Research Journal of Environmental Toxicology

ISSN 1819-3420

Academic Journals Inc.

www.academicjournals.com

Research Journal of Environmental Toxicology 10 (1): 60-67, 2016
ISSN 1819-3420 / DOI: 10.3923/rjet.2016.60.67

Effect of Acid and Alkaline Pretreatment on the Production of Biosurfactant from Rice Husk Using *Mucor indicus*

[1]Obinna A. Oje, [2]Victor E. Okpashi, [2]Jane C. Uzor, [2]Ugochukwu O. Uma, [2]Anthony O. Irogbolu and [3]Ikechukwu N.E. Onwurah

[1]Department of Chemistry/Biochemistry/Molecular Biology, Federal University Ndufu Alike Ikwo (FUNAI), Ikwo, Ebonyi State, Nigeria

[2]Department of Biochemistry, University of Nigeria, Nsukka (UNN), Nsukka, Enugu State, Nigeria

[3]Biotechnology and Pollution Control Unit, Department of Biochemistry, University of Nigeria, Nsukka (UNN), Nsukka, Enugu State, Nigeria

Corresponding Author: Obinna A. Oje, Department of Chemistry/Biochemistry/Molecular Biology, Federal University Ndufu Alike Ikwo (FUNAI), Ikwo, Ebonyi State, Nigeria

ABSTRACT

This study was designed to determine the effect of acid and alkaline pretreatment of rice husk on the production of biosurfactant using *Mucor indicus*. Rice husk was pretreated at 120°C in a mineral nutrient medium at variable pH of 2, 7 and 12. The media were adjusted to pH7 after pretreatment to provide suitable environmental pH for microbial growth. *Mucor indicus* isolated from the rice husk dump site was used to inoculate the pretreated rice rusk media and the set-up was allowed to stand for 28 days with intermittent shaking. At the expiration of 28 days, the broth was filtered and centrifuged to remove all suspended cells. The cell free broth was collected for the extraction of biosurfactant using a mixture of chloroform/methanol at ratio 2:1 (mixture/broth). The results revealed that the biosurfactants yield were 0.59±0.078, 0.40±0.042 and 0.78±0.050 g in 100 mL of broth for pH 2, 7 and 12 pretreated rice husk, respectively. The biosurfactants produced showed highest emulsification index for automotive gas oil and lowest for premium motor spirit. The results also suggested that the biosurfactants produced have glycolipids properties. Therefore, it will be correct to suggest that for the conversion of rice husk into useful material such as biosurfactant, the heat pretreatment should be carried out at an alkaline pH.

Key words: Biosurfactant, emulsification index, *Mucor indicus*, pretreatment, rice husk, waste

INTRODUCTION

Recently, emphasis has been on environmental impacts caused by chemical surfactants due to toxicity and the inability for recalcitrant contaminants to degrade in the environment. The increase in rice husk dumps at different rice mill locations is an issue of environmental concerns. Advances in biotechnology and the emergence of stringent laws has led to biosurfactants potential as an alternative to the chemical surfactants (Muller *et al*., 2012). Biosurfactants are amphiphilic compounds produced by mostly microbial cell surfaces or excreted extracellularly. They contain hydrophobic and hydrophilic moieties which help to reduce surface and interfacial tensions between individual molecules at the surfaces and interfaces (Muller *et al*., 2012). Biosurfactants have several applications, for example in agriculture, biosurfactants are used as mobilizing agents due to their ability to increase apparent solubility of Hydrophobic Organic Contaminants (HOC) in the environment and increases the ability of microbes to adsorb and degrade these compounds. Also, they are used for hydrophilization of heavy in compacted soils to achieve wettability and facilitate

even distribution of fertilizer in the soil. Their antifungal activities have been employed in bio-control of plant diseases (Kachholz and Schlingmann, 1987). Biosurfactants are applied in commercial laundry as detergents (Das and Mukherjee, 2007) and also as bio-pesticide (Mulligan, 2005). In medicine, biosurfactants are used as antimicrobial agents (Zhao *et al*., 2010), antibacterial, antifungal, anti-adhesive agents, antivirus agents and as immunological adjuvants (Gharaei-Fathabad, 2011; Muthusamy *et al*., 2008). Report has shown that they also possess anti-cancer activity (Zhao *et al*., 2010). Biosurfactant has been used in food processing industry as food formulation ingredient and anti-adhesive agents (Muthusamy *et al*., 2008). They are used in the cosmetic industry (Gharaei-Fathabad, 2011), as well as microbial enhanced oil recovery (Das and Mukherjee, 2007).

With recent advancement in biotechnology, attention has been paid to the alternate environmental friendly processes for production of different types of biosurfactants from microorganisms (Lotfabad *et al*., 2009). Although, biosurfactants have promising applications in bioremediation, their production by industries in large scale is difficult due to high raw-material costs, high processing costs and poor output of manufacturing (Pacheco *et al*., 2010). As a result, current research challenges are to evoke an increase in yield and reduce the cost of raw materials. Therefore, this research sets out to determine the effect of acid and alkaline pretreatment of rice husk on biosurfactant production using *Mucor indicus* isolated from dump site.

MATERIALS AND METHODS

Materials

Rice husk: The rice husk used in this study was collected from a rice dump site located at Adani in Uzo-Uwani local government Area of Enugu State during the rainy season (June, 2014). Two (2 kg) kilogram of rice husk was collected from the dump site and put in a polyethene bag before transporting to the laboratory where it was stored at 4°C before analysis.

Methods

Preparation of agar for isolation of fungi: A modified czapek agar medium was prepared as follows: Agar (15.0 g), $NaNO_3$ (3.0 g), K_2HPO_4 (1.0 g), KCl (0.5 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), $FeSO_4{\cdot}7H_2O$. (0.01 g), Rice husk (10.0 g) and Chloramphenicol (0.1 g) were put in a 1 L conical flask and 400 mL of distilled was added to dissolve compound and then made to mark with distilled water. The pH of the mixture was adjusted to 7.0 and the mixture was autoclaved at 121°C and at a pressure of 15 psi for 30 min. The autoclaved mixture was allowed to cool to 5°C before aseptically dispensed into petri dishes.

Fungi isolation: One gram of rice husk from the dump site was put in a test-tube and 10 mL of distilled water was added. The mixture was vortex for ten minutes and allow to stand for 10 min. This process was repeated three times. The vortex mixture (0.5 mL) was aseptically inoculated into the petri dishes containing the modified czapek agar media using the spread plate technique. The petri dishes were incubated at 28°C for 4 days, then the organism with the highest visible growth was isolated.

Organism identification: The isolated organism was smeared on a slide glass and lactophenol staining blue was dropped on it and a cover glass was used to place on it. The prepared organism was viewed via microscope for morphological appearances. The image was matched with that of the Color Atlas of diagnostic Microbiology.

Preparation of media for pre-treatment/fermentation of rice husk: The modified Czapek broth was prepared as follows: $NaNO_3$ (3.0 g), K_2HPO_4 (1.0 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), Rice husk (50.0 g) and Chloramphenicol (0.1 g) were put in a 1 L conical flask and 400 mL of distilled was added to dissolve compound and was made to mark with distilled water. The pH of the mixture was adjusted to 12.0 and the mixture was autoclaved at 121°C and at a pressure of 15 psi for 1 h. The autoclaved mixture was allowed to cool.

Rice husk fermentation: The fungi (*Mucor indicus*) were aseptically inoculated into the pretreated broth and the setup was allowed to stand for 28 days with intermittent shaken to bring the fungi in contact with the pretreated rice husk. The growth of the organisms was monitored via the observed CO_2 production channel into a container of $Ca(OH)_2$.

Biosurfactant isolation: The crude biosurfactants produced by the organisms was isolated using the method of Sen and Swaminathan (1997). The broth was centrifuged at 4000 rpm for 30 min and the supernatant collected. The 6 M HCl was used to adjust the pH to 2. The precipitate was extracted with 2:1 chloroform-ethanol mixture at ratio 1:1 broth: extracting mixture 2:1 chloroform-ethanol mixture. The organic phase was removed and the biosurfactant was concentrated using ovum at 40°C. The solvents was evaporated, leaving biosurfactant and weighed.

Determination of carbohydrate: The extracted biosurfactant 50 mg was put in boiling tube and 2.5 mL of 2.5 N HCl was added into the tube. The tube was placed in boiling water bath for 3 h and cooled to room temperature. Solid sodium trioxocarbonate (Na_2CO_3) was added until effervescence ceases. The solution was made up to 50 mL with distilled water and centrifuged. A 1 mL of the supernatant was put in a test tube and 4 mL of anthrone reagent was added. The mixture was placed in a boiling water bath for 8 min. The tube was allowed to cool and the absorbance was read at 630 nm. The carbohydrate concentration was calculated using a calibration curve.

Preparation of protein: Biosurfactant (10 mg) was dissolved in 1 mL of water, 5 mL of solution D (Freshly prepared alkaline solution was made by mixing 50 mL of solution A, 1 mL of solution B and 1 mL of solution C) was added to the diluents. The mixtures were allowed to stand for 10 min, after which 0.5 mL of solution E (Folin-ciocauteau phenol reagent was made by diluting the commercial reagent with water in the ratio 1:1 freshly prepared) was added to the test tubes and allowed for 30 min. Finally, the absorbance was taken at a wavelength 740 nm using a spectrophotometer, while protein concentration was determined using a calibration curve.

Determination of lipids: One gram of extracted biosurfactant was dissolved in 10 mL of distilled water and 1 mL of the solution was used for lipid determination. About 1 mL of biosurfactant solution was put in a test tube and 4 mL of chloroform was added. The mixture was placed in a heating block at 90-110°C to evaporate the solvent. 0.2 mL of H_2SO_4 was added while heating continued for 10 min at 90-110°C. Vanillin reagent 5 mL was added and the mixture was removed from the heating block and allowed to cool. A reddish color develops after 5 min. The absorbance of the mixture was taken at 625 nm and the lipid concentration extrapolated from the standard curve.

Determination of cellulose content (Kurschner and Hoffer, 1933): A quantity 0.7 g designated as weight of the air dried, milled fibre already treated with n-hexane and methanol was added to a 95% solution of nitric acid (HNO_3) and ethanol mixture. The cellulose corresponds to the insoluble fraction of the sample. The mixture was filtered and the residue washed first with hot water followed by absolute ethanol to completely remove the residual acids. The residue was oven dried at 100°C to constant weight W1. The test was run in triplicates and the mean taken.

Experimental design: The rice husk was collected from the dump site and put in polyethene bag and brought to the laboratory. About 50 g of the rice husk was put in a conical flask containing 1 L of prepared media. The pH of the media was adjusted to pH 7 before autoclaving which served the purpose of heat pretreatment. After that the organism was inoculated and the experiment was monitored for 28 days.

Statistical analysis: The result was analyzed statistically using the ANOVA and presented as Mean±Standard Deviation. The graph pad version 6 was used for this analysis.

RESULTS

Isolation and characterization of organism: The organism was characterized as *Mucor indicus*.

Figure 1 is the structure of *Mucor indicus* as viewed in the microscope and matched with the image in the color atlas of diagnostic microbiology.

Determination of percentage of cellulose in rice husk: The result showed higher percentage of cellulose in the normal rice husk. There was a significant decrease ($p<0.05$) in the percentage of cellulose in pre-treated rice husk and fermented rice husk compared to the normal rice husk. The result also showed a significant increase ($p<0.05$) in cellulose (%) in pre-treated filtrate (broth) compared with the fermented filtrate as shown in Fig. 2.

The result in Fig. 2 showed a high percentage of cellulose in the husk when compared to the filtrated. It also showed higher concentrations of cellulose in the samples pretreated at pH 12.

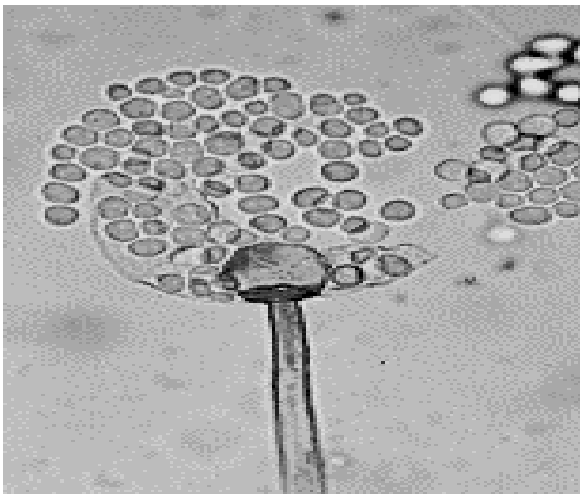

Fig. 1: Structure of *Mucor indicus*

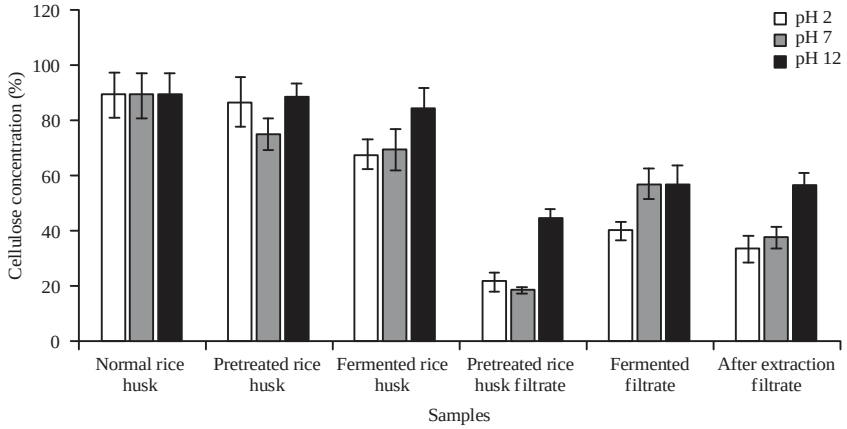

Fig. 2: Percentage of cellulose in the untreated rice husk, pre-treated rice and filtrate (liquid part) and the fermented rice husk and filtrate and also the filtrated after the extraction of biosurfactant

Table 1: Biosurfactant percentage yield

	Volume of cell free broth (mL)	Biosurfactant (mg/100 mL) of cell free broth	Biosurfactant (%) yield
pH 2	100	0.59±0.078	0.59±0.078
pH 7	100	0.40±0.042	0.40±0.042
pH 12	100	0.78±0.050	0.78±0.050

Table 2: Determination of lipid, protein and carbohydrate content of the biosurfactant

Parameters pH	After fermentation of biosurfactants (μg mL^{-1})	After extraction of biosurfactant (μg mL^{-1})	Content in extracted biosurfactant (μg g^{-1})
Lipid			
pH 2	92.50±8.95	32.50±4.79	60.00±6.57
pH 7	146.25±8.98	55.00±5.73	91.25±8.43
pH 12	145.00±27.01	83.75±9.37	61.25±5.79
Protein			
pH 2	30.70±0.05	29.79±0.25	0.91±0.20
pH 7	14.57±0.22	14.36±0.28	0.21±0.06
pH 12	13.43±0.07	12.74±0.24	0.69±0.31
Carbohydrate			
pH 2	2.33±0.15	1.19±0.15	1.14±0.06
pH 7	1.60±0.17	1.00±0.10	0.60±0.07
pH 12	2.91±0.14	1.65±0.29	1.26±0.18

Production of biosurfactant: The Table 1 shows the percentage yield of biosurfactant produced from rice husk pre-treated at pH 2, 7 and 12. The results revealed that the pre-treated at pH 12 produced 0.78±0.050 g of biosurfactant which was higher when compared to the biosurfactant produced at pH 7 (0.40±0.042) as seen in the Table 1.

Biochemical characterization of biosurfactant: The Table 2 shows the lipid, protein and carbohydrate content of the biosurfactant produced. The result revealed that the lipid content of the cell free broth after fermentation, after extraction of biosurfactant and in the biosurfactant were 92.50±8.95 μg mL^{-1}, 32.50±4.79 μg mL^{-1} and 60.00±6.57 μg g^{-1}, respectively for pH 2 and 145.00±27.01 μg mL^{-1}, 83.75±9.37 μg mL^{-1} and 61.25±5.79 μg g^{-1}, respectively for pH 12. It also showed that lipid content of the extracted biosurfactant were higher in samples pre-treated at pH7.

The result revealed that the protein content of the cell free broth after fermentation, after extraction of biosurfactant and in the biosurfactant were 30.70±0.05 mg/100 mL, 29.79±0.25 mg/100 mL and 0.91±0.20 mg g^{-1}, respectively for pH 2 and 14.57±0.22 mg/100 mL,

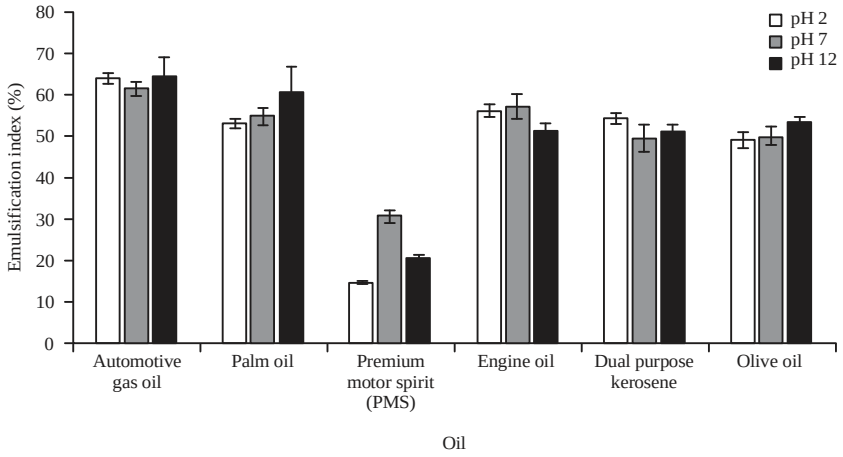

Fig. 3: Emulsification index of biosurfactant produced from rice husk pre-treated at various pHs

14.36±0.28 mg/100 mL and 0.21±0.06 mg g^{-1}, respectively for pH 7. It also showed that protein content of the extracted biosurfactant were lower in samples pre-treated at pH7. The result revealed that the carbohydrate content of the cell free broth after fermentation, after extraction of biosurfactant and in the biosurfactant were 1.60±0.17 mg/100 mL, 1.00±0.10 mg/100 mL and 0.60±0.07 mg g^{-1}, respectively for pH 7 and 2.91±0.14 mg/100 mL, 1.65±0.29 mg/100 mg and 1.26±0.18 mg g^{-1}, respectively for pH 12. It also showed that carbohydrate content of the extracted biosurfactant were higher in samples pre-treated at pH12.

Emulsification index: The result reveals that biosurfactant have the capacity to emulsify AMO, palm oil, Engine oil, DPK and Olive oil exception of PMS. The emulsification index at pH 12 pre-treated husk was higher for AMO, palm oil, DPK and olive oil compared the emulsification index of husk pre-treated at pH 7 in Fig. 3.

DISCUSSION

Rice husk was largely considered as waste material, always dumped and burned in landfills, this usually lead to environmental pollution, which contributes to the greenhouse effect (Ubwa *et al*., 2014; Nwofe, 2015), due to the introduction of CO_2 to the atmosphere (Isa, 2014). In this study, therefore, fungi species isolated from the dump site were used to convert this environmental waste and pollutant (rice husk) to useful product (biosurfactant). Due to the structure of the structural carbohydrate in rice husk, it makes it difficult for organisms to direct utilization of rice husk for energy and biomass generation. Therefore, pre-treatment of the rice husk is necessary to disrupt recalcitrant structure prior to the conversion of the biomass. Alkaline pre-treatment at pH 12 showed a greater effect on the degradation of rice husk lignocellulose structure, thereby releasing most of cellulose into the media solution as seen in the results. This enables the organism access to the cellulose which is utilized in the formation of biosurfactant (Diaz *et al*., 2013). The organism used in this study was a fungi strain which was adapted to the husk and is suspected to have the ability of breaking down the husk dumped in site. The morphological characterization of the organism identified the organism as *Mucor indicus,* shown in Fig. 1. It is suggested that *Mucor inducus* was able metabolize the rice husk a structural carbohydrate that is made up of cellulose, hemicellulose and lignin. The growth of this organism at different environmental condition (pHs) shows its ability to utilize these lignocellulose material for energy (Taherzadeh and Karimi, 2007). The result also revealed that heat and alkaline

pre-treatment cause the release of more cellulose into the solution, although, other component of the rice husk could possibly be released into the media during pre-treatment. This is because pretreatment is essential to breakdown lignocellulosic biomass for enhancing cellulose component to enzymatic hydrolysis (Sridevi *et al*., 2015). Although, it was obvious that the organisms utilizes the cellulose in the solution for growth, it is possible that the organism still breakdown rice husk which cause a rise in the cellulose concentration after the aerobic fermentation. The percentage of cellulose in normal rice husk (89.24±8.21%), pre-treated rice husk (78.57±5.32%), pre-treated filtrate (19.56±3.28%), also fermented rice husk (84.62±7.32%), fermented filtrate (71.56±7.34%) and the filtrate (39.67±4.28%) after extraction of the biosurfactant at pH 7. Comparing the percentage of cellulose in normal rice husk (89.247±8.21%), pre-treated rice husk (88.571±5.32%), pre-treated filtrate (44.440±3.28%) and the fermented rice husk (84.615±7.32%), fermented filtrate (56.667±7.34%) and the filtrate (56.667±4.28%) upon extraction of the biosurfactant at pH 12. From the above result, it can be inferred that the organism (*Mucor indicus*) has the ability to digest and release different amounts of cellulose in the husk at different conditions (pH). After the extraction of biosurfactant, the fungi were able to produce 0.59±0.078, 0.40±0.042 and 0.78±0.05% of biosurfactants for pHs 2, 7 and 12 respectively. Which showed that *Mucor indicus* can produce more biosurfactants in alkaline pre-treated rice husk. The higher concentration of carbohydrate and protein in the biosurfactants showed that the biosurfactant could belong to the glycoprotein kind of biosurfactants.

The result of the emulsification index of the biosurfactant produced from rice husk reveals that at pH 7 the biosurfactant was able to emulsify AGO (61.47), Palm oil (54.83), Engine oil (57.117), DPK (49.5), Olive oil (50.09) and PMS (30.75) while at pH 12 AGO (64.45), Palm oil (60.63), Engine oil (51.45), DPK (51.00) and Olive oil (53.59) with PMS (20.87). The result showed that at pH 12 the emulsification of AGO, palm oil, DPK, PMS increased considerably compared to pH 7. There was an increase in emulsification of AGO and PMS compare to pH7. This shows that the produced biosurfactant can be used in treating area where accidental discharges and spillage of kerosene, diesel, engine oil, PMS and palm oil occurred. The extracted biosurfactant was characterized at pH 12 and the result showed carbohydrate concentration (1.264±0.179 mg/100 mL), lipids (61.25±35.79 μg mL^{-1}). Protein concentration (0.688±0.31/100 mg mL^{-1}) cell free broth and at pH 12 and the carbohydrate concentration (0.603±0.073 mg mL^{-1}), lipids (91.25±11.43 g mL^{-1}), protein concentration (0.207±0.06 mg mL^{-1}) of cell free broth see results. From this result, it can be said that biosurfactant produced belongs to glycolipids class because of the possibility that the protein content was low and may be part of the cellulase which the organism used in breaking the rice husk.

CONCLUSION

The results show that pre-treatment of rice husk with alkaline help increase the quantity/amount of biosurfactant produced compared pre-treatment at pH 7. Therefore, it is better to use alkaline pre-treatment during biosurfactant production to be used in spilled oil remediation due to it higher emulsification index.

REFERENCES

Das, K. and A.K. Mukherjee, 2007. Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresour. Technol., 98: 1339-1345.

Diaz, A., J. le Toullec, A. Blandino, I. de Ory and I. Caro, 2013. Pretreatment of rice hulls with alkaline peroxide to enhance enzyme hydrolysis for ethanol production. Chem. Eng. Trans., 32: 949-954.

Gharaei-Fathabad, E., 2011. Biosurfactants in pharmaceutical industry (A mini-review). Am. J. Drug Discovery Dev., 1: 58-69.

Isa, R., 2014. Greenhouse Gas (GHG) emissions and oil and gas revenue in Nigeria. Acad. J. Interdisciplin. Stud., 3: 127-132.

Kachholz, T. and M. Schlingmann, 1987. Possible Food and Agricultural Applications of Microbial Surfactants: An Assessment. In: Biosurfactants and Biotechnology, Kosaric, N., W.L. Carns and N.C.C. Gray (Eds.). Marcel Dekker, New York, USA., pp: 183-210.

Kurschner, K. and A. Hoffer, 1933. Cellulose and cellulose derivatives. Fresenius' J. Anal. Chem., 92: 145-154.

Lotfabad, T.B., M. Shourian, R. Roostaazad, A.R. Najafabadi, M.R. Adelzadeh and K.A. Noghabi, 2009. An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in South of Iran. Colloids Surf. B: Biointerfaces, 69: 183-193.

Muller, M.M., J.H. Kugler, M. Henkel, M. Gerlitzki and B. Hormann *et al*., 2012. Rhamnolipids-next generation surfactants? J. Biotechnol., 162: 366-380.

Mulligan, C.N., 2005. Environmental applications for biosurfactants. Environ. Pollut., 133: 183-198.

Muthusamy, K., S. Gopalakrishnan, T.K. Ravi and P. Sivachidambaram, 2008. Biosurfactants: Properties, commercial production and application. Curr. Sci., 94: 736-747.

Nwofe, P.A., 2015. Management and disposal of municipal solid wastes in Abakaliki metropolis, Ebonyi State, Nigeria. Int. J. Scient. Res. Environ. Sci., 3: 107-118.

Pacheco, G.J., E.M.P. Ciapina, E.B. Gomes and N. Pereira Jr., 2010. Biosurfactant production by *Rhodococcus erythropolis* and its application to oil removal. Braz. J. Microbiol., 41: 685-693.

Sen, R. and T. Swaminathan, 1997. Application of response-surface methodology to evaluate the optimum environmental conditions for the enhanced production of surfactin. Applied Microbiol. Biotechnol., 47: 358-363.

Sridevi, A., G. Narasimha, G. Ramanjaneyulu, K. Dileepkumar, B.R. Reddy and P.S. Devi, 2015. Saccharification of pretreated sawdust by *Aspergillus niger* cellulase. Biotech. 10.1007/s13205-015-0284-7

Taherzadeh, M.J. and K. Karimi, 2007. Enzymatic-based hydrolysis processes for ethanol from lignocellulosic materials: A review. BioResources, 2: 707-738.

Ubwa, S.T., J. Abah, B.A. Oshido and E. Otokpa, 2014. Studies on urea treated rice milling waste and its application as animal feed. Afr. J. Pure Applied Chem., 8: 23-31.

Zhao, Z., Q. Wang, K. Wang, K. Brian, C. Liu and Y. Gu, 2010. Study of the antifungal activity of *Bacillus vallismortis* ZZ185 *in vitro* and identification of its antifungal components. Bioresour. Technol., 101: 292-297.