Fish waste, fruit waste and sugarcane waste did not support biosurfactant production much. Oily wastes are known to be preferred substrates for biosurfactant production due to their resemblance to

Table 1 — Results of the screening tests for biosurfactant production

Sr. No.	Screening Test	Result
1	Drop Collapse Test	0.4 cm
2	Oil Spread Assay	0.6 cm^2
3	Hemolysis	No Clear Zone (β hemolysis)
4	Blue Agar Plate Test	Blue colonies
5	Surface Tension Reduction	40 dynes/cm
6	Emulsification Index (E_{24})	53 %

Table 2 — Biochemical characteristics of the isolate L1

Sr. No	Biochemical test	Result
1	Catalase Production	Positive
2	Oxidase Test	Positive
3	Indole Production	Negative
4	Methyl Red Test	Negative
5	Voges Proskauer Test	Negative
6	Citrate Utilization	Positive

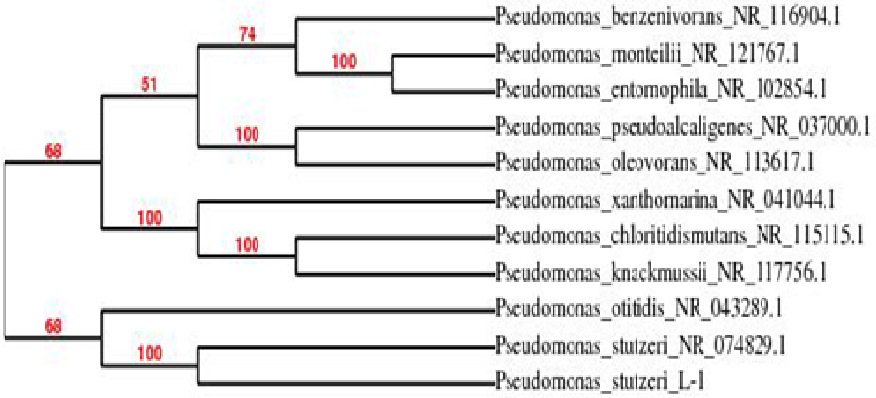


Fig. 2 — Phylogenetic tree showing the relationship among *Pseudomonas* sp. based on 16S rRNA gene sequences.

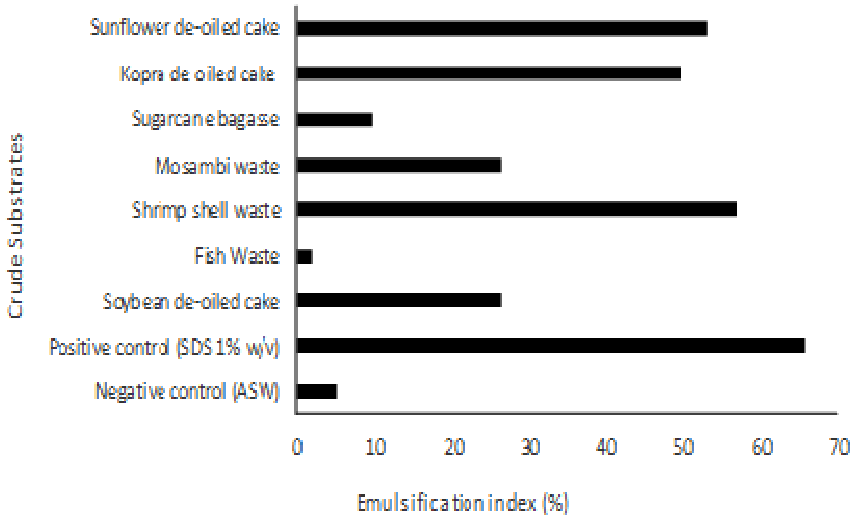


Fig. 3 — Utilization of crude substrates by isolate L1

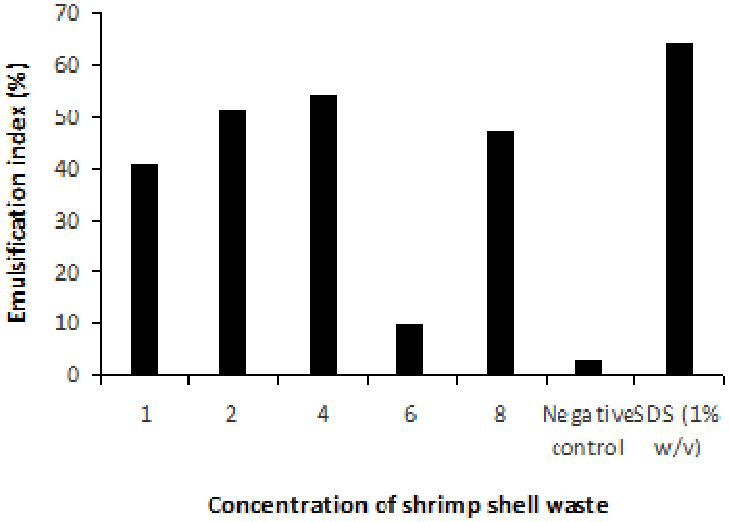


Fig. 4 — Effect of concentration of shrimp shell waste on biosurfactant production

hydrocarbon substrates[24]. For de oiled cakes, emulsification activity was in order of sunflower> coconut (kopra) > soybean. These results supported the findings of Ferraz *et al*, 2002[25] which suggested that the linoleic acid in sunflower oil actually enhances the biosurfactant production. The best biosurfactant production, however, was obtained with shrimp shell waste as the substrate with 45% increase in emulsification activity with respect to artificial sea water medium. Shrimp shell waste was further tested at different concentrations ranging 1% - 8% w/v. The optimum concentration to achieve maximum emulsification index was 4% w/v (Refer Fig. 4).

Shrimp shell waste is a low cost bio-resource abundantly available in coastal areas. It is estimated that the shell-fish industry produces about 8.5 million tonnes of waste every year, with shrimp processing accounting for more than one lakh tonnes of industrial waste. During processing, generally, the meat part is taken while the shell and head portions are discarded as wastes. Generally, more than 50-80% raw material results in the generation of a waste[26]. Although this waste is biodegradable, its disposal is a serious environmental concern as the rate of generation is high and causes obnoxious smell, attract pathogenic insects, flies and rodents, thus creating an unhygienic atmosphere[27] However, it is a highly nutritious material. It consists of 38-40% protein, very little carbohydrate, 1-10% fat content, 10-20% fibre and 20-30% ash. It contains minerals like Na, K, Ca, Mg, Cu, Fe, Co, Mn, Cr and P[28,29]. It is used as the poultry feed, as fertilizer in agriculture, production of chitin,

collagen, gelatin, chitosan, glucosamine hydrochloride, pearl essence, fish food, antioxidants, pigment astaxanthin and chitinase enzyme[30,31]. This is probably the first report of biosurfactant production from shrimp shell waste. As shrimp shell waste is abundantly available in the coastal region and is cheaper than any other hydrocarbon substrates, this method of biosurfactant production will not only assist municipal corporations in management of this waste but it can also be used by fish processing industries for waste treatment along with some revenue generation and can be used as an alternative livelihood method by the fishermen community.

Carbon and nitrogen source are two crucial factors for biosurfactant production. Sucrose gave best results as shown in Fig. 5 (a). The optimum concentration of sucrose was 4% w/v as shown in Fig. 5(b). These results support earlier findings of Persson and Molin (1987) who showed biosurfactant production by *Pseudomonas* in presence of sucrose[32] and Bayoumi *et al*, 2011 and Khopade *et al*, 2012 who also showed good growth and biosurfactant production using sucrose as carbon source[33,34].

It is well known that inorganic nitrogen sources give better results for biosurfactant production. Fig 6 shows more emulsification index for inorganic nitrogen sources with maximum emulsification with ammonium sulphate. Humzah *et al*, 2013 also obtained the similar results for *Pseudomonas* using ammonium sulphate[35.] All the organic nitrogen sources also supported biosurfactant production with maximum emulsification with peptone.

Figure 7(a) shows effect of pH on biosurfactant production by *Pseudomonas stutzeri*, it implies that it can grow and produce biosurfactant in limited range of pH and its optimum activity is at pH of 7. There are many reports on optimal growth of *Pseudomonas* species at pH range of 6 to 7 [36.]

With respect to temperature, it was observed that the isolate is sensitive to temperature fluctuations and

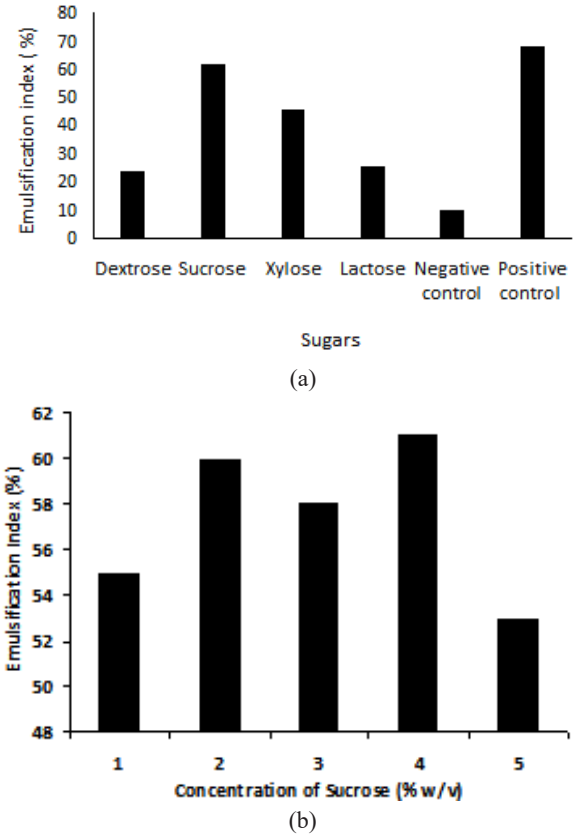


Fig. 5 — (a) Utilization of various sugars on biosurfactant production (b) effect of sucrose concentration on biosurfactant production

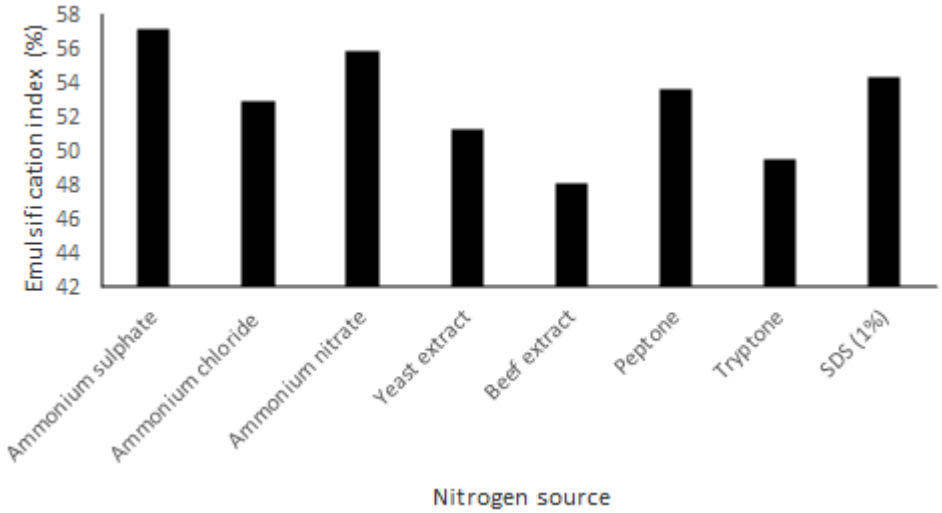
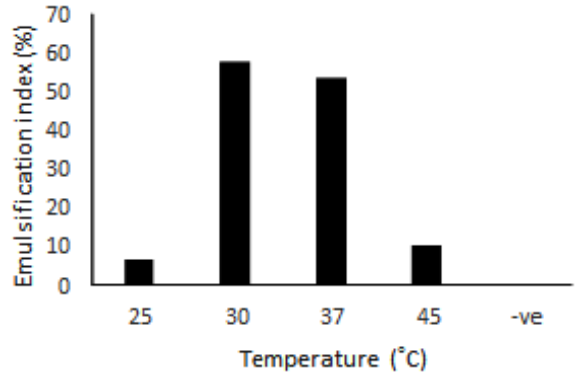


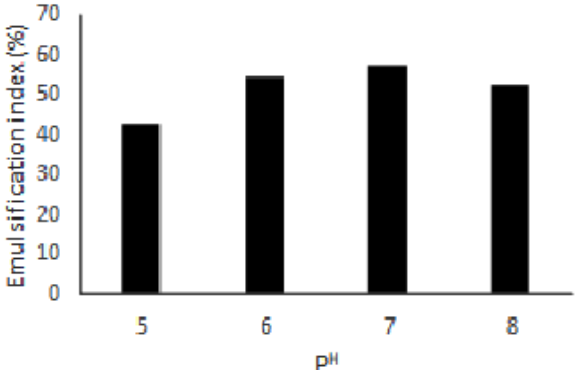
Fig. 6 — Effect of various nitrogen sources on biosurfactant production

its growth and biosurfactant production both were restricted to temperatures between 25 °C and 45 °C. It gave optimum results at 30 °C and almost equal at 37 °C as seen in Fig. 7(b). Previous reports also indicated optimal growth and biosurfactant production from *Pseudomonas* spp. in the range 30-37 °C[37].

Figure 7 (c) shows that the isolate L-1 could grow in presence of NaCl concentrations of up to 8% w/v. But its biosurfactant production activity was highest at 2 % w/v concentration of salt. These observations suggest that L1 is not a true halophile; however, it survives and produces biosurfactant in the marine environment[22,38].



(a)



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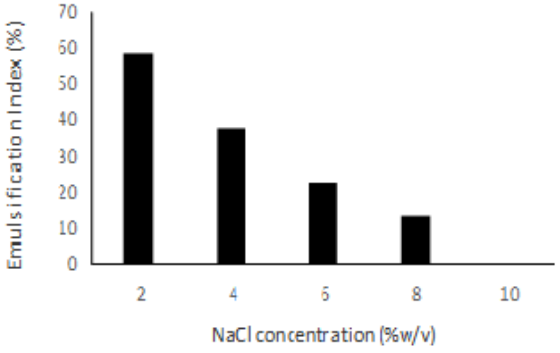
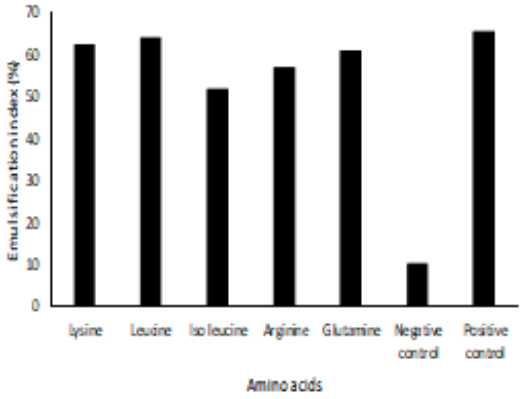


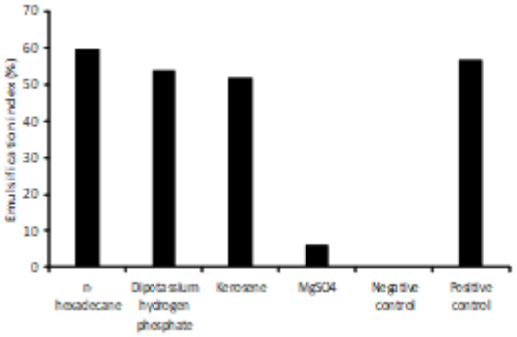
(c)

Fig. 7 — Effect of (a) pH (b) temperature (c) NaCl concentration on biosurfactant production

Fermentation was carried out with addition of different amino acids, surfactants, hydrocarbons and some chemicals in the fermentation medium. The results showed that very good emulsification activity could be obtained with n-hexadecane (Fig. 8). Poor



(a)



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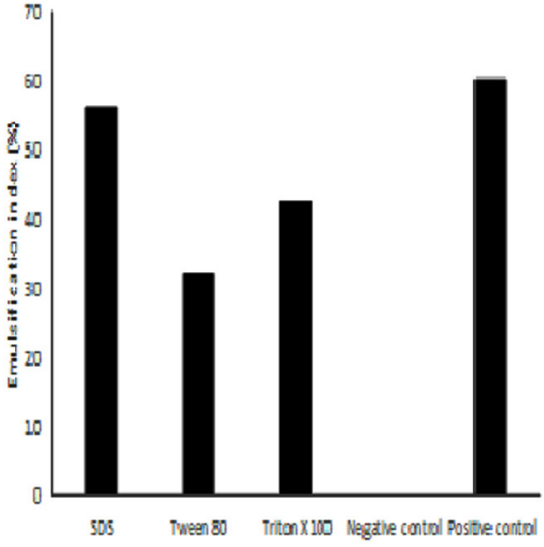

(c)

Fig. 8 — Effect of (a) amino acids (b) hydrocarbons and chemical stimulators (c) surfactants on biosurfactant production

emulsification in magnesium sulphate may be due to high amount of $MgCl_2$ already present in the medium. It was observed that amino acids were stimulatory and showed maximum emulsification with leucine and supported earlier observations by Huszczaa and Burczykb[39] and Dubey[40]. Amongst the surfactants, SDS showed maximum activity as a stimulator. Celik et al, 2007 had obtained similar results with Tween-80 and Triton X-100 which enhanced crude oil biodegradation and rhamnolipid production by a *Pseudomonas stutzeri* strain[41].

The biosurfactant was obtained as a yellowish brown liquid. About 4 -6 g/l biosurfactant could be produced in the optimized medium as against less than 0.1 g/l in ASW medium. It showed emulsification index of 65 % and could reduce surface tension upto 40 dynes/cm.

Conclusion

Shrimp shell waste could be used as an efficient substrate for production of biosurfactant from *Pseudomonas stutzeri*. The optimum conditions for biosurfactant production include 4% shrimp shell waste, 4% sucrose, 0.4 g/l ammonium sulfate as nitrogen source, 2% NaCl, leucine, n-hexadecane and SDS as stimulators (1g/l each), pH 7.0, temperature 30 °C, 2 % v/v inoculum and an incubation period of 5 days. About 4-6 g/l biosurfactant could be obtained under these conditions. The yield can be further increased using statistical strategies such as multivariate analysis and response surface methodology.

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References

1 Gunkel, W. & Gassmann, G., Oil, oil dispersanants and related substances in the marine environment, *Helgolander Meeresunters,* 33 (1980) 164-181.

2 Nansingh, P. & Jurawan, S., Environmental sensitivity of a tropical coastline (Trinidad, West Indies) to oil spills, *Spill Sci. Technol. Bull.*, 5 (1999) 161-172.

3 Santra, S. C., *Environmental Science* (New Central Book Agency (P) Ltd., Kolkata) 2013, pp. 278-282.

4 Aguilera, F., Mendez, J., Pasaro, E. & Laffon, B., Review on the effects of exposure to spilled oils on human health, *J. Appl. Toxicol.,* 30 (2010) 291-301.

5 Venosa, A.D. & Zhu, X., Bioremediation of crude oil contaminating marine shorelines and fresh water wetlands. *Spill Sci. Technol. Bull.,* 8(2) (2003) 163-178.

6 Adebajo, M.O., Frost, R.L., Kloprogge, J.T., Carmody, O. & Kokot, S., Porous materials for oil spill cleanup: a review of synthesis and absorbing properties, *J. Porous Mat.*, 10(2003) 159-170.

7 Desai, J.D. & Banat, I.M., Microbial production of surfactants and their commercial potential, *Microbiol. Mol. Biol. Rev*., 61(1997) 47–64.

8 Mukherjee, S., Das, P. & Sen, R., Towards commercial production of microbial surfactants, *Trends in Biotech*, 24(2006) 509-515.

9 Makkar, R.S., Cameotra, S.S. & Banat, I.M., Advances in utilization of renewable substrates for biosurfactant production, *AMB Express,* 1(5) (2011) 1-19.

10 Perfumo, A., Rancich, I. & Banat, I.M., Possibilities and challenges for biosurfactants use in petroleum industry, in: Biosurfactants (Springer, New York) 2010, pp. 135-145.

11 Henkel, M., Müller, M.M., Kügler, J.H., Lovaglio, R.B., Contiero, J., Syldatk, C. & Hausmann, R., Rhamnolipids as biosurfactants from renewable resources: concepts for next-generation rhamnolipid production, *Process Biochem.,* 47 (2012) 1207-1219.

12 Maneerat, S., Production of biosurfactants using substrates from renewable-resources, *Songklanakarin J. Sci. Technol.*, 27(2005) 675-683.

13 Satpute, S.K., Banat, I.M., Dhakephalkar, P.K., Banpurkar, A.G. & Chopade, B.A., Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms, *Biotech Adv.*, *28*(2010) 436-450.

14 Berges, J.A., Franklin, D.J. & Harrison, P.J., Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the last two decades, *J. Phycol.,* 37(2001) 138-1145.

15 Morikawa, M., Hirata, Y. & Imanaka, T., A study on the structure–function relationship of lipopeptide biosurfactants, *Biochim. Biophy. Acta (BBA)-Mol.Cell Biol Lipids*, 1488(2000) 211-218.

16 Abouseoud, M., Maachi, R. & Amrane, A., Biosurfactant production from olive oil by *Pseudomonas fluorescens*, *Commun. Curr. Res. Edu. Topics Trends Appl. Microbiol.*, 1(2007) 340-347.

17 Cooper, D.G. & Goldenberg, B.G., Surface-active agents from two *Bacillus* species. *Appl. Environ. Microbiol.,* 52(1987) 224-229.

18 Youssef, N.H., Duncan, K.E., Nagle, D.P., Savage, K.N., Knapp, R.M. & Inerney, M.J. Comparison of methods to detect biosurfactant production by diverse microorganisms, *J. Microbiol. Methods*, 56(2004) 339-347.

19 Abu-Ruwaida, A.S., Banat, I.M., Haditirto, S. & Khamis, A., Nutritional requirements and growth characteristics of a biosurfactant-producing *Rhodococcus* bacterium, *World J. Microbiol. Biotechnol.*, 7(1991) 53-60.

20 Carrillo, P.G., Mardaraz, C., Pitta-Alvarez, S.I. & Giulietti, A.M., Isolation and selection of biosurfactant-producing bacteria. *World J.Microbiol. Biotechnol.,* 12(1996) 82-84.

21 Siegmund, I. & Wagner, F., New method for detecting rhamnolipids excreted by *Pseudomonas* spp. during growth on mineral agar, *Biotechnol. Techniques,* 5(1991) 265-268.

22 Buchanan, R.E., Gibbons, N.E., Cowan, S.T., Holt, T.G., Liston, J., Murry, R.G., Niven, C.F., Ravin, A.W. & Stanier, RY., Bergey's Manual of Determinative Bacteriology, (Williams and Wilkins Co., Baltimore), 1974, 1246.

23 Dubey, K. & Juwarkar, A., Distillery and curd whey wastes as viable alternative sources for biosurfactant production, *World J. Microbiol. Biotechnol.,* 17(2001) 61-69.

24 Sousa, M., Melo, V.M.M., Rodrigues, S., Sant'ana, H.B. & Gonçalves, L.R.B., Screening of biosurfactant-producing *Bacillus* strains using glycerol from the biodiesel synthesis as main carbon source, *Bioprocess Biosyst Engg*., 35 (2012) 897-906.

25 Ferraz, C., De Araújo, Á.A. & Pastore, G.M., The influence of vegetable oils on biosurfactant production by *Serratia marcescens,* in: *Biotechnology for Fuels and Chemicals* , edited by (Humana Press), 2002, pp. 841-847.

26 Zynudheen, A.A., Utilisation of fishery waste in India (CIFT Publication, Cochin), 2010.

27 Tarafdar A. & Biswas, G., Extraction of chitosan from prawn shell wastes and examination of its viable commercial applications, *Int. J. Theor. Appl. Res. Mech Res.,* 2(2013) 2319 – 3182.

28 Adeyeye, E.I., Adubiaro, H.O. & Awodola, O.J. Comparability of chemical composition and functional properties of shell and flesh of *Penaeus notabilis*, *Pakistan J. Nutrition*., 7(2008) 741-747.

29 Ravichandran, S., Rameshkumar, G. & Prince, A.R., Biochemical composition of shell and flesh of the Indian white shrimp *Penaeus indicus* (H. milne Edwards 1837), *American-Eurasian J. Sci. Res.,* 4(2009) 191-194.

30 Muzzarelli, R.A., Jeuniaux, C. & Gooday, G.W., Chitin in nature and technology, in: International Conference on Chitin and Chitosan (Senigallia, Italy) 1985, Plenum Press.

31 Healy, M.G., Romo, C.R. & Bustos, R., Bioconversion of marine crustacean shell waste, *Resources, Conservation and Recycling*, 11(1994) 139-147.

32 Persson, A. & Molin, G., Capacity for biosurfactant production of environmental *Pseudomonas* and *Vibrionaceae* growing on carbohydrates, *Appl. Microbiol. Biotechnol.,* 26 (1987) 439-442.

33 Bayoumi, R.A., Atta, H.A., El-Sehrawey, M.A. & Selim, S.M., Microbial production of biosurfactants from some El-Khorma governorate microbial isolates for bioremediation of crude oil spills in the different environments, *J. Basic Appl. Sci. Res.,* 1(2011) 1541-1555.

34 Khopade, A., Ren, B., Liu, X-Y, Mahadik, K., Zhang, L. & Kokare, C., Production and characterization of biosurfactant from marine *Streptomyces* species B3, *J. Colloid Interface Sci.* 367(2012) 311–318.

35 Hamzah, A., Sabturani, N. & Radiman, S., Screening and optimization of biosurfactant production by the hydrocarbon-degrading bacteria, *Sains Malaysiana* 42(2013) 615–623.

36 Guerra-Santos, L., Kappeli, O. & Fiechter, A., *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source, *Appl. Environ. Microbiol.,* 48(1984*)* 301–305.

37 Wei, Y.H., Chou, C.L. & Chang, J.S., Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater, *Biochem. Eng. J.*, 27(2005) 146-154.

38 Maneerat, S. & Phetrong, K., Isolation of biosurfactant-producing marine bacteria and characteristics of selected biosurfactant, *Songklanakarin J. Sci. Technol.*, 29(2007) 781-791.

39 Huszczaa, E. & Burczykb, B., Biosurfactant production by *Bacillus coagulans*, *J. Surfactants Detergents*, 6(2003) 61-64.

40 Dubey, K., Biosurfactant production by new microbial isolates from soil co-contaminated with lube oil and distillery spent wash, *Int. J. Sci. Res.,* 2(2013) 17-19.

41 Celik, G.Y., Aslim, B. & Beyatli, Y., Enhanced crude oil biodegradation and rhamnolipid production by *Pseudomonas stutzeri* strain G11 in the presence of Tween-80 and Triton X-100, *J. Environ. Biol.,* 29(2008) 867-870.