Journal of Ecobiotechnology 2/3: 33-39, 2010
ISSN 2077-0464

Journal of Ecobiotechnology
http://journal-ecobiotechnology.com/

Enhancement Effects of Biosurfactant Produced by *Pseudomonas aeruginosa* MTCC 2297 and *Pseudomonas fluorescens* on Sugar Cane Bagasse Composting

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Keywords

Biosurfactant
Rhamnolipid
Pseudomonas
Compost pile
Sugarcane bagasse
Decomposing

Abstract

Biosurfactant produced by *Pseudomonas aeruginosa* MTCC2297 and *Pseudomonas fluorescens* (a Mangrove forest isolate) was added to the Sugar cane bagasse decomposing process to initiate and enhance the production of reducing sugars. Both isolates produced a glycolipid biosurfactant, namely a rhamnolipid that was confirmed by biochemical and analytical studies. The surface tension of fermentation broth reduced from 73 to 34.2 mN/m by *P. aeruginosa* MTCC 2297 and from 76 to 29 mN/m by *P. fluorescens*. Sugar cane bagasse decomposing process was attempted by two different methods, adding pure rhamnolipid and on-site production of it were compared. The study revealed that rhamnolipid concentration of 0.75gl-1 was optimum for composting process and the optimum temperature for compost pile production was 32°Cfor the first 48h subsequently 35°C for the next 48h. At the optimum temperature for this two-stage fermentation, the production of reducing sugar could be increased to 15.73 gl-1 for P. aeruginosa MTCC 2297 and for the *P. fluorescens* reducing sugar increased to 15.88 gl-1, both experiments resulted in higher values than that of the treatment without rhamnolipid sample. The results indicate that application biosurfactant could possibly reduce the composting period. As the compost pile production of rhamnolipid does not require the purification process, the production cost reduced significantly.

1. Introduction

Environmental pollution is a great concern to the world today, mainly due to rapid industrialization, urbanization and utilization. Since large amount of CO_2 are let out by the combustion of fossil fuel, development of alternative energy resources that have minimal environmental impact is an urgent need of the hour. Several review papers have been published covering wide range of topics related to reduction of carbon dioxide emission by mixing bioethanol in to gasoline. Alternative energy sources that are being currently used for the production of bioethanol are lignocellulosic agricultural wastes. Lignocellulosic residues such as sugarcane bagasse is available in large quantity used in industry to produce power, make paper, building materials, as a fuel and even as feedstock. Bagasse is among the world's most widely used and available non wood fibres (Laszlo, 1998).

Enzymatic hydrolysis of sugar cane bagasse rich in cellulose can be converted to reducing sugars, which can subsequently be fermented to target products such as ethanol; the hydrolytic cleavage of cellulose to reducing sugar are catalyzed by cellulases. However, the problem in the enzymatic hydrolysis of cellulose is the significant enzyme deactivation. The partially irreversible adsorption of cellulase on cellulose is usually proposed as a decisive mechanism (Wu and Ju, 1998; Eriksson *et al.,* 2002). Many reports have shown that surfactants can modify the cellulose surface property and minimize irreversible binding, thus promoting the production of cellulase (Reese and Manguire, 1969; Pardo, 1996) and enhancing the enzymatic hydrolysis of cellulose (Helle *et al.,* 1993; Eriksson *et al.,* 2002). Enhancement of cellulose hydrolysis by adding surfactants to the hydrolysis mixture has been reported by several authors (Castanon and Wilke, 1981; Ooshima *et al.,* 1986; Park *et al.,* 1992). Different cellulose substrates have been studied. Castanon and Wilke (1981) showed that conversion of newspaper increased by 14% after 48 h hydrolysis by the addition of Tween 80. Ooshima *et al.* (1986) compared amorphous cellulose with different types of crystalline celluloses (Avicel, tissue paper and reclaimed paper). They showed that the higher the crystallinity of the substrate, the more positive was the effect of the added surfactant. Increased hydrolysis by addition of surfactants has also been reported for delignified steam-exploded wood,

bagasse and corn stover (Helle *et al.,* 1993; Kurakake *et al.,* 1994; Kaar and Holtzapple, 1998).

Surfactants, both chemically-synthesized surfactants and biosurfactants, are extensively used in bioremediation, pharmaceutical, cosmetic, and food industry because of their surface activity. Among them, chemically synthesized surfactants are not biodegradable and can be toxic to the environment, biosurfactants have attracted a lot more attention because of their specificity, biodegradability, and biocompatibility (Wang and Mulligan, 2009).

Biosurfactants are amphiphilic compounds produced by a number of microorganisms, including bacteria, yeasts, and fungi. On the basis of the types of biosurfactant producing microbial species and the nature of their chemical structures, biosurfactants can be categorized as glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, peptides, phospholipids, and neutral lipids. It has been reported that addition of biosurfactants, particularly rhamnolipid, effectively improves the cellulase activity as well as preserves them for being recycled in the course of cellulose decomposing (Kaya *et al.,* 1995; Park *et al.,* 1992). Liu *et al.* (2006) discovered that rhamnolipid at 0.018% (W/W) can noticeably increase the production of xylanase in solid substrate fermentation (SSF), which is 119.6% higher than that of the control. However, the core problem for commercial application of the biosurfactant are low yield and high production cost (Wei *et al.,* 2005). Therefore, the research has been focused to develop efficient biosurfactant producer and a cost-effective production bioprocess.

In the current study, *P. chrysosporium* and *P. aeruginosa* MTCC 2297 and *P. fluorescens* (Parthasarathi and Sivakumaar, 2009) preserved in the laboratory are used for biosurfactant production and sugar cane bagasse decomposing respectively. The enhancement effects derived from adding biosurfactant to the sugar cane bagasse saccharification hydrolytic medium was mainly studied in current work. The two different methods of adding biosurfactant have been compared. One is by purifying the biosurfactant from fermentation broth of *P. aeruginosa* MTCC2297 and *P. fluorescens* separately first, and then adding it to the sugar cane bagasse decomposing process, which is used in many observations. Another is by making *P. chrysosporium* and *P. aeruginosa* MTCC2297 or *P. fluorescens* work together. This is a compost pile production of the biosurfactant and can eliminate the cost of purification of biosurfactant.

2. Materials and Methods

Chemicals and Microbial cultures used in the study

Chemical-surfactants such as poly polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether, Octyl phenol ethoxylate (Triton X-100), Sodium dodecyl sulphate (SDS), and Polyoxyethylene (80) sorbitan monooleate (Tween 80), were all obtained from Hi-Media, India. *P. chrysosporium*, a compost strain obtained from the Department of Plant Pathology, Faculty of Agriculture, Annamalai University, India. *P. aeruginosa* MTCC 2297 and a *P. fluorescens* isolate from mangroves forest Pichavaram (Parthasarathi and Sivakumaar, 2009) were used in the study. The surface tension of fermentation broth reduced from 73 to 34.2 mN/m by *P. aeruginosa* MTCC 2297 and from 76 to 29 mN/m by *P. fluorescens*. The compost culture *P. chrysosporium* maintained at 4°C on slants of PDA and the bacterial cultures were maintained in LB broth and the cultures were sub cultured once in every 3 weeks.

Enzymatic hydrolysis

Sugar cane bagasse was pretreated with 2% NaOH at 85°C for 1h before enzymatic hydrolysis. Then, the pretreated sugar cane bagasse was decomposed by *P. chrysosporium* at 32°C for 4 d in a shaking bed (120 r/ min). After fermentation, the culture broth was centrifuged at 8,600 r/min for 1 5 min and the supernatant was analyzed for reducing sugar production. All the experiments were performed in triplicate and the mean values were recorded.

Biosurfactant production and purification

The inoculum, was prepared by suspending colonies in 2 ml medium (10^6 CFU/ml) and then pipetted into a 250-ml Erlenmeyer flask containing 50 ml of inoculum growth medium, followed by incubation in a shaking bed (120 r/min) at 35°C. Sterile supernatant served as the source of the crude biosurfactant. Nutrient broth was used for preparation of the inoculum. For biosurfactant synthesis a mineral salt medium with the following composition (gl^{-1}) was used: Na_2HPO_4 (2.2), KH_2PO_4 (1.4), $MgSO_4 \cdot 7H_2O$ (0.6), $FeSO_4 \cdot 7H_2O$ (0.01), NaCl (0.05), $CaCl_2$ (0.02), yeast extract (0.02) and 0.1 ml of trace element solution containing (g/-L): 2.32 g $ZnSO_4 \cdot 7H_2O$, 1.78 g $MnSO_4 \cdot 4H_2O$, 0.56 g H_3BO_3, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.42 g $CoCl_2 \cdot 6H_2O$, 1.0 g EDTA, 0.004 g $NiCl_2 \cdot 6H_2O$ and 0.66 g KI. pH of the medium was adjusted to 7.0 ± 0.2. The carbon sources used glucose (20 gl^{-1}) (Sigma), with NH_4Cl

1 gl^{-1} as nitrogen source. Cultivations were performed in 250 ml flasks containing 100 ml medium at room temperature, and stirred in a rotary shaker at 110 rpm for 3 days. The culture broth was centrifuged (10,000*g*, 15 min) to remove the cells and thereafter sterilized with Millipore membrane filter. The clear biosurfactant was recovered from the cell-free culture supernatant by cold acetone precipitation as described by Pruthi and Cameotra (1995). Three volumes of chilled acetone was added and allowed to stand for 10h at 40°C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone after which it was re-dissolved in sterile water. Solid obtained after frozen lyophilization was the coarse biosurfactant product. To further purify the biosurfactant, it was then extracted by chloroform and methanol in the ratio of 2:1 (V/V). Ultimately, the solvent layer was pooled and concentrated under vacuum using a rotoevaporator at 65°C, thus obtaining an aqueous solution of pure biosurfactant.

Biochemical and analytical methods

Reducing sugars, produced by *P. chrysosporium* in the course of sugarcane bagasse hydrolysis, were determined by the DNS method (Miller, 1959). The cultures namely, *P. aeruginosa* MTCC 2297 and *P. fluorescens* ability to produce biosurfactants and the activity was determined by the following methods, The equilibrated surface tension (ST) and interfacial tension (IFT) of Cell free culture broth (CFCB) were measured by the ring method (Margaritis *et al.,* 1979) using a digital tensiometer (K10T; Krüss, Germany). The IFT was measured by submerging the tensiometer ring in 10 mL CFCB and adding an equal volume of kerosene oil in the tensiometer vessel, so that the ring did not break through the upper oil layer before the interfacial film was ruptured (Akit *et al.,* 1981). The change in pH value of the culture broth was also monitored by a digital pH-meter. Emulsification activity (E_{24}) was determined by Cooper and Goldenberg (1987) method at zero time and at the end of 10 h of incubation.

Biosurfactant analysis

The purified biosurfactant extracts were dissolved in distilled water to determine rhamnolipids in terms of rhamnose equivalents by the orcinol method (Chandrasekaran and Bemiller, 1980). A 333 μl^{-1} sample of the supernatant, obtained after centrifugation, was extracted twice with 1 ml^{-1} diethyl ether. Ether fractions were evaporated to dryness and 0.5 ml^{-1} of distilled H_2O was added. To 100 μl^{-1} of each sample, 900 μl^{-1} of a solution containing 0.19% orcinol (in 53% H_2SO_4) were added. After heating (at 80°C for 30 min), the samples were cooled to room temperature and the absorbance was measured at 421 nm. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg ml^{-1}). The rhamnolipid contents were calculated as 3.4 times the rhamnose contents (Benincasa *et al.,* 2004).

Statistical analysis

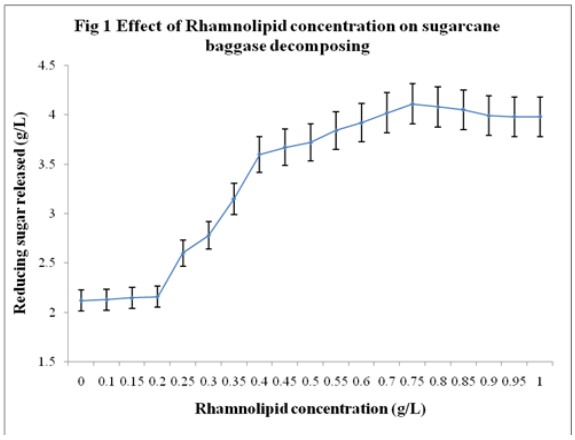
All experiments were carried out at least three times and were highly reproducible. Therefore, data from one of each experiment are presented. The treatments in the figures represent significant differences at P= 0.05 according to one way analysis of variance.

3. Results and Discussion

The biosurfactant produced by *P. aeruginosa* MTCC 2297 and *P. fluorescens* was then purified. The surface tension of fermentation broth reduced from 73 to 34.2 mN/m by *P. aeruginosa* MTCC2297 and from 76 to 29 mN/m by *P. fluorescens*. IFT in the CFCB of both the cultures in kerosene oil resulted in the values from about 26 mN/m to 0.6 (*P. aeruginosa* MTCC 2297) and to 0.5 (*P. fluorescens*) on the same period of study respectively. The pH values of the CFCB of both the cultures grown on hydrocarbon media was lowered from 7.2 to about 6.0. E_{24}, emulsification activity was 76% and71% by *P. aeruginosa* MTCC 2297 and *P. fluorescens* in n-Hexadecane respectively. The *P. aeruginosa* MTCC 2297 yielded about 8.366 g/l and *P. fluorescens*, mangrove isolate yielded 9.35 gl^{-1} of glycolipid biosurfactant, namely rhamnolipid. Several reports have earlier suggested about the ability of *P. aeruginosa* and *P. fluorescens* to produce glycolipid type biosurfactant namely, rhamnolipid and it has the surface tension reduction, IFT reduction and emulsification activity (Matsufugi *et al.* 1997, Banat *et al* .2000; Tahzibi *et al* .2004; Wang and Mulligan, 2009).

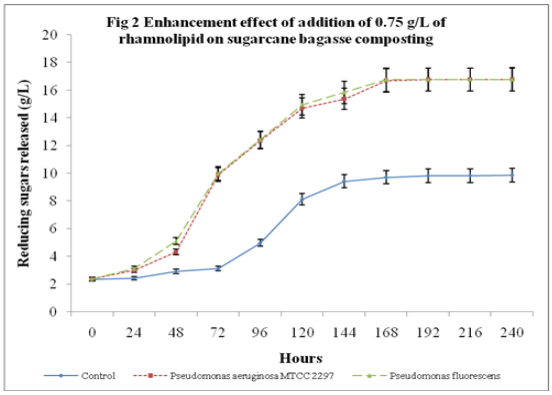
Enhancement effects of pure biosurfactant on Sugar cane bagasse decomposing

Enhancement effects were studied by the addition of purified rhamnolipid with varied concentrations to the sugarcane bagasse decomposing course shown in Fig. 1. It was evident that as more rhamnolipid was added, more reducing sugars were produced by *P. chrysosporium*. When the concentration of rhamnolipid was below 0.25 gl^{-1}, there was only a slight increase in the reducing sugars. When 0.25 gl^{-1} rhamnolipid was added, the production of reducing sugar was only 18.46%, higher than that of the control.



However, when the concentration of rhamnolipid was increased to 0.75 gl^{-1}, the production of reducing sugar was 48.54% higher than that of the control (*P. aeruginosa* MTCC 2297), which signifies that rhamnolipid had an enhancement effect on sugarcane bagasse decomposing. When the concentration of rhamnolipid was above 0.75 gl^{-1}, the increase of reducing sugar started to stabilize. Thus, 0.75 gl^{-1} was chosen as the optimum adding concentration of rhamnolipid.

The enhancement effect of rhamnolipid further proved on sugarcane bagasse hydrolysis, the influence of fermentation time on the production of reducing sugar, by *P. chrysosporium* was compared with control without rhamnolipid samples and rhamnolipid-added (0.75 gl^{-1}) samples, as illustrated in Fig.2. In the control without rhamnolipid samples, 8.12 gl^{-1} reducing sugar produced by *P. chrysosporium* in 120h, which was considered as the optimal fermentation time. However, in the rhamnolipid-added samples, the production of reducing sugar could achieve 9.92 gl^{-1} in 72h (*P. aeruginosa* MTCC 2297) and 9.91 gl^{-1} in 72 h (*P. fluorescens*). Therefore, that the addition of rhamnolipid to the sugarcane bagasse decomposing course could not only increase the production of reducing sugars, but could also reduce the fermentation time significantly, thus reducing the production cost effectively.



Low concentration of cellulose, non ionic character of the biosurfactant produced and possible delignification mechanism by the biosurfactant are may be one among the reasons of the effects in the current study .The results were in accordance with previous reports (Ooshima *et al.,* 1986 and Kim *et al.,* 1982). The biosurfactant produced could also increase the stability of the enzymes and thus, reduce enzyme denaturation during the hydrolysis of cellulose.

When the enzymes adsorbed in the cellulose matrix as limitation of hydrolysis of cellulose substrate described by several authors may be prevented by the facilitative desorption effect of the added biosurfactant (Kaar and Holtzapple, 1998 and Park *et al.,* 1992).The facilitative desorption effect could be seen in the current study form the enhancement of sugar cane bagasse decomposing process in the presence of pure rhamnolipid biosurfactant.

Compost pile production of biosurfactant and its enhancement effects on sugarcane bagasse decomposition

To avoid the complex purification process and reduce the cost of sugarcane bagasse decomposing, Compost pile production of the rhamnolipid bioprocess was thought. As the growth periods for *P. chrysosporium* and *P. aeruginosa* MTCC 2297, *P. fluorescens* are 120 and 48 h (bacterial cultures), respectively. *P. chrysosporium* was first cultivated on sugarcane bagasse decomposing for 48 h at 32°C subsequently after the next 48 h, the exponential inoculum of *P. aeruginosa* MTCC 2297 (Experiment -I) and *P. fluorescens* (Experiment - II) inoculated into it in order to keep these two microorganisms working together.

The result showed that 35°C was the optimum temperature for the next 48 h. however; the optimum temperature for *P. chrysosporium* is 32°C for sugarcane bagasse decomposing. The rhamnolipid production bioprocess requires compatible non competitive fermentation conditions for both microorganisms. As temperature is one of the most important factors, *P. chrysosporium* is considered as the microorganism in the first 48 h fermentation. As shown in Fig. 2 the production of reducing sugar increased with an increase in temperature.

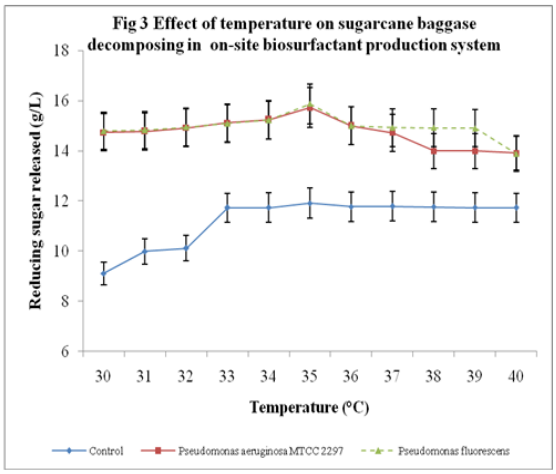
This might be because of the stronger temperature tolerance ability of *P. aeruginosa* MTCC 2297 and *P. fluorescens* than that *P. chrysosporium.* At the optimum temperature for this two-stage fermentation, namely, 32°C for the first 48 h and 35°C for the next 48 h, the production of reducing sugar increases to 16.43 gl^{-1}, for *P. aeruginosa* MTCC

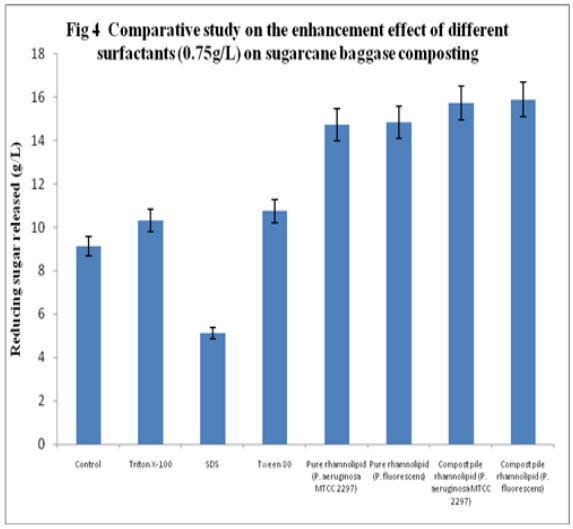
2297 (27% higher than control) and for the *P. fluorescens* reducing sugar increases to 16.48 gl^{-1} (27.66% higher than control), both experiments resulted in higher values than that of the control. The inherent ability of biosurfactant production by the *P. aeruginosa* MTCC 2297 and *P. fluorescens* revealed that biosurfactant mediated hydrolysis of cellulose could be possible. The stability, prevention of enzyme adherence to the cellulose matrix could be improved in the presence of biosurfactants. The results were supportive of the mechanism of surfactant mediated enhancement proposed by Kaar and Holtzapple (1998) and Park *et al.* (1992).

Comparative study on enhancement effect with chemical surfactants

The effects of chemically synthesized surfactants and two different adding methods of biosurfactants on sugarcane bagasse decomposing were studied as follows. Four different surfactants, including Triton X-100, SDS, Tween 80, and Pure rhamnolipid (*P. aeruginosa* MTCC 2297 and *P. fluorescens*), were evaluated for their ability to enhance the enzymatic hydrolysis of sugarcane bagasse.

Moreover, for adding rhamnolipid, compost pile production, was also compared, as described in Fig.1. For each surfactant, the adding dosage was 0.75 gl^{-1}. whereas, for the production of rhamnolipid, the adding inoculation concentration ratio of *P. aeruginosa* MTCC2297 or *P. fluorescens* to *P. chrysosporium* was 4%. As seen in Fig. 3. Reducing sugar of 9.13 gl^{-1} was obtained in enzymatic hydrolysis of sugarcane bagasse without surfactant (control) and also revealed that all nonionic surfactants, including Triton X- 100 and Tween 80, improved the hydrolysis slightly.





Among them, Tween 80 showed the best ability to improve sugarcane bagasse decomposing, which could increase the production of reducing sugar to 10.5 gl^{-1}, 15% higher than that of the control. However, the negatively charged surfactant SDS reduced the production of reducing sugars (5.13 gl^{-1}). Moreover, it could obviously be seen that these two adding methods of rhamnolipid greatly increased the production of reducing sugars, both of which were better than any other kind of chemically synthesized surfactant used in the present study.

The pure rhamnolipid adding method and compost pile production of rhamnolipid could increase the production of reducing sugar to 9.13 to 14..72 gl^{-1} (*P. aeruginosa* MTCC 2297) and 14.83gl^{-1} (*P. fluorescens*), which is 37.97% and 38.43% higher than that of the control in pure rhamnolipid application method and 9.13 to 16.43 gl^{-1} (*P. aeruginosa* MTCC 2297*)* and 16.48 g l^{-1} (*P. fluorescens*), which is 41.95% and 42.5% higher than that of the control in compost pile production of rhamnolipid application method respectively, these results provide a promising future for the biosurfactant in sugarcane bagasse decomposing, especially for the compost pile production method.

SDS is well known to be an effective surfactant for protein denaturation. It unfolds the secondary and tertiary protein structure. Current study revealed that SDS concentration of 0.75 gl^{-1} was resulted in the negative effect on reducing sugars released. The negative effect of enzymatic action when SDS (1.0 gl^{-1}) mixed was reported by Erikkson *et al.* (2002). Since the surfactant inactivates enzymes released results in lesser reducing sugar produced in the sugar cane bagasse composing.

4. Conclusion

The enhancement effect of the biosurfactant produced *P. aeruginosa* MTCC 2297 and *P. fluorescens* on sugarcane bagasse decomposing was studied in this article. The results obviously showed that both the adding methods of rhamnolipid were more effective than the control. As the production of rhamnolipid could leave out the purification process, thus reducing the production cost effectively, it seemed to be a prospective adding method of the biosurfactant, for enhancing the hydrolysis of sugarcane bagasse. The enhancement effect of biosurfactants may be due to the improvement of permeability of the cell membrane and led to the secretion extracellular enzymes. However, the surfactants might improve the cellulase stability and prevent the denaturation of enzymes during hydrolysis by desorbing it from the cellulose substrate. The facilitative desorption effect could be seen in the current study form the enhancement of sugar cane bagasse decomposing process in the presence of pure rhamnolipid biosurfactant. Therefore, the mechanism of facilitative enhancement of rhamnolipid on the sugarcane bagasse decomposing should be observed in depth in the future study. Moreover, as *P. chrysosporium* and *P. aeruginosa* MTCC 2297 and *P. fluorescens* were not compatible with fermentation conditions, such as pH and substrate concentration. Hence the optimum fermentation conditions for these two strains should be studied in the future extensively.

Acknowledgements

The author would like to thank the Department of Microbiology, Faculty of Agriculture, Annamalai University for providing the infrastructure and lab facility to successfully conduct the research work. Special thanks to Department of Plant Pathology, Faculty of Agriculture for providing the compost strain.

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