RESILIENT INFRASTRUCTURE
June 1–4, 2016

PRODUCTION OF BIOSURFACTANT BY *RHODOCOCCUS ERYTHROPOLIS SP.* CULTIVATED IN A NOVEL FISH WASTE COMPOST EXTRACT SUBSTRATE

Khoshrooz Kazemi
Memorial University, Canada

Baiyu Zhang
Memorial University, Canada

Leonard M. Lye
Memorial University, Canada

Abstract

Compost generated through fish waste composting could provide an effective source of nutrient-rich organic matter for microbial growth, leading to the production of valuable products such as biosurfactants. Existing biosurfactant production is a relative expensive process and raw materials contribute about 30% of the production cost. Utilizing waste streams such as fish waste compost (FWC) as a substrate is an economically viable alternative. In this study, biosurfactant was produced by *Rhodococcus erythropolis* sp. P6-4P, a strain isolated from the North Atlantic Ocean. Biosurfactant production with FWC extract was compared with other soluble and insoluble carbon and nitrogen sources using emulsification assay and surface tension measurement. FWC extract showed good potential as an unconventional source of nutrient for microbial growth. The produced biosurfactant under optimum condition obtained via response surface methodology was further characterized for total carbohydrate, total lipid and total protein content. The results provided evidence for using FWC extract as a novel substrate for biosurfactant production.

Keywords: Fish waste compost, biosurfactant production, novel substrate, response surface methodology

1. INTRODUCTION

Biosurfactants are amphiphilic compounds produced by a wide variety of microorganisms containing both hydrophilic and hydrophobic moieties that allow them to array at the interface of polar and nonpolar media (Sen, 2010). They have been identified for several industrial applications in cosmetic, pharmaceutical, food processes, and environmental engineering as emulsifiers, humectants, preservatives, and detergents. Because of their structural diversity (i.e., glycolipids, lipopeptides, fatty acid esters), low toxicity thus ecologically safe, and high biodegradability, biosurfactants have the potential for replacing synthetic surfactants in bioremediation and waste treatments (Pal et al., 2009). Despite all the advantages that biosurfactant have, low yields and high production cost limit the extension of biosurfactant applications (Makkar, Cameotra, and Banat, 2011). Raw material can account for almost 30% of the overall cost of a microbial surfactant production, therefore the economical production of biosurfactant depends on the development of low-cost raw material and optimization of the production processes (George and Jayachandran, 2013). The use of alternative substrates such as industrial and/or municipal wastes is one of the possible attractive strategies for economical biosurfactants production to minimize pollutants and produce valuable products (Kosaric, 1992). Improvement of efficiency of the production process (e.g., optimization of cultural condition) can also help to overcome the economic constraints associated with biosurfactant production (Mukherjee, Das, and Sen, 2006).

From about 267,959 tonnes of fish landed in Newfoundland and Labrador, 54% were classified as fish waste in 2001(Ghaly AE, 2013). Composting is considered to be a viable solution to the problems of waste disposal experienced by fish processing plants and fish farms (Liao, May, and Chieng, 1995). Compost made from fish waste is rich in nutrients, particularly nitrogen and phosphorous (Benhabiles et al., 2012; Illera-Vives, Labandeira, and López-Mosquera, 2013; Laos et al., 2002). It can be used to generate substrates for bacterial growth and production of valuable products such as biosurfactants.

Medium compositions such as carbon sources, nitrogen sources, and inorganic salts strongly influence cell growth and the accumulation of metabolic products (Li, Bai, Cai, and Ouyang, 2002). Environmental factors and growth conditions such as pH and time of cultivation also affect biosurfactant production through their effects on cellular growth or activity (Desai and Banat, 1997). Through studying the effect of these factors on production process and optimizing media condition, the yield of biosurfactant production can be elevated (Kiran et al., 2009; Mukherjee et al., 2006). Among various statistical methods, response surface methodology (RSM) is the most widely used method in system optimization. By integrating a collection of statistical tools and techniques, RSM leads to constructing and exploring an approximate functional relationship between a response variable and a set of design variables (Venter, 1998).

This paper investigates the potential usage of fish waste compost (FWC) extract as a novel substrate for biosurfactant production by a strain *Rhodococcus* (P6-4P) isolated from the Atlantic Ocean. RSM based on a central composite design will be used to develop an empirical model of the process and to optimize media conditions to enhance the yield of Rhodococcus biosurfactant using FWC extract as factor.

2. MATERIAL AND METHODS

2.1 Strain and culture condition

Biosurfactant producing microorganism P6-4P (*Rhodococcus erythropolis* sp.) isolated as an effective microorganisms from petroleum hydrocarbon contaminated marine sources in the North Atlantic was selected to produced biosurfactant (Cai et al., 2015). Bacteria colony was transferred from agar plate to 125-ml Erlenmeyer flask containing 50 ml BD 23400 nutrient broths (Fisher scientific company, Ottawa, Canada) to growth the culture on a rotary shaker for 24h at 37 °C and 180rpm to reach the optical density of the culture at 600 nm (OD600) of 0.8. Growth and biosurfactant production by the isolate was evaluated using media which is adopted and modified from (Peng, Liu, Wang, and Shao, 2007) including NaCl, 2.2 g; $FeSO_4 \cdot 7H_2O$, 2.8×10^{-4} g; KH_2PO_4, 3.4 g;$K_2HPO_4 \cdot 3H_2O$, 4.4 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; yeast extract, 0.5 g, N-hexadecane 30 ml/L, $(NH_4)_2SO_4$ 15 g, and 0.5 mL/L trace element solution in 125 mL conical flasks. The trace element solution contained ZnSO4, 0.29 g; CaCl2, 0.24 g; CuSO4, 0.25 g; MnSO4, 0.17 g L^{-1} and was sterilized separately. The chemicals used were analytical grade, unless otherwise specified. Incubation was conducted at 30°C, 200 rpm for 2 days. After 2 days, before inoculation, purity check was conducted by spreading the medium over nutrition broth agar plate to avoid cross contamination. Nutrition broth composed of peptone, 8 g; yeast extract, 3 g; NaCl, 6 g; Glucose, 1 g: and agar. 15 g. Difference carbon and nitrogen sources has been used to compare the efficiency of FWC extract as substrate for biosurfactant producing bacteria.

2.2 Effect of carbon and nitrogen sources on biosurfactant production

The effect of different carbon sources was studied by replacing the n-hexadecane with sucrose, starch, glucose, and fish waste compost extract (FWCC). The different carbon sources were added to the media at a concentration of 5 g l^{-1}. To evaluate the nitrogen sources, ammonium sulphate was replaced by an equivalent amount of different nitrogen sources, namely yeast, ammonium nitrate and FWC extract (FWCN). The different nitrogen sources were added to the media at a concentration of 15 g l^{-1}. A 1% bacterial cell suspension from a 24-h culture was used as inoculum. 15 ml medium has been prepared in the 50 ml conical flask and incubated at 30°C, 200 rpm for 5 days. Cells were removed from the culture by centrifugation at 12, 000 rpm for 20 min. Cell- free culture broth was used for analytical measurements.

2.3 Surface tension and CMD measurement

Surface tension measurements of culture broth supernatants were performed according to the Ring method with a surface tensiometer (DuNouyTensiometer, Interfacial, CSC Scientific). To increase the accuracy of the surface tension measurements, an average of triplicates was determined. All measurements were performed at room temperature (20 °C). Critical micelle concentration (CMD) is the dilution of the culture broth upon reaching the critical micelle concentration (Shavandi et al., 2011). After centrifuging at 12,000 rpm for 20 min and discarding the pellet, the cell free broth were diluted with distilled water, while the surface tension of each dilution was measured. The CMD was determined as the highest dilution with which the surface tension did not significantly increase. As the broth consists of both aqueous and oil phases, each dilution was conducted with sonication to ensure homogeneity. Before each measurement, the sonicated solution was allowed to stand for 15-20 min to achieve equilibrium.

2.4 Emulsifying activity

Emulsifying activity was determined by the addition of 2 ml of *n*-hexadecane to the same volume of cell-free culture broth supernatant in glass test tubes. The tubes were mixed with vortex at high speed for 2 min and subsequently incubated at 25 °C for 24 h. The stability of the emulsion was determined after 24 h, and the emulsification index (E24) was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). All emulsification indexes were performed in triplicate.

2.5 RSM experimental design

To examine the combined effect of three different medium conditions and to obtain the functional relationship between incubation component including time, pH and FWC concentration and the response namely CMD, a Central Composite Design (CCD) with 3 factors was used. A total of 2^3=8 factorial points, 6 center points, one replicate of star points and one replicate of factorial points leading to a total of 20 experiments was designed. The value of the response (CMD) was the mean of three replications. Design Expert software (version 8.0, Stat-Ease, USA) was used to design and analyse the data collected from the experiment. Table 1 shows the variables and their high and low levels and Table 2 shows the factor combinations of the CCD, the corresponding observed responses and model predictions.

Table 1: Biosurfactant production variables and their high and low levels

Independent variables	Coded	Low	High
Time (d)	A	3	7
pH	B	6	8
FWC concentration (%)	C	20	80

2.6 Biosurfactant extraction and assay

To extract the produced biosurfactant, the cell free culture were mixed with an equal volume of chloroform/ methanol (1:2 v/v) and shaken on an orbital shaker (200 rpm) for 24 hours. The solvent was then evaporated by a rotary evaporator and kept at 4°C. For total lipid and total carbohydrate, (Pande, Khan, and Venkitasubramanian, 1963) and (Dubois, Gilles, Hamilton, Rebers, and Smith, 1956) methods were used, respectively. The total carbohydrate in the sample solution was expressed in terms of D-Glucose (g/ 100 mL) and the total lipid in the sample solution was expressed in terms of Palmitic acid (g/ 100 mL). The surface tension of 10 mL diluted biosurfactant solution at various concentrations was determined in triplicate with a surface tensiometer at 25°C for CMC estimation. The CMC was determined by plotting the surface tension versus the concentration of biosurfactants in the solution. Total lipid and fatty acids test was conducted at the Ocean Science Centre (OSC) of Memorial University of Newfoundland. Lipid samples were extracted according to (Parrish, 1999). Lipid class composition was determined using an Iatroscan Mark VI TLC-FID, silica coated Chromarods following a three-step development method (Parrish, 1987). The fatty acids composition of surfactant extracts was analysed by GC–FID.

Table 2: CCD for biosurfactant production

Run	Factor A	Factor B	Factor C	Y_0 observed CMD	Y predicted CMD
1	5	8.5	50	10.8	8.757
2	5	7	50	14.2	13.34
3	2	7	50	20.8	21.99
4	5	7	50	12.2	13.34
5	3	8	20	15.5	11.927
6	5	7	50	12.4	13.34
7	7	6	20	2.2	0
8	3	8	80	21.5	23.64
9	7	8	20	0	0
10	7	8	80	8.13	12.112
11	5	7	5	0	4.56
12	5	7	95	23	22.13
13	5	5.5	50	0	2.35
14	5	7	50	17.6	13.34
15	7	6	80	9.13	7.845
16	8	7	50	0	4.7
17	5	7	50	16.2	13.34
18	3	6	20	2.83	7.659
19	3	6	80	20.5	19.37
20	5	7	50	16.7	13.34

2.7 Biosurfactant stability test

Stability studies were done using cell-free broth obtained after 72h of cultivation. Broth samples were incubated in a water bath at different temperatures: 4, 20, 40, 60, 80 and 100°C and cooled at room temperature. The pH stability was performed by adjusting the broth to different pH (3, 6, 9, and 12) values by adding 1N NaOH or 1 N HCl. Different concentrations of NaCl comprise 0, 5 and 10 % (W/V) were added to broth samples and mixed until complete dissolution to study the effect of salt addition on biosurfactant.

3. RESULTS AND DISCUSSION

3.1 Effect of Carbon and nitrogen sources on biosurfactant production

The genus *Rhodococcus* bacteria has diverse and efficient metabolism which enables it to transform, biodegrade or utilize several hydrophobic compounds such as hydrocarbons, chlorinated phenols, steroids, lignin, coal and crude oil as a carbon source. This capability could be of great commercial and industrial importance (Bicca, Fleck, and Ayub, 1999). Biosurfactants produced by some *Rhodococcus* species have been reported to be more effective and efficient in reduction of surface and interfacial tensions than many synthetic surfactants (Bell, Philp, Aw, and Christofi, 1998). Therefore, *Rhodococcus erythropolis* sp has been selected for this study. The type of carbon and nitrogen source affected biosurfactant yield which is depicted through ST, E24 and production rate shown in Figure1. Biosurfactant produced with FWC as a carbon and nitrogen sources showed excellent surface tension reduction activity and they reduce water surface tension to 29.33 and 28.95 mN/m, respectively. The lowest surface tension was recorded for sucrose (24.61 mN/m). All carbon and nitrogen sources except glycerol reduced water surface tension to under 40 mN/m. The highest emulsification activity was observed for n-hexadecane. Also, FWCN

showed well emulsification activity while it was less than n-hexadecane and yeast. Yeast yielded the highest production rate, while yeast ammonium nitrate and FWCN produced higher biosurfactant. The lowest production rate was due to glycerol. According to surface tension, emulsification activity, and biosurfactant production, FWC was able to promote the production of biosurfactant as a nitrogen source and carbon source and it can be considered as a promising nutrient source for the selected *Rhodococcus* strain.

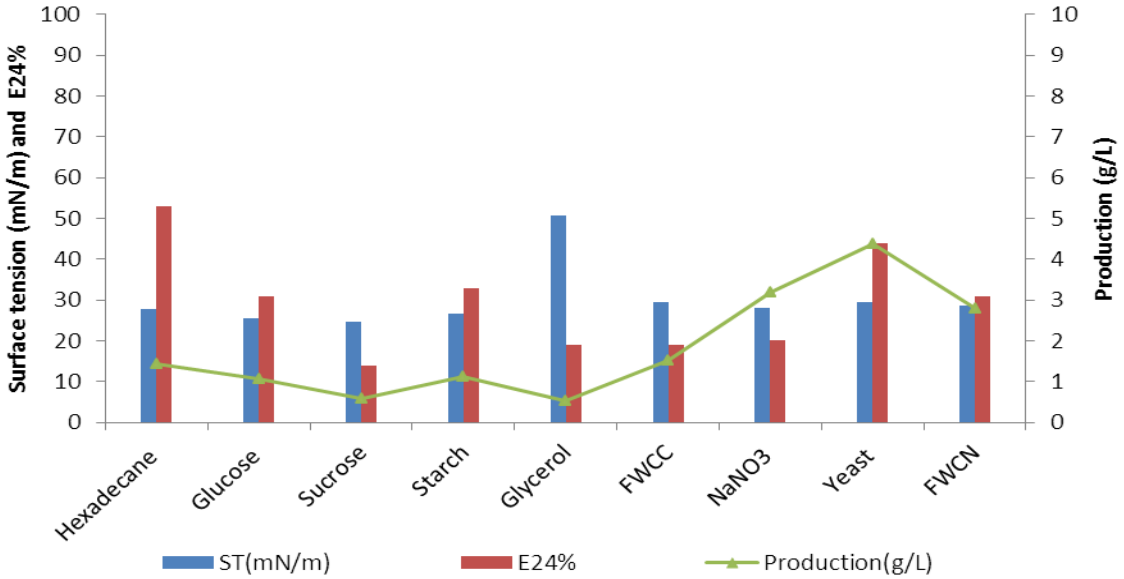

Figure 1: Effect of carbon and nitrogen sources on the biosurfactant production by *Rhodococcus* P6-4P

3.2 Biosurfactant production optimization

The ANOVA table from the CCD results of Table 2 is shown in Table 3.

Table 3: ANOVA table for biosurfactant production

Source	Sum of Squares	df	Mean Square	F Value	p-value
Model	1025.13998	4	256.285	20.05758	< 0.0001
A-Time	415.526792	1	415.5268	32.52029	< 0.0001
B-pH	56.903112	1	56.90311	4.453397	0.0520
C-FWC (%)	429.010632	1	429.0106	33.57558	< 0.0001
B^2	123.6994438	1	123.6994	9.681066	0.0071
Residual	191.6619152	15	12.77746		
Lack of Fit	165.4135819	10	16.54136	3.150935	0.1086
Pure Error	26.24833333	5	5.249667		
Cor Total	1216.801895	19			

$R^2 = 0.843$	Adj $R^2 = 0.801$	Pred $R^2 = 0.697$	Adeq Precision = 15.39

From Table 3, all model terms are tested at the 5% significance level. The coefficients and the corresponding p-values suggest that, among the input variables time, pH, and FWC concentration are significant model terms. It can be seen that there is a highly significant quadratic effect of pH (B). The lack-of-fit p-value is greater than 5% indicating that the lack of fit is not significant. This means all significant model terms have been included. The resulting regression equation is given by:

[1] $$Y = 13.35 - 5.77\,A + 2.13\,B + 5.86\,C - 3.46\,B^2$$

Where: Y is the response (CMD) of the produced biosurfactant and A, B and C are coded values of the test variables, time, pH and FWC concentration (%), respectively. Goodness-of-fit statistics show that the regression model provided a fairly good fit to the experimental data. Figure 2 shows the response surface plots as a function of Time and pH, and as a function of pH and FWC.

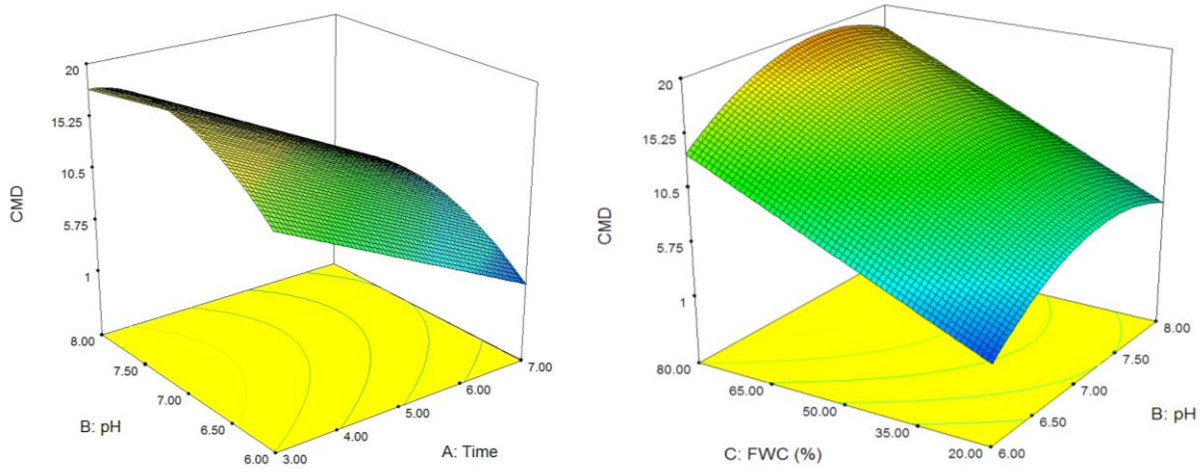

Figure 2: Response surface plot for CMD of biosurfactant production (1) as a function of time and pH, (2) pH and FWC concentration

Since linear effect of FWC concentration is significant which means that it can act as limiting nutrient source and variation in its concentration will change the product CMD. Increase of time has a negative effect on CMD. pH has a positive linear effect and negative quadratic effect on CMD of the produced biosurfactant, therefore pH changes alter the CMD. To achieve the highest CMD the time of 3.31 days, pH of 7.38 and FWC concentration of 73.97 were suggested as optimal conditions to generate biosurfactant for further tests.

3.3 Biosurfactant production assay

Biosurfactant production over the course of 72 h was investigated by means of ST, E24% and production rate measurement. As expressed by decrease in surface tension, the *Rhodococcus* (P6-4P) started to produce biosurfactant after 12 hours of cultivation. Dramatic decrease was observed in surface tension until 24 hours and then it reached its minimum values at 31 (mN/m). The emulsification index increased continuously until it reached its maximum value after 30 h. The maximum production rate of biosurfactant has been observed after 60 h of cultivation which was 3.2 g/l.

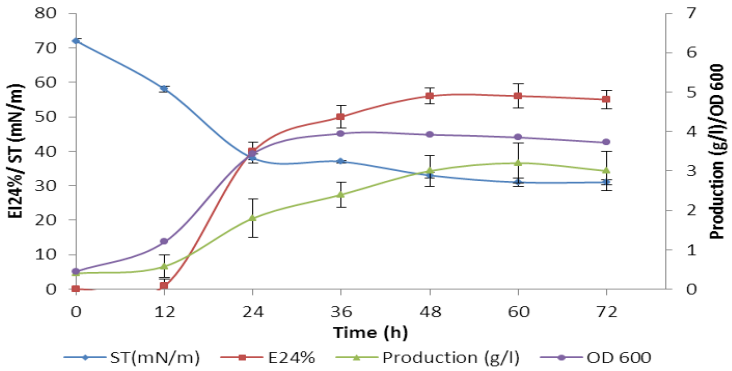

Figure 3: Times course of growth and biosurfactant production

A direct relationship between biosurfactant production and cell growth was observed during the biosurfactant production by *Rhodococcus* (P6-4P). The biosurfactant production with FWC started soon after inoculation and increased progressively, then remained constant during stationary phase and reached its maximum value at the end of the stationary phase. Cell growth stayed at stationary phase form 24 h to 60h of cultivation. Although it can be stated that FWC is a promising substrate for bacterial growth and biosurfactant production by the tested microorganism, it cannot be claimed that biosurfactant production by *Rhodococcus* (P6-4P) with FWC is a metabolic process and it is growth-associated, since biosurfactant production continued during the stationary phase.

3.4 Critical micelle concentration determination

When biosurfactants were produced in the water, the surface tension changes with increasing concentration of biosurfactants until it reaches the critical micelle concentration (CMC), at this point surface tension remains constant and biosurfactant molecules start to form aggregates like micelles because of the chemical interactions between the polar head groups and the non-polar tail groups including hydrophobic, Van der Waals' force, and hydrogen bonding (Mulligan, 2005; Schramm, 2000; Soberón-Chávez and Maier, 2011). To evaluate biosurfactant content in the cell free broth, the CMC was determined by measuring the surface tension of the supernatant at various dilutions (Mulligan, Yong, and Gibbs, 2001). The CMC can be determined by plotting surface tension as a function of biosurfactant (or broth) concentration since the slope of the curve abruptly changes at the CMC. However, the abruptness is a function of both the particular surfactant and the presence of impurities in the system (Sheppard and Mulligan, 1987). The method of (Sheppard and Mulligan, 1987) has been followed to determine CMC of the produced biosurfactant. In the Figure 4 the minimum effective concentration of biosurfactants corresponds to 0.0155 g/ml. CMC varies with the structure of surfactants, pH, ionic strength, temperature, and the polarity of the solvent (Desai and Banat, 1997). Improving the downstream process for biosurfactant extraction and reducing the impurities can reduce the CMC.

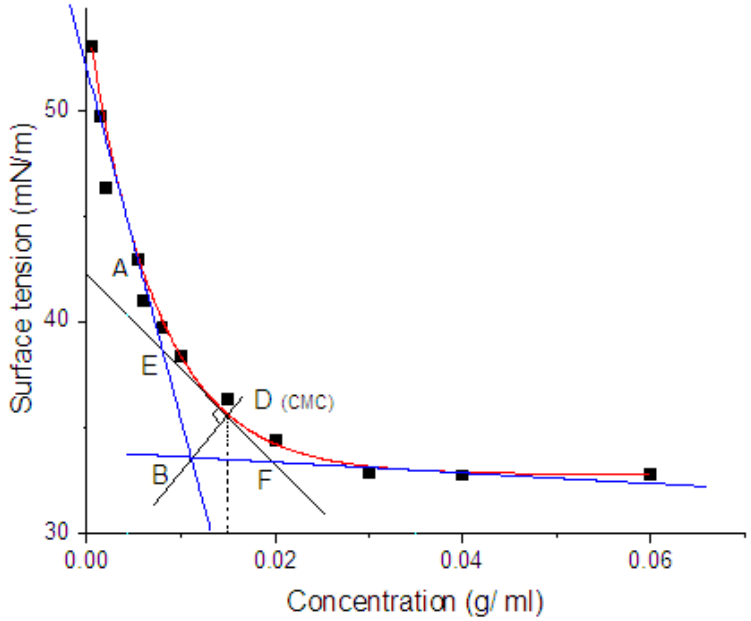

Figure 4: Method for determining the critical micelle concentration (CMC) from measurements of surface tension of the broth after dilution with distilled water. A line is drawn from B to intersect the tangent E--F at 90 degrees

Biochemical composition of the produced biosurfactant revealed that the total carbohydrate content in 1 g biosurfactant was 18.2 mg in term of D-glucose and the total lipid content was 48.6 mg in term of Palmitic acid in 1g product. Certain species of *Rhodococcus*, such as *Rhodococcus erythropolis* are an important biosurfactant producer. These species produce biosurfactants through the utilization of water-insoluble hydrocarbons. Most of the biosurfactants produced in this way are lipids containing trehalose. Although the diversity of *Rhodococcus* glycolipids has been reported, free fatty acids are rarely reported as major biosurfactant products of *Rhodococcus erythropolis* (Peng et al., 2007). The major component of produced biosurfactant was fatty acid and accounted for 64% of total lipid composition.

Table 3: Lipid composition of the biosurfactant (percent of the total amount of lipid)

% Lipid Composition in biosurfactant	
Hydrocarbons	2.71
Steryl Esters/Wax Esters	0.00
Ethyl Esters	0.79
Methyl Esters	0.00
Ethyl Ketones	1.99
Methyl Ketones	0.00
Glycerol Ethers	0.00
Triacylglycerols	0.00
Free Fatty Acids	63.84
Alcohols	0.93
Sterols	6.64
Diacylglycerols	0.00
Acetone Mobile Polar Lipids	9.80
Phospholipids	13.31

Table 4: Fatty acid composition of the biosurfactant (percent of the total amount of fatty acids)

% ID'ed Fatty acids in biosurfactant	
14:0	1.30
Trimethyltridecanoic acid (TMTD)	1.04
16:0	8.26
16:1w7	1.77
i17:0	1.29
18:0	7.88
18:1w9	5.36
18:1w7	7.30
18:2w4	3.22
20:1w11	1.67
20:1w9	23.06
20:1w7	2.71
22:1w11(13)	17.50
22:1w9	4.86
21:5w3	1.99
24:1	1.72
Sums	100.00

3.5 Biosurfactant stability

Surface tension of the produced biosurfactant was measured under wide range of temperature, pH and salinity to study the stability of the biosurfactant. The biosurfactant showed stability at all temperature ranges and salinity ranges and its surface tension changed slightly. At low pH, biosurfactant precipitated and surface tension was high. Biosurfactant stayed stable for 4 days when salt concentration was 5% (w/v) at 80 °C in the pH range of 6.5–10.5.

Stability studies demonstrated that the biosurfactant is stable under extreme temperature and salinity and wide range of pH.

4. CONCLUSION

This study investigated the novel use of FWC as substrates for the production of biosurfactant and the use of response surface methodology to study the influence of media condition on biosurfactant production, screening of experimental significant factors on media conditions, and optimization of biosurfactant production. The effort of using FWC as a cheap substrate and optimization of the production condition to enhance the biosurfactant production rate and decrease the cost can help to make the microbial surfactant competitive with synthetic surfactants. The significance of the present work lies in the fact that FWC can be used as a cheap and novel source of nutrient to produce biosurfactants.

5. REFERENCES

Bell, K., Philp, J., Aw, D., and Christofi, N. 1998. The genus *Rhodococcus*. *Journal of Applied Microbiology, 85*,(2), 195-210.

Benhabiles, M. S., Abdi, N., Drouiche, N., Lounici, H., Pauss, A., Goosen, M. F. A., and Mameri, N. 2012. Fish protein hydrolysate production from sardine solid waste by crude pepsin enzymatic hydrolysis in a bioreactor coupled to an ultrafiltration unit. *Materials Science & Engineering C-Materials for Biological Applications, 32*,(4), 922-928. doi: 10.1016/j.msec.2012.02.013

Bicca, F. C., Fleck, L. C., and Ayub, M. A. Z. 1999. Production of biosurfactant by hydrocarbon degrading *Rhodococcus* ruber and *Rhodococcus* erythropolis. *Revista de Microbiologia, 30*,(3), 231-236.

Cai, Q., Zhang, B., Chen, B., Song, X., Zhu, Z., and Cao, T. 2015. Screening of biosurfactant-producing bacteria from offshore oil and gas platforms in North Atlantic Canada. *Environmental Monitoring and Assessment, 187*,(5), 1-12.

Desai, J. D., and Banat, I. M. 1997. Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews, 61*,(1), 47-64.

Dubois, M., Gilles, K., Hamilton, J., Rebers, P., and Smith, F. 1956. Phenol sulphuric acid method for total carbohydrate. *Annual Chemistry, 26*350.

George, S., and Jayachandran, K. 2013. Production and characterization of rhamnolipid biosurfactant from waste frying coconut oil using a novel Pseudomonas aeruginosa D. *Journal of Applied Microbiology, 114*,(2), 373-383.

Ghaly AE, R. V., Brooks MS, Budge SM, Dave D 2013. Fish Processing Wastes as a Potential Source of Proteins, Amino Acids and Oils: A Critical Review. *J Microb Biochem Technol, 5*107-129.

Illera-Vives, M., Labandeira, S. S., and López-Mosquera, M. 2013. Production of compost from marine waste: evaluation of the product for use in ecological agriculture. *Journal of Applied Phycology, 25*,(5), 1395-1403.

Kiran, G. S., Hema, T., Gandhimathi, R., Selvin, J., Thomas, T. A., Ravji, T. R., and Natarajaseenivasan, K. 2009. Optimization and production of a biosurfactant from the sponge-associated marine fungus Aspergillus ustus MSF3. *Colloids and Surfaces B: Biointerfaces, 73*,(2), 250-256.

Kosaric, N. 1992. Biosurfactants in industry. *Pure and Applied Chemistry, 64*,(11), 1731-1737.

Laos, F., Mazzarino, M., Walter, I., Roselli, L., Satti, P., and Moyano, S. 2002. Composting of fish offal and biosolids in northwestern Patagonia. *Bioresource Technology, 81*,(3), 179-186.

Li, C., Bai, J., Cai, Z., and Ouyang, F. 2002. Optimization of a cultural medium for bacteriocin production by Lactococcus lactis using response surface methodology. *Journal of Biotechnology, 93*,(1), 27-34.

Liao, P., May, A., and Chieng, S. 1995. Monitoring process efficiency of a full-scale invessel system for composting fisheries wastes. *Bioresource Technology, 54*,(2), 159-163.

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

Mukherjee, S., Das, P., and Sen, R. 2006. Towards commercial production of microbial surfactants. *Trends in Biotechnology, 24*,(11), 509-515.

Mulligan, C. N. 2005. Environmental applications for biosurfactants. *Environmental Pollution, 133*,(2), 183-198.

Mulligan, C. N., Yong, R. N., and Gibbs, B. F. 2001. Heavy metal removal from sediments by biosurfactants. *Journal of Hazardous Materials, 85*,(1), 111-125.

Pal, M. P., Vaidya, B. K., Desai, K. M., Joshi, R. M., Nene, S. N., and Kulkarni, B. D. 2009. Media optimization for biosurfactant production by *Rhodococcus* erythropolis MTCC 2794: artificial intelligence versus a statistical approach. *Journal of Industrial Microbiology & Biotechnology, 36*,(5), 747-756.

Pande, S., Khan, R. P., and Venkitasubramanian, T. 1963. Microdetermination of lipids and serum total fatty acids. *Analytical Biochemistry, 6*,(5), 415-423.

Parrish, C. C. 1987. Separation of aquatic lipid classes by chromarod thin-layer chromatography with measurement by latroscan flame ionization detection. *Canadian Journal of Fisheries and Aquatic Sciences, 44*,(4), 722-731.

Parrish, C. C. (1999). Determination of total lipid, lipid classes, and fatty acids in aquatic samples *Lipids in freshwater ecosystems* (pp. 4-20): Springer.

Peng, F., Liu, Z., Wang, L., and Shao, Z. 2007. An oil-degrading bacterium: *Rhodococcus* erythropolis strain 3C-9 and its biosurfactants. *Journal of Applied Microbiology, 102*,(6), 1603-1611.

Schramm, L. L. 2000. *Surfactants: fundamentals and applications in the petroleum industry*: Cambridge University Press.

Sen, R. (2010). *Biosurfactants*. Springer Science & Business Media.

Sheppard, J. D., and Mulligan, C. N. 1987. The production of surfactin by *Bacillus* subtilis grown on peat hydrolysate. *Applied Microbiology and Biotechnology, 27*,(2), 110-116.

Soberón-Chávez, G., and Maier, R. M. (2011). Biosurfactants: a general overview *Biosurfactants* (pp. 1-11): Springer.

Venter, G. 1998. *Non-dimensional response surfaces for structural optimization with uncertainty*.