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Optimum Medium Components for Biosurfactant Production by *Klebsiella pneumoniae* WMF02 Utilizing Sludge Palm Oil as a Substrate

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Abstract: In the present study, optimizing critical nutritional constituents was attempted as a primary strategy to improve biosurfactant production from *Klebsiella pneumoniae* WMF02 in liquid state fermentation utilizing sludge palm oil as a substrate. One-factor-at-a-time (OFAT) optimization was employed to evaluate the effects of sludge palm oil (SPO), sucrose, $MgSO_4$, $FeSO_4$, $NaNO_3$ and K_2HPO_4 on surface tension reductivity. Sucrose was selected as a co-substrate over glucose in the production media. The optimal levels of the aforementioned variables were (g/l) sucrose 5.0, $MgSO_4$ 0.4, $FeSO_4$ 0.3, $NaNO_3$ 2.0, and K_2HPO_4 4.0, with SPO concentration of 4% (v/v). The optimized medium shows surface tension reduction from 36.2 mN/m (non-optimized medium) to 25.70 mN/m. Preliminary biosurfactant identification indicated that the biosurfactant produced was phospholipids in nature.

Key words: biosurfactant; sludge palm oil, one-factor-at-a-time optimization (OFAT); *Klebsiella pneumoniae*.

INTRODUCTION

Biosurfactants are valuable surface active molecules produced by wide variety of microorganisms. Due to its amphipathic nature, these biomolecules are capable of lowering the surface tension, interfacial tension and forming microemulsion to enable mixing of two immiscible solutions. Such properties exhibit excellent detergency, emulsifying, foaming, and dispersing traits. Some of the features, which make them promising alternatives to chemically synthesized surfactants, are their lower toxicity, higher biodegradability, greater stability at wide range of pH and temperature, and better environmental compatibility (Desai and Banat 1997) Thus, interest towards these biomolecules has increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries.

Despite such advantages, biosurfactant have not been fully commercialized due to expensive raw material and low yields. One of the strategies to improve production is to optimize the growth media in order to get maximum production. Formulation of an optimized production medium involves selection of the right nutrients at their correct levels to provide an ideal microenvironment for supporting growth and metabolite production (Mukherjee *et al*., 2008) The classical method of medium optimization involves changing one variable at a time, keeping the others at fixed levels. This approach usually termed as one-factor-at-a-time (OFAT) technique. Standard textbooks and scholarly papers currently favor multivariate optimization such as fractional factorial designs and response surface methodology where multiple factors are changed at once. Despite these criticisms, some researchers have articulated a role for OFAT and showed they can be more effective than fractional factorials under certain conditions: number of runs is limited, primary goal is to attain improvements in the system, and experimental error is not large compared to factor effects, which must be additive and independent of each other (Daniel 1973) The reality that OFAT is inferior in certain situations does not eliminate the possibility that this traditional technique has a useful place in the experimental toolbox. For example, some situations are limited by time and resource pressure and only free experimentation, such as OFAT, is possible.

Many experiments are conducted on processes and factors where little is known. It is then impossible to determine the variable ranges for the experiment with a reasonable degree of confidence for multivariate statistical optimization. The only way to determine the possible ranges is to experiment on the system, and OFAT framework can determine the maximum and minimum settings (Friedman and Savage, 1947: Mutalik *et al*., 2008) have use OFAT method during their preliminary screening experiments to identify critical medium components for biosurfactant production from *Rhodococcus* spp. MTCC 2574 before subjected obtained result to statistical multivariate optimization. Similiarly, Sivapathasekaran *et al*., (2010) optimized nutritional medium for biosurfactant production from *Bacillus circulans* using response surface methodology (RSM) only after identify the critical component in modified marine medium using OFAT technique.

In the present work, OFAT optimization was used to optimize critical nutritional requirement obtained from previous Plackett Burman screening studies (Nawawi *et al*., 2010) for biosurfactant production utilizing sludge

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palm oil (SPO) as a substrate. SPO is the floating residual oil that is separated during initial stage of palm oil mill effluent (POME) discharge. Due to its low grade quality, this residual oil cannot be used for a wide variety application. By utilizing SPO, cheaper raw material was used as a substrate and simultaneously helped in managing palm industry waste with less cost and high production of value added end product. The results obtained from this study will be the basis of further statistical multivariate optimization such as response surface methodology in the future.

MATERIALS AND METHODS

Microorganism and Raw Material:

The bacterial strain *Klebsiella pneumoniae* WMF02 (IMI 398784, CABI, Europe-UK) was selected for this study through a series of experiments such as isolation, purification and screening based on surface tension activities on liquid state fermentation using sludge palm oil (SPO) as a substrate. Initial isolation was carried out at various contaminated hydrocarbon region at West Palm Oil Mill, Carey Island, Selangor, Malaysia. The culture was maintained on nutrient agar and sub cultured for every two weeks.

Samples of SPO were collected from West Palm Oil Mill, Carey Island, Selangor, Malaysia. It was orange in color and existed in semi-solid form at room temperature. A separate study of the same samples showed that the moisture content of SPO was 1.2% and the solid part was attributed by oil rich fibrous residue that resulted from crude palm oil processing (Hayyan *et al*., 2010) The percentage of free fatty acid content was in the range of 21-25%, which consisted mainly of palmitic acid (42.8%), oleic acid (39.6%), and linoleic acid (9.9%).The samples were stored at 4^0C and brought to room temperature before use.

Preparation of Seed Culture:

A loopful of isolated bacterial colony, previously maintained on nutrient agar, was transferred to 50 ml nutrient broth. The culture was grown on a rotary incubator shaker for 12 h at 37^0C and 180 rpm. This primary inoculum was grown until the optical density at 600nm wavelength (OD_{600}) reached to 1.85-1.87 absorbance unit (AU) and was used to inoculate the production media at 2% (v/v).

Optimization Study:

Critical nutritional components that were obtained from previous Plackett Burman study (Nawawi *et al*., 2010) were subjected to one-factor-at-a-time (OFAT) optimization in order to determine the central value for future work on statistical optimization experiments. This is carried out by changing only one factor at a time while fixing others factors. The comparison of contribution towards surface tension reduction between glucose and sucrose were first evaluated in order to introduce a possibility of utilizing only one co-substrate in the experiments. Selected co-substrate was then subjected to OFAT optimization along with other nutritional components i.e. sludge palm oil (SPO), $MgSO_4$, $FeSO_4$, $NaNO_3$, and K_2HPO_4.

Fermentation was carried out in 100 ml Erlenmeyer flasks with a 50 ml working volume. For inoculation, the flasks were allowed to cool down to room temperature before transferring 2% (v/v) primary inocula into the production media. The cultures were incubated in a rotary incubator shaker for 30 hours at 37^0C and 180 rpm. All experiments were carried out in triplicate. Reduction of surface tension was used as an indirect method to determine the amount of biosurfactant after the fermentation. Lowering of surface tension is directly correlated with higher biosurfactant concentration.

Analysis of Biosurfactant:

The analysis was done by first centrifuging the 30 hours fermentation broth at 8000 rpm for 15 min. Residual sludge palm oil in the culture supernatant was removed by extracting it with an equal volume of hexane in separatory funnel (Raza *et al*., 2006) The supernatant was then analyzed by surface tension measurement. The surface tension that obtained using a digital surface tensiometer (KSV Sigma 702) working on the principle of the Du Nuoy ring method (Bodour and Miller-Maier 1998) Ten milliliters volume of each surfactant was transferred into a clean 20 ml beaker and placed onto the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled through the liquid-air interface, to measure the surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with water, flamed and was allowed to dry. The calibration was done using water (ST=71.5 mN/m ±0.5) before taking samples measurement.

Extraction of Crude Biosurfactant:

Ten milliliter of supernatant was treated by acidification to pH 2.0 using 6 M hydrochloric acid solution, and the acidified supernatant was left overnight at 4^0C for the complete precipitation of the biosurfactant. The precipitate was extracted with a solvent having a 2:1 chloroform-to-methanol ratio in a separatory funnel at room temperature. The organic phase was transferred to a round bottom flask connected to a rotary evaporator

to remove the solvent. The evaporation process was carried out under reduced pressure of 400 mbar at 40^0C until the viscous and oily honey colored residue was observed.

Preliminary Identification of Biosurfactant:

a) CTAB/methylene-blue Agar Test:

Blue agar plates composing of 15 g/l agar bacteriological no. 1, 0.2 g/l N-Cetyl-N,N,N-trimethylammonium bromide, and 0.005 g/l methylene blue were used to detect extracellular glycolipid production. Formation of dark blue halos around the colonies indicates rhamnolipid biosurfactant production (Siegmund. and Wagner 1991).

b) Biuret Test:

Two milliliter of crude extract solution was first heated at 70^0C before mixed with 1 M NaOH solution. Drops of 1% $CuSO_4$ were added slowly and a change in color was observed. A positive result is indicated by a violet or pink ring, due to the reaction of peptide bond proteins or short-chain polypeptides, respectively. This test was used to detect lipopeptide biosurfactant (Feigner Besson and Michel 1995).

c) Phosphate Test:

Ten drops of 6 M HNO_3 was added to 2 ml of crude extract solution followed by heating at 70^0C. Drops of 5% ammonium molybdate were added slowly and observation was recorded. Formation of yellow color, which may be followed by slow formation of a fine yellow precipitate, indicates the presence of phospholipid biosurfactant (Okpokwasili and Ibiene 2006).

RESULT AND DISCUSSIONS

Determination of Co-substrate:

During previous Plackett-Burman screening studies (Nawawi *et al.,* 2010) both glucose and sucrose were identified among the critical media components. Glucose is a simple sugar, which is a mono-saccharide. It is easier for the bacteria to use it as an energy source. Sucrose is a di-saccharide, which is made up of one molecule of glucose and one molecule of fructose. It takes an enzymatic reaction to break apart the sucrose into an usable form of energy. It is assumed that both simple sugars play a vital role in feeding the microorganisms during the initial phase of growth. After the population matured, a complex carbon source, sludge palm oil was utilized as the main substrate in the production medium.

Isolated *Klebsiella pneumoniae* WMF02 that was used in this study was known as both glucose and sucrose fermenter. In this study, post-analysis showed that the contribution of both co-substrates as co-carbon source was minimal relative to others critical media components. This would introduce the possibility of using either sucrose or glucose as a co-carbon sources. Fig. 1 shows the results obtained when the production media consisting of glucose only and sucrose only as their co-carbon sources. It was observed that there is not much difference in reduction of surface activity when using either one of co-substrate at lower concentration (below 5 g/l). Sepahy *et al*., (2005) had drawn same conclusion when they studied the effect of glucose and sucrose on biosurfactant production by isolates from Iranian oil. It was found that the amount of surface tension and interfacial tension reduction in glucose containing medium almost close to the surface reduction activity in sucrose medium. Due to the high cost associated with pure commercial glucose, raw table sugar (sucrose) was selected as a co-carbon source for further study.

One-Factor-at-a-Time Optimization:

Due to limited information available in the literature on the production of biosurfactant by *Klebsiella* sp. genus, one-factor-at-a-time (OFAT) optimization was used to determine the possible optimum level of significant variables obtained from previous Plackett-Burman results.

a) Effect of SPO:

Fig. 2 shows the effect of SPO on surface tension activity while other variables fixed at following concentration: 2 g/l sucrose, 0.3 g/l $MgSO_4$, 0.1 g/l $FeSO_4$, 0.2 g/l $NaNO_3$, and 3.3 g/l K_2HPO_4. Sludge palm oil was added at five levels of concentration varying from 0 to 8% (v/v).

Lowest measurement of surface tension was observed at 4% (v/v) SPO. Concentration at 4% was chosen as optimal value, although similar reduction occurred also at 6% concentration. This is because the difficulties of separating the semi-solid phase in the broth supernatant after fermentation increased as the concentration of SPO increased. Furthermore, excess SPO in fermentation broth will reduce dissolved oxygen in cultures as oxygen transfer from outside is prevented.

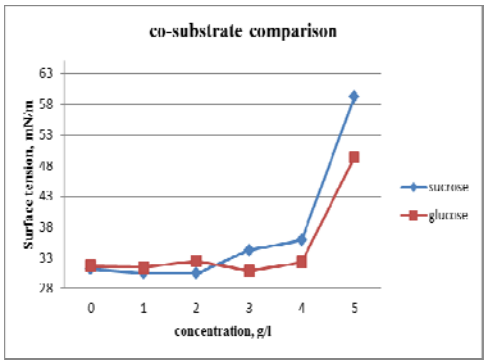

Fig. 1: Comparison of glucose and sucrose at different concentration for surface reducing capabilities.

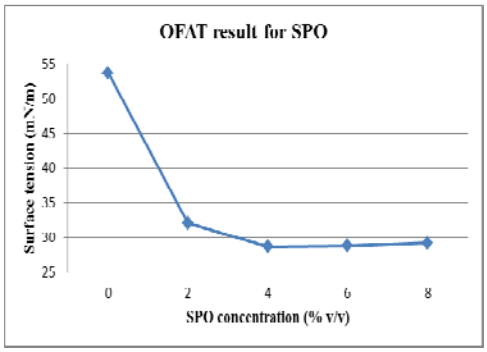

Fig. 2: Effect of SPO concentration on surface tension activity.

b) Effect of Sucrose:

In order to study the effect of sucrose on biosurfactant production, five sucrose levels ranging from 0 g/l to 20 g/l were used to evaluate the reduction of surface tension of fermentation broth after 30 hour (Fig. 3). SPO concentration was fixed at optimal value of 4% v/v according to previous OFAT result, while concentration of $MgSO_4$, $FeSO_4$, $NaNO_3$, and K_2HPO_4 were fixed at 0.3 g/l, 0.1 g/l, 0.2 g/l, and 3.3 g/l respectively. The lowest surface tension 26.6 mN/m was obtained at a concentration of 5 g/l. The addition of co-carbon source helped in reducing the surface tension from 28.05 mN/m (without sucrose) to 26.2 mN/m. Growth was also observed at zero concentration of sucrose, indicating the ability of strain to utilize SPO as sole carbon source. Duvnjak and Kosaric (1985) reported that the existence of co-substrate from simple sugar like glucose or sucrose in addition of water-immiscible substrate helps to facilitate the release of surfactant from cells.

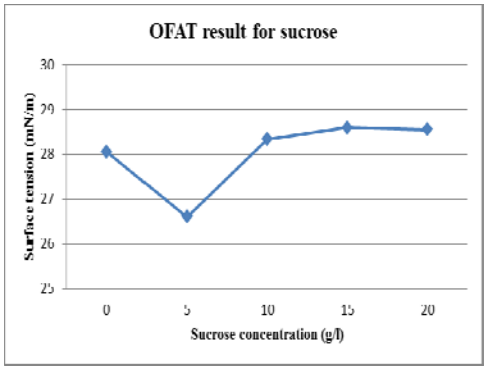

Fig. 3: Effect of sucrose on surface tension activity.

c) Effect of $MgSO_4$:

Different levels of $MgSO_4$ from 0 g/l to 0.8 g/l were tested to determine the optimal concentration for biosurfactant production (Fig. 4).SPO and sucrose concentration was fixed at optimal value of 4% v/v and 5 g/l according to previous OFAT results, while concentration of $FeSO_4$, $NaNO_3$, and K_2HPO_4 were fixed at 0.1 g/l,

0.2 g/l, and 3.3 g/l respectively. No bacterial growth was observed at zero concentration of $MgSO_4$ indicating the vitality of this mineral for strain survival. The surface tension reduced to its lowest value (27.16 mN/m) at 0.4 g/l concentration of $MgSO_4$. However, the surface activity increased significantly beyond 0.8 g/l indicating possible inhibitory effect of $MgSO_4$ at high concentration during biosurfactant production. This inhibitory effect also reported by Mukherjee *et al*., (2008) when evaluating important nutrient requirement for marine bacterium.

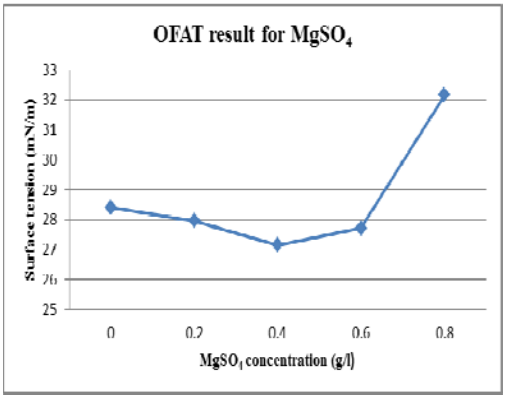

Fig. 4: Effect of $MgSO_4$ on surface tension activity.

d) Effect of $FeSO_4$:

Fig. 5 shows the effect of $FeSO_4$ on surface tension activity while SPO, sucrose, and $MgSO_4$ concentration were fixed according to their optimal concentration of 4% v/v, 5 g/l, and 0.4 g/l respectively. Previous Plackett-Burman studies (Nawawi *et al*., 2010) showed that a lower amount of $FeSO_4$ is preferred due to consequent broth acidification that may be caused by excess iron. Thus the selected range of five different levels used in OFAT optimization of $FeSO_4$ were much smaller (0.05 g/l to 0.4 g/l). Lowest measurement of surface tension (25.90 mN/m) was obtained at concentration of 0.3g/l. According to Glick *et al*., (2010) this iron-limiting phenomenon serves as an environmental signal for biofilm development in biosurfactant producer strain. He reported that under iron limitation, the timing of rhamnolipid expression by Pseudomonas aeruginosa is shifted to the initial stages of biofilm formation (vs. later in biofilm development under iron-replete conditions) and results in increased bacterial surface motility thus yield more biosurfactant.

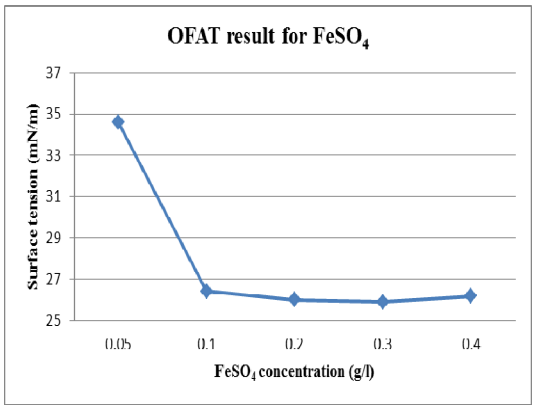

Fig. 5: Effect of $FeSO_4$ concentration on surface tension activity.

e) Effect of $NaNO_3$:

The effect of different $NaNO_3$ concentration upon the ability to reduce surface tension was evaluated at 0 g/l, 1 g/l, 2 g/l, 3 g/l, and 4 g/l (Fig. 6). Concentration of SPO, sucrose, $MgSO_4$, and $FeSO_4$ were fixed according to optimal value obtained from previous OFAT results while K_2HPO_4 concentration was fixed at 3.3 g/l.

At first, the surface tension of fermentation broth decreased steadily parallel to addition of $NaNO_3$, and reached its lowest value (26.09 mN/m) at 2 g/l concentration, but then again increased beyond 3 g/l concentration. Similar nitrogen limiting phenomena observed in various literature. Syldatk *et al*., (1985) reported that nitrogen limitation not only causes overproduction of biosurfactant but also changes the composition of the biosurfactant produced. Guerra-Santos *et al*., (1984) also found higher rhamnolipid

production at C/N ratio of 16 to 18 and no surfactant production below a C/N ratio of 9, where the culture was not nitrogen limited.

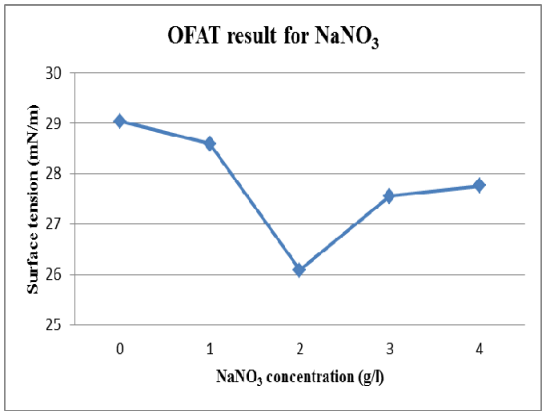

Fig. 6: Effect of $NaNO_3$ concentration on surface tension activity.

f) Effect of K_2HPO_4:

Five different levels ranging from 0 g/l to 10 g/l were used to determine the optimal concentration needed for biosurfactant production (Fig. 7). All other variables were fixed at their respective optimal value obtained from previous OFAT experiments.Very high surface tension(45.22 mN/m) was obtained and no bacterial growth was observed when the fermentation was carried out in the absence of K_2HPO_4 indicating the importance of this mineral for supporting bacterial growth and biosurfactant production.

In this study, the surface tension reduced to its lowest value (25.70 mN/m) at 4 g/l concentration of K_2HPO_4. Pruthi and Cameotra (2003) claimed that although phosphate is essential for bacterial growth, an increase in the concentration of phosphorus in the growth medium was not associated with any remarkable change in growth, emulsification activity, and surface tension reduction of the culture broth.

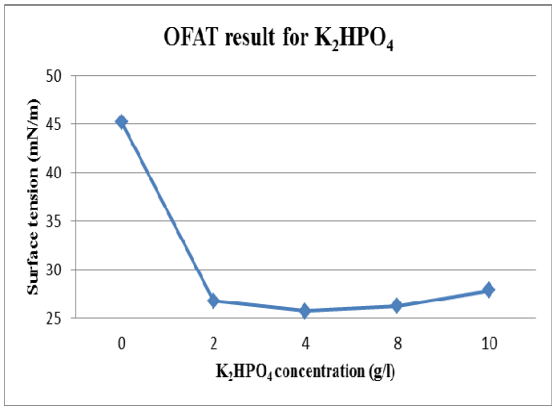

Fig. 7: Effect of K_2HPO_4 concentration on surface tension activity.

Preliminary Biosurfactant Identification:

Chloroform:methanol (2:1) extraction was carried out in order to separate extracellular biosurfactant from other constituents, proteins, polysaccharides, cell debris, sugars, etc. Acidification of the sample was done (pH 2) prior to extraction to enhance the extraction yield of biosurfactant. At low pH, biosurfactant will present in their protonated form, hence, are less soluble in aqueous solution (Heyd *et al*., 2008).

Three biochemical tests were carried out in order to determine the type of biosurfactant produced in this study. CTAB/methylene-blue agar is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactant. If glycolipid biosurfactants are secreted by the microbes growing on the plate, they form dark blue halos (Fig. 8-a). In this study, negative result was obtained when the strain cultivated on a light blue mineral salts plate containing the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue (Fig. 8-b).

Biuret reagent turned to violet or pink ring due to reaction of peptide bond proteins or short-chain polypeptides. This test was applied in order to detect lipopeptide biosurfactant in the sample. In this study, negative result obtained (no color change to violet) when crude biosurfactant extract was dissolved in biuret reagent (Fig. 9-b).

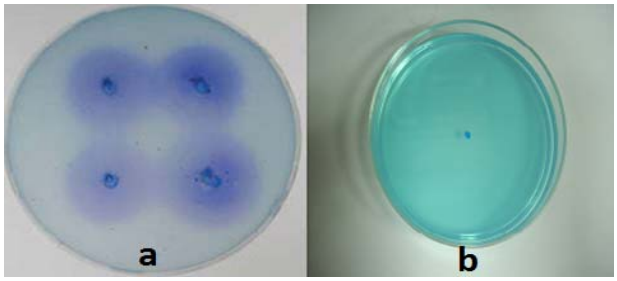

Fig. 8: CTAB/methylene-blue agar test for glycolipid identification. (a) positive result (b) negative result obtained during this study.

Okpokwasili and Ibiene used phosphate test to determine phospholipid biosurfactant of crude extract from a culture of *Pseudomonas* sp. grown on kerosene-supplemented mineral salts medium (Okpokwasili and Ibiene 2006).

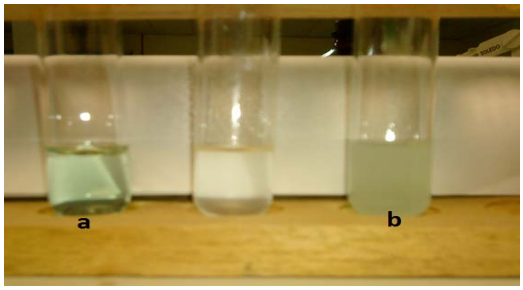

Fig. 9: Biuret test for lipopeptide biosurfactant test. (a) biuret reagent (b) negative result obtained during this study.

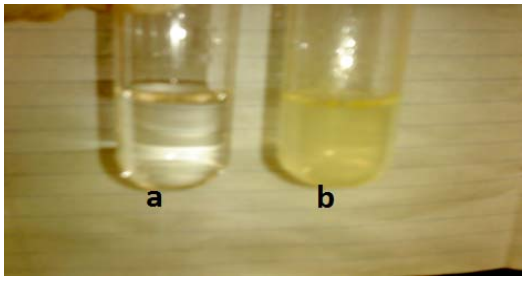

Fig. 10: Phosphate test for phospholipid biosurfactant. (a) negative control (b) positive results obtained when crude biosurfactant extract dissolved in phosphate reagent.

In this study, colorless phosphate assay solution changed to yellow indicated positive result for phospholipid (Fig. 10-b). In addition, formation of fine yellow precipitate was also observed after few minutes. The result of preliminary identification revealed that the biosurfactant produced by *Klebsiella pneumoniae* WMF02 in this study was phospholipid type.

Conclusions:

In this study, sludge palm oil was introduced as a novel substrate for biosurfactant production by locally isolated *Klebsiella pneumoniae* WMF02. One-factor-at-a-time (OFAT) optimization was carried out in order to determine the optimal concentration of critical nutritional components which is obtained from previous Plackett-Burman screening study (Nawawi *et al*., 2010) Sucrose was selected as co-substrate over glucose due to small difference in reduction of surface activity when using either one of them in the production media. Media

optimization suggested an optimal concentration of nutrients, which were found at (g/l): Sucrose (5), $MgSO_4$ (0.4), $FeSO_4$ (0.3), $NaNO_3$ (2), K_2HPO_4 (4), and 4 % (v/v) of SPO. This step greatly reduced the surface tension of non-optimized control from 36.2 mN/m to 25.70 mN/m. Preliminary identification using CTAB/methylene blue agar test, biuret test, and phosphate test indicated that the biosurfactant produced was phospholipids.

ACKNOWLEDGMENT

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