4.0 RESULTS

4.1 Process Optimization

4.1.1 Temperature optimization for the growth of *B. licheniformis* JF-2

Growth of *B. licheniformis* JF-2 was investigated at 35°, 40° and 45°C in shake flasks containing Nutrient broth at pH 7.0 for biomass production and surface tension reduction. The optimum temperature for growth of *B. licheniformis* JF-2 was 40°C, which showed a lag phase of 3 hours and a log phase of 5 hours. The data shown in figures 7 and 8, between the 8th and 16th hour of fermentation show the general trend for all parameters measured,therefore,a break is shown on the graph. Biomass averaged a maximum of 1.13 g/L (Fig. 7). This was higher to that obtained at the other 2 temperatures (35°C and 45°C).

The absorbance profile of the culture in Nutrient broth read at 480 nm showed a similar trend to biomass production (Fig. 7). Maximum absorbance averaging 1.14 was obtained at the end of the log phase after 8 hours of fermentation. The stationary phase was reached at the 8th hour fermentation period onwards until the 24th hour. There were no drastic fluctuations observed in the average absorbance values during the 8th and 16th hour of fermentation. The break between the 8th and 16th hour observed on the graph is to shorten the time scale only. This was similar to the maximum absorbance between 1.0 to 1.2 obtained after 20 hours of growth in Medium E at 40°C by Jenneman *et al.* (1983). The lag phase reported by Jenneman *et al.* (1983) of *B. licheniformis* JF-2 grown in Medium E was 6 hours, which was twice that compared to the growth in Nutrient broth in our study. The log phase

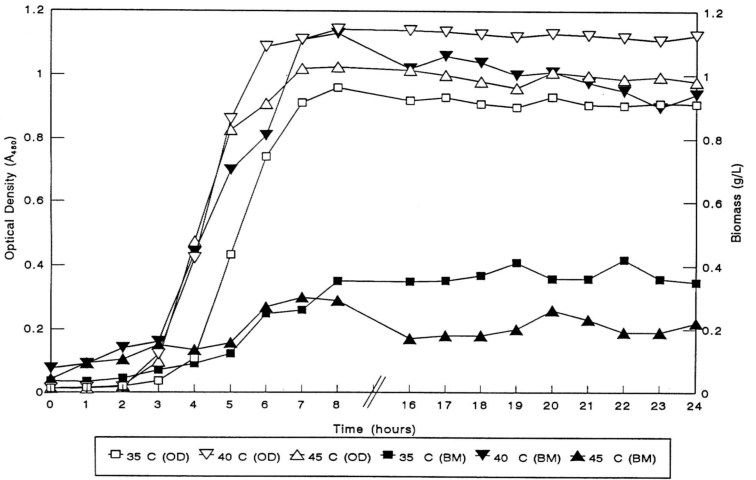


Fig. 7: Biomass and Optical density profiles of the growth of *B. licheniformis* JF-2 in Nutrient Broth, incubated at 35°C, 40°C and 45°C, and shaken at 250 rpm.

lasted only 5 hours in our study (Fig. 7). The pH of Nutrient broth decreased from 7.1 to 6.8 during the first 4 hours of growth of B. licheniformis JF-2 at 40°C. Thereafter, it increased gradually to 8.5 (Fig.8) after 24 hours. Similar findings were observed at 35°C and 45°C.

Surface tension of the Nutrient broth was measured in the whole broth (Fig. 8). There was a marked decrease in surface tension at 40°C from 60.8 to 31.5 mN/m, which constituted a drop of 48.2% between the 4th and 5th hour of growth. The lowest surface tension recorded at 40°C was 29.9 mN/m at the 7th hour. There was no significant increase in the surface tension values between the 8th and 16th hour of fermentation for all three temperatures of 35°C, 40°C and 45°C. Only from the 16th hour onwards of fermentation, could we see marked increases in the measured surface tension values which peaked to 57.0 mN/m (at the 24th hour) at 35°C and at 45°C, it peaked to a value of 52.7 mN/m (at the 24th hour) whereas at 40°C, the increase was only marginal until the end of the fermentation period of 24 hours where it peaked to a value of 37.3 mN/m, which is depicted in Figure 8.

4.1.2 Determination of optimum pH for growth and production of the lipopeptide by *B. licheniformis* JF-2

pH optimization studies for the growth and production of the lipopeptide by *B. licheniformis* JF-2 was conducted in shake flasks using Nutrient broth as the cultivation medium. The maximum biomass obtained was 1.14 g/L at a pH of 7.0 which was the highest biomass obtained compared to that at pH of 2.0 - 9.0 (Fig. 9). Growth measured by optical density was similar to that of

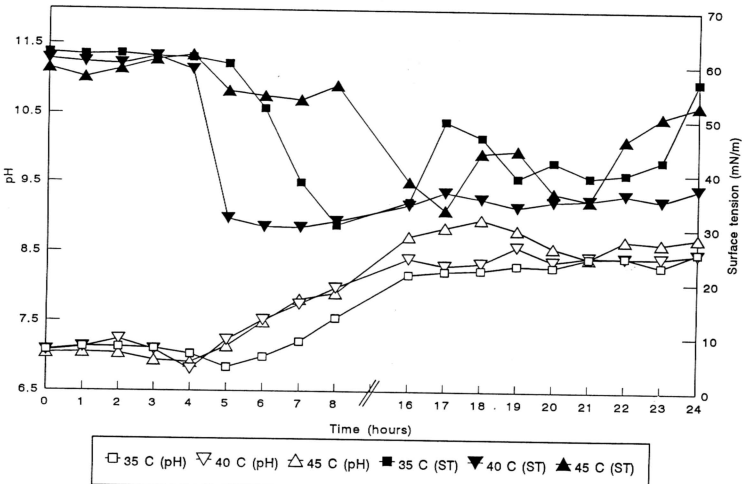


Fig. 8: Final pH and Surface tension (ST) profiles of *B. licheniformis* JF-2 in a time course study at 35°C, 40°C and 45°C, and shaken at 250 rpm.

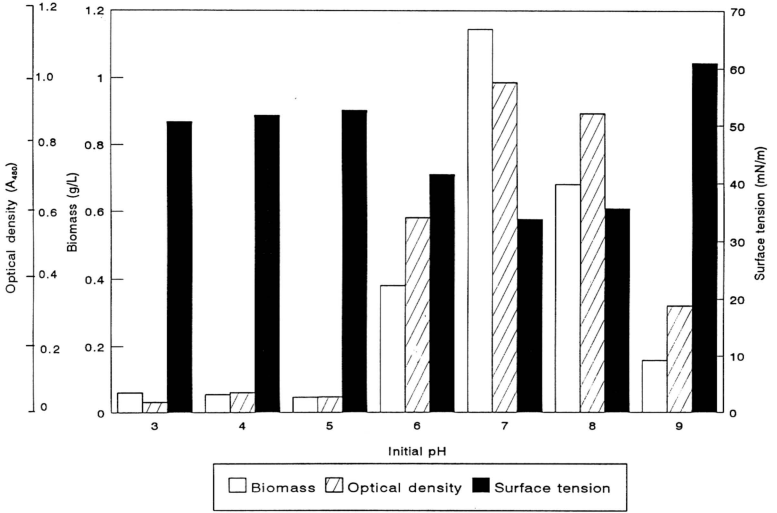


Fig. 9: pH optimization with respect to biomass, optical density and surface tension measurements of the cultures of *B. licheniformis* JF-2 grown in Nutrient broth in shake flasks at 40^{0}C and 250 rpm after 8 hours of growth.

the biomass profile with a maximum absorbance of 0.99 (Fig. 9) at the 8th hour of fermentation. The maximum biomass achieved at an initial pH of 7.0 also resulted in the maximum decrease in the surface tension of the cultivation medium to 33.7 mN/m (Fig. 9) indicating that the presence of the biosurfactant at a higher concentration was due to a higher concentration of biomass as compared to all the other cultivation medium with initial pH values of between 2.0 - 6.0 and 8.0 - 9.0.

4.2 Media Optimization

4.2.1 Effect of sodium chloride (NaCl) on the growth and lipopeptide synthesis by *B. licheniformis* JF-2

Halophilic characteristic is a prerequisite for an organism to be used in MEOR. Due to the halophilic nature of *B. licheniformis* JF-2, NaCl was added at various levels in the medium to determine its effect on biomass and lipopeptide production. Sodium chloride is generally used in the cultivation media of many microorganisms, including *B. licheniformis* JF-2. Lin *et al.* (1993 and 1994a) included 0.5% of NaCl in the cultivation medium in production and deactivation studies of the lipopeptide biosurfactant by *B. licheniformis* JF-2.

Our study indicated an increase in the biomass yield to 1.40 g/L at 3% NaCl (w/v) (Fig. 10) compared to the control (0% NaCl) which only produced 1.05 g/L after 8 hours of fermentation. The increase in the concentration of NaCl of up to 3% (w/v) in the medium resulted in a concomitant increase in the yield of the crude lipopeptide (Fig. 10). The maximum yield of crude lipopeptide obtained averaged 0.31 g/L at 3% (w/v) NaCl, which was higher compared to the control (0% NaCl) which produced only 0.22 g/L. However,

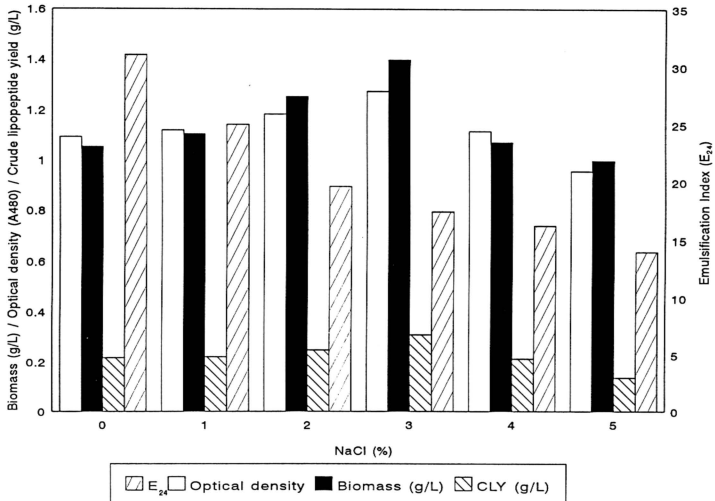


Fig. 10: The Biomass, Optical density and Crude Lipopeptide Yield (CLY) and the Emulsification Index profiles in various concentrations of NaCl after 8 hours of fermentation with *B. licheniformis* JF-2 in Nutrient broth at 40^{0}C and shaken at 250 rpm.

the lowest surface tension was measured in the control (30.2 mN/m) containing no NaCl (Table 2).

Table 2: Surface tension of the cultivation media containing various concentrations of NaCl after 8 hours of fermentation with *B. licheniformis* JF-2 in shake flask culture

% Sodium Chloride (w/v)	Surface tension (mN/m)
0	30.2
1.0	34.4
2.0	36.9
3.0	42.8
4.0	44.5
5.0	47.2

The Emulsification Index (E_{24}) was determined in the cultivation medium containing various levels of NaCl after 8 hours of fermentation (Fig.10 and Plate 5). The increasing NaCl concentration in the cultivation medium was found to decrease the surfactant activity.The maximum activity of the surfactant was at 0% (w/v) NaCl concentration with a mean E_{24} value of 3.1 and gradually decreased to an average of 14.0. The decreasing values of the Emulsion Index clearly shows that the NaCl present in the cultivation medium acts to dampen the activity of the lipopeptide biosurfactant. The surface tension of the cultivation medium increased from 42.8 to 47.2 mN/m as the NaCl concentration increased from 3.0 % (w/v) to 5.0 % (w/v).

4.2.2 Effect of 5% and 10% glucose in the cultivation medium containing NaCl on the growth and lipopeptide production by *B. licheniformis* JF-2

The addition of 5% and 10% (w/v) glucose with various concentrations

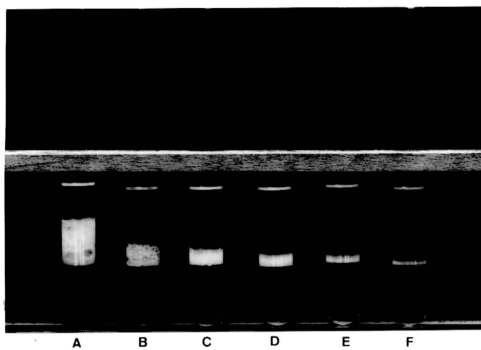


Plate 5: The Emulsification Index (E_{24}) measured at six concentrations of NaCl in Nutrient broth after 8 hours of growth with *B. licheniformis* JF-2 at 40°C and shaken at 250 rpm. The Emulsification Index was measured after 24 hours of settling at room temperature. NaCl (w/v) : A - 0%; B - 1%; C - 2%; D - 3%; E - 4%; F - 5% .

of NaCl on biomass and lipopeptide biosurfactant synthesis were studied (Figs. 11 and 12). At 5% (w/v) glucose concentration, the maximum absorbance measured was averaging 0.96 with a NaCl concentration of 5% (w/v), whereas at 10% (w/v) glucose concentration, the maximum absorbance measured was 1.10 at a NaCl concentration of 4% (w/v). The maximum biomass averaging 1.74 g/L was obtained at 4% NaCl in 10% (w/v) glucose medium compared to 1.05 g/L in the 5% (w/v) glucose medium at 5% (w/v) NaCl concentration (Fig. 11). Figure 12 indicates the increase in surface tension of the cultivation medium and then begins to stabilize between 2% and 5% (w/v) NaCl concentration in the 5% and 10% (w/v) glucose containing medium.

The crude lipopeptide yield reached a maximum at 3% (w/v) NaCl concentration for both the 5% and 10% (w/v) glucose containing medium with a maximum yield averaging 1.17 g/L for 5% (w/v) glucose concentration and 0.87 g/L, for the 10% (w/v) glucose concentration medium, respectively (Fig. 12). Maximum biomass synthesis does not lead to maximum lipopeptide production but depends largely on the optimum quantity of glucose and NaCl.

4.2.3 Effect of various concetrations of Yeast extract (YE) on the growth and lipopeptide yield of *B. licheniformis* JF-2

In this study, the amount of yeast extract in the medium had a significant effect on biomass production which increased with increasing concentrations of yeast extract of up to 0.5% (w/v) (Fig. 13). The trend was similar in the absorbance readings of the cultivation medium, with a maximum value FIG.13 averaging 1.55 at 0.5% (w/v) YE. Although higher concentrations of YE

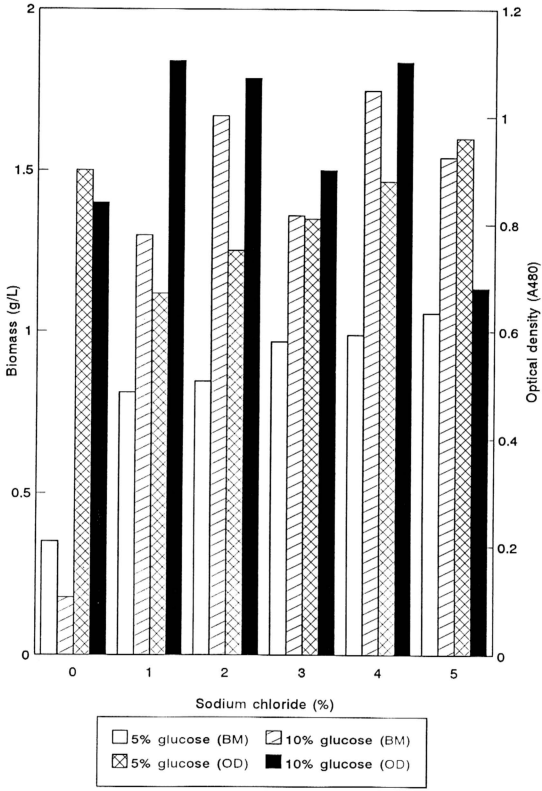


Fig. 11: Optical density and Biomass profiles of *B. licheniformis* JF-2 after 8 hours of growth in Nutrient broth containing various concentrations of NaCl and glucose at 40°C and shaken at 250 rpm (BM - Biomass, OD - Optical density).

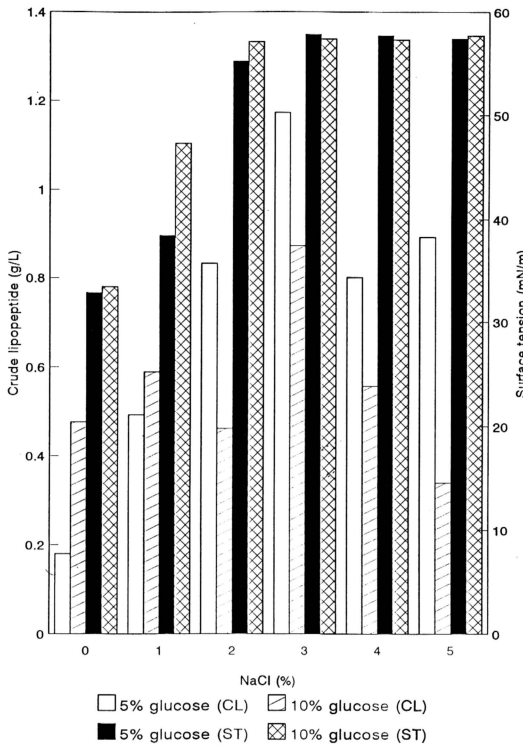


Fig. 12: Surface tension (ST) measurements and Crude lipopeptide yield (CL) by *B. licheniformis* JF-2 in Nutrient broth containing NaCl between 1-5% (w/v) and glucose at 5% and 10% in shake flask at 40^0C and shaken at 250 rpm.

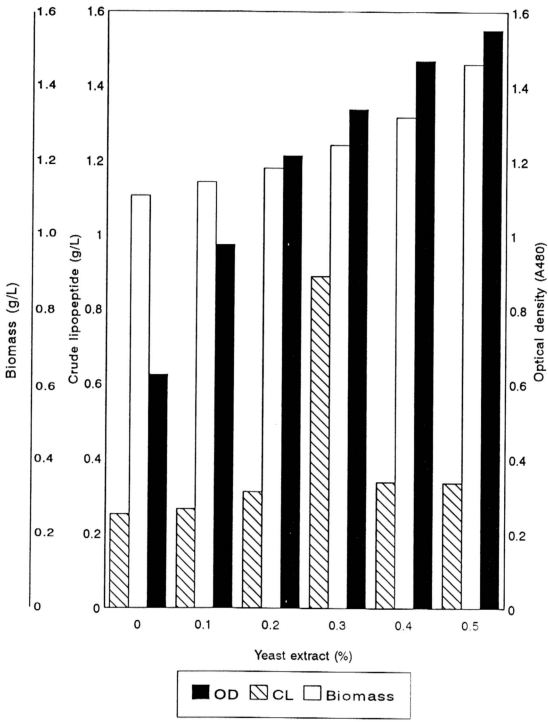


Fig. 13: The effect of yeast extract on cell density (OD), Biomass and Crude lipopeptide yield (CL) in Nutrient broth at pH 7.0, 40^{0}C and shaken at 250 rpm in shake flasks.

increased the biomass yield it did not increase the synthesis of the extracellular lipopeptide. The maximum yield of crude lipopeptide averaged 0.89 g/L at 0.3% (w/v) YE which was higher than the control (0% YE) which produced an average of 0.25 g/L after 8 hours of fermentation.

The cultivation medium containing 0.3% (w/v) YE showed a surface tension measurement of 36.8 mN/m which was higher than the control (0% YE) which gave the lowest reading averaging 32.3 mN/m (Table 3).

Table 3: Surface tension of the Nutrient broth containing various concentration of yeast extract after 8 hours fermentation with *B. licheniformis* JF-2 at 40°C and shaken at 250 rpm.

% Yeast Extract	Surface tension (mN/m)
0	32.3
0.1	42.8
0.2	42.8
0.3	36.8
0.4	38.4
0.5	35.0

From this study, we found that the maximum lipopeptide yield averaging 0.89 g/L depended on the critical yeast extract concentration of 0.3% (w/v).

From our media optimization studies, we found that *B. licheniformis* JF-2 grew optimally at 40°C and gives high yields of the crude lipopeptide biosurfactant. The newly formulated medium now termed as the Optimized Medium consist of (g/L); Nutrient broth, 8.0; yeast extract, 3.0; NaCl, 30.0 and glucose, 50.0. Therefore, the next stage of our study focuses on the maximum biomass and crude lipopeptide yield under the optimized conditions.

4.2.4 Production of biomass and lipopeptide by *B. licheniformis* JF-2 in shake flask cultures using the Optimized medium (OM)

The Optimized medium was tested for growth of *B. licheniformis* JF-2 in terms of biomass and optical density (A_{480}) and correlated with lipopeptide production in a time course study. The biomass and optical density depicted in Figure 14 show a similar trend. The lag phase lasted for about 4 hours with an average biomass yield of 0.29 g/L while the log phase extended for 4 hours, with a maximum yield of 1.61 g/L at the 8th hour of fermentation. The maximum absorbance averaging 1.58 was also achieved after 8 hours of growth in the optimized medium.

The crude lipopeptide yield which is indicated in Fig. 14 showed a maximum value averaging 1.82 g/L after 8 hours of fermentation. This correlates with the maximum biomass obtained after 8 hours. As the biomass decreased during the stationary phase, the lipopeptide yield also decreased to a minimum of 1.59 g/L after 24 hours of fermentation.

The surface tension of the cultivation medium decreased from an initial value of 48.6 mN/m to 29.1 mN/m after 6 hours of fermentation (Fig.14). Subsequently, it increased to 36.2 mN/m at the 24th hour. Throughout the fermentation period, the pH of the cultivation medium decreased gradually from 7.0 at 0 hour to 5.0 at the 24th hour. The maximum biomass which averaged 1.61 g/L is the highest achieved thus far in shake flask cultures according to literature, whereas, the crude lipopeptide yield averaged 1.82 g/L after 8 hours of growth.

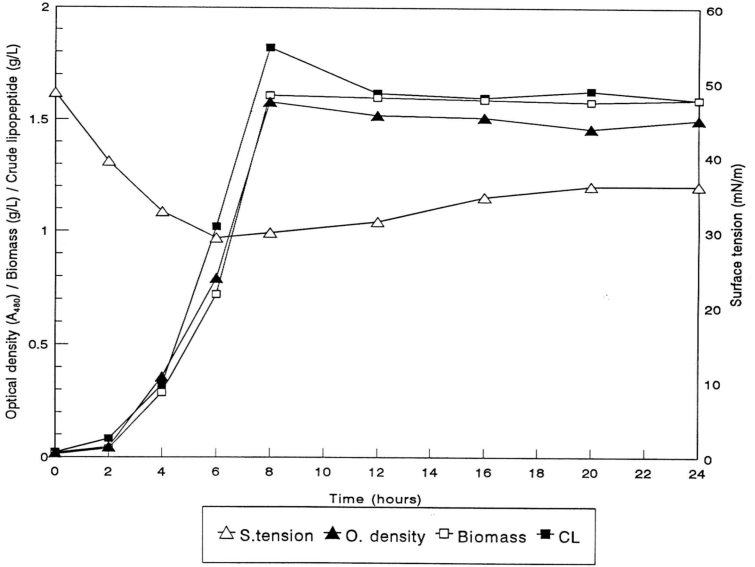


Fig. 14: Fermentation kinetic profiles of *B. licheniformis* JF-2 in the Optimized Medium in shake flasks at 40°C and shaken at 250 rpm (CL - Crude lipopeptide yield).

4.2.5 A comparison of Medium E (ME) and our Optimized Medium (OM) in shake flask culture for the production of biomass and lipoptide from *B. licheniformis* JF-2

Our optimized medium consisting of Nutrient broth with 0.3% (w/v) yeast extract, 5% (w/v) glucose and 3% (w/v) NaCl was found to be superior to Medium E (ME) which was used by McInerney *et al.* (1985). The cell biomass in a time course study was determined by measuring the absorbance (A_{480}) of the cultivation medium containing *B. licheniformis* JF-2 while reduction in the surface tension was used to determine the biosynthesis of the biosurfactant. The break observed in the time (hour) scale in Fig. 15 between the 8th and 16th hour of fermentation is done to shorten the scale as the general trend observed is similar. There were no significant changes observed in the various parameters measured as the *B. licheniformis* JF-2 culture had already entered the stationary phase (after the 8th hour of fermentation).

The pH of ME decreased from an initial value of 7.0 to 6.9 after 4 hours of fermentation. Then it increased gradually to a maximum of 8.6 after 24 hours (Fig. 15) whereas this was not the case in the OM (Fig. 15) which had an initial pH of 7.0 and decreasing to 5.0 after 24 hours of fermentation.

Higher absorbance (A_{480}) values were observed in the OM compared to ME in a time course study (Fig. 15). Maximum absorbance in the OM averaged 1.58 at the 8th hour of fermentation, after which the culture enters the stationary phase which lasts up to the 24th hour. However, the maximum absorbance of 1.02 was achieved at the 20th hour of fermentation in ME. Thus, our study indicates that the growth of *B. licheniformis* JF-2 was superior in the OM compared to ME with a shorter growth period.

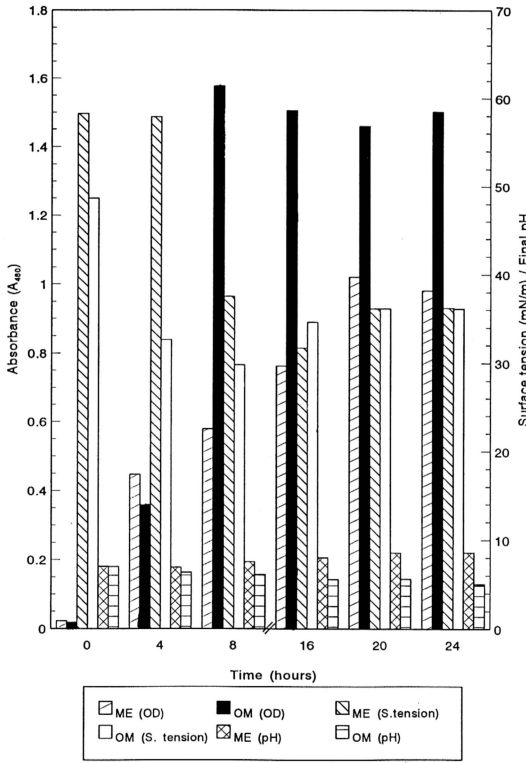


Fig. 15: The Optical density, Surface tension and final pH profile of Medium E (ME) and the Optimized Medium (OM) during the growth of *B. licheniformis* JF-2 in shake flask culture at 40^0C and 250 rpm (The data shown for the OM is the same as in Fig. 14).

The growth of *B. licheniformis* JF-2 resulted in the reduction of the surface tension in both the cultivation media (Fig. 15). Our study indicates that the reduction in surface tension is correlated linearly with the production of the lipopeptide up to a critical concentration of the product. The CMC of the crude lipopeptide obtained from the OM was 165.9 mg/L (Section 4.4) with a minimum average surface tension of 29.8 mN/m after 8 hours of fermentation compared to a minimum average value of 31.7 mN/m obtained after 16 hours of growth in Medium E. The surface tension of both the cultivation medium increased after 24 hours of growth to 36.1 mN/m and 36.2 mN/m in the OM and Medium E, respectively.

4.3 Identification of the crude lipopeptide biosurfactant by thin layer chromatography

The lipopeptide was extracted separately in methanol, chloroform or dichloromethane and detected by thin layer chromatography using Silica Gel-60 F_{254} precoated plates (Merck, Germany). A 10 μL sample of 20 mg/mL solution were spotted on to the baseline of the plates and allowed to dry. The plates were developed in a standard developing tank, 9.5 cm x 29.0 cm x 27.5 cm, containing chloroform - methanol - 28% NH_4OH (65:35:5:v/v/v) (McInerney *et al.*, 1990). The plates were allowed to dry and stained with anisaldehyde reagent which contained a mixture of anisaldehyde/sulphuric acid/acetic acid at 0.5/1.0/50 (v/v/v) or sprayed with concentrated sulphuric acid and placed at 100°C until charred to visualize the migrated spots (Sasidharan, 1995). The R_f of each spot was determined according to the

following formula:

$$R_f = \frac{\text{Distance of spot from the origin}}{\text{Distance of the solvent from the origin}}$$

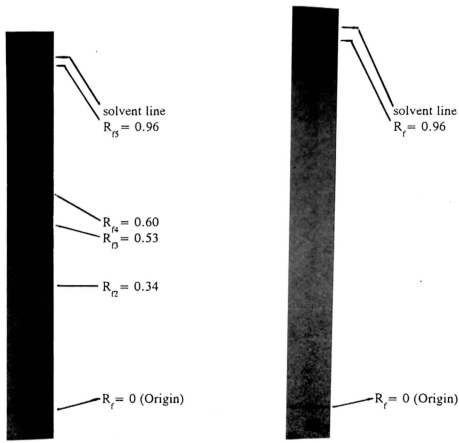
Table 4 shows the R_f values of the extracted crude lipopeptide from *B. licheniformis* JF-2. The chloroform soluble fraction contained one component with an R_f value of 0.95 which was comparable to the R_f value obtained by McInerney *et al.* (1990).

The methanol soluble fraction consisted of four spots (Plate 6) with R_f values of 0.34, 0.53, 0.60 and 0.96 (Table 4). The spot at 0.45 in McInerney's study was absent in ours indicating that the crude lipopeptide obtained in the optimized medium was slightly different from that obtained by McInerney *et al.* (1990). The common spots with similar R_f values with reference to McInerney *et al.* (1990) study have R_f values of 0.34 and 0.96. This may be attributed to the use of a different growth media for the cultivation of *B. licheniformis* JF-2.

Table 4: R_f values of the migrated spots and stained with anisaldehyde reagent in TLC

Solvent	R_f	
	This study	McInerney *et al.* (1990)
(a) Chloroform	0.95	0.97
(b) Methanol	0.34	0.31
	Nd	0.45
	0.53	Nd
	0.60	Nd
	0.96	0.97
(c) Dichloromethane	Nd	0.55
	0.96	0.97

Nd: Not detected



a) Methanol (b) Dichloromethane

Plate 6: Plates stained with anisaldehyde reagent to visualise the spots from extracts using Thin Layer Chromatography (TLC).
(a) Methanol and (b) Dichloromethane

The dichloromethane fraction obtained by McInerney *et al.* (1990) contained 2 components with R_f values of 0.55 and 0.95. This study only showed the presence of one component with an R_f value of 0.96 (Table 4 and Plate 6). McInerney *et al.* (1990) showed that the chloroform extract contained one component which contained 23% of activity while the methanol contained four components which together contained 94% of the activity whereas dichloromethane had 56%. The combination of the individual components was more active than the individual components.

4.4 Critical Micelles Concentration (CMC) of the crude lipopeptide obtained from the growth of *B. licheniformis* JF-2 after 8 hours of fermentation in the Optimized Medium in shake flask cultures

The biosurfactant produced by *B. licheniformis* JF-2 showed a significant reduction in surface tension of deionized water to values below 29.0 mN/m. This is comparable to surfactin, a biosurfactant produced by *B. subtilis* (Arima *et al.*, 1968) and *B. licheniformis* JF-2 described by McInerney *et al.* (1985).

The critical micelle concentration (CMC) was determined by measuring the surface tension at various concentrations of the crude lipopeptide biosurfactant at 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240 and 260 mg/L in deionized water. The crude biosurfactant was obtained by acid precipitation of the cell free culture medium after 8 hours growth in the Optimized Medium by *B. licheniformis* Jf-2 at 40°C and shaken at 250 rpm. A graph was plotted with surface tension against surfactant concentration. The slope of the curve changed from a steep non-micellar to a relatively flat curve

where micelles predominated the transition point on the curve which is defined as the CMC. The CMC for the lipopeptide from *B. licheniformis* JF-2 was determined at 165.9 mg/L (Fig. 16).

4.5 Fermentation studies on *B. licheniformis* JF-2 in a 1.5L Biolab Fermenter

4.5.1 The production of biomass and lipopeptide from *B. licheniformis* JF-2 using the optimized medium (OM) in a 1.5L capacity fermenter (B. Braun Biolab)

Initial studies on the growth of *B. licheniformis* JF-2 using the optimized medium (OM) in shake flask cultures for biomass and lipopeptide production (Section 4.2.4) proved to be very successful compared to the growth in Nutrient broth and Medium E. Therefore, a 1.5 litre Biolab fermenter was used to culture *B. licheniformis* JF-2 in order to increase the biomass and crude lipopeptide yield.

The biomass profile of *B. licheniformis* JF-2 grown in the OM showed a lag phase of 1 hour with an average yield of 0.29 g/L (Fig. 17). The log phase began from the 2nd hour onwards and continued up to the 24th hour. The maximum biomass achieved averaged 3.15 g/L which was 48.89% higher than that of 1.61 g/L obtained after 8 hours of growth in the OM conducted in shake flask cultures (Section 4.2.4). Improved mass transfer in the medium obviously contributed to the increase in the biomass production.

The optical density profile was similar to the biomass curve (Fig. 17). Maximum absorbance recorded at the 24th hour averaged 2.90. 4.2.4). Plate 7 shows the amount of cells present during different sampling periods from 0 hour

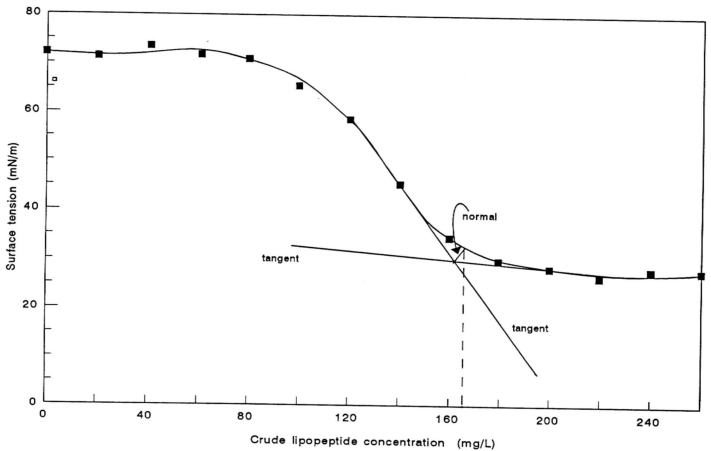


Fig. 16: Critical Micelle Concentrations (CMC) of the crude biosurfactant from *B. licheniformis* JF-2 after 8 hours of growth in the Optimized Medium (OM) at 40^0C and shaken at 250 rpm.

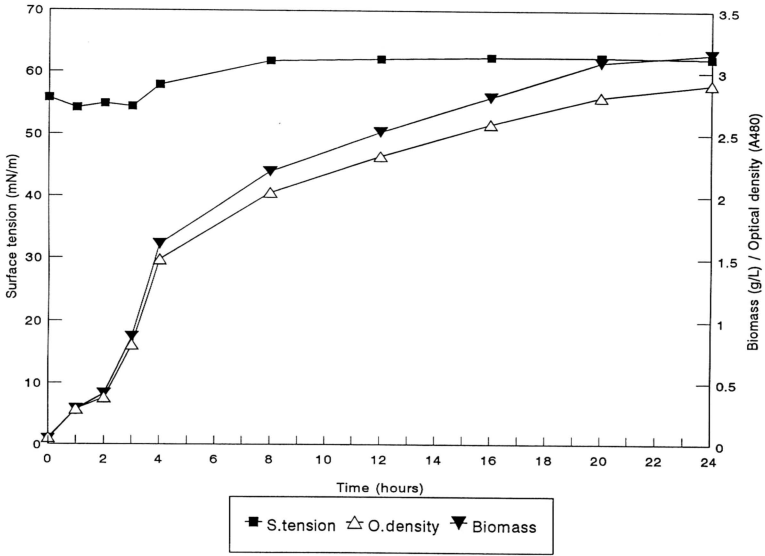


Fig. 17: Biomass, Optical density, pH and Surface tension monitored during the growth of *B. licheniformis* JF-2 using the Optimized Medium (OM) in a 1.5L Biolab fermenter at 40^{0}C, initial pH of 7.0 and agitation speed of 400 rpm. There is no significant drop in the surface tension of the cultivation medium as the lipopeptide biosurfactant is effectively removed from the cultivation medium by the foam fractionation method.

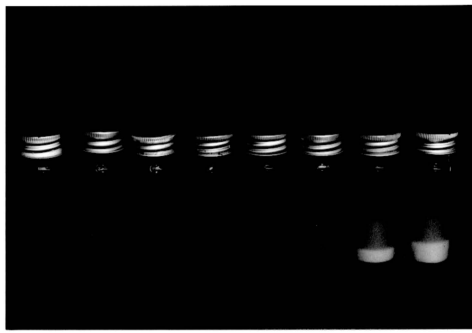


Plate 7: The amount of cells present during different sampling periods (harvest time) from 0 hour to 24 hours (with half an hour of settling). These samples were obtained during the growth of *B. licheniformis* JF-2 in the Optimized medium, using a 1.5L capacity Biolab fermenter at 40^{0}C, with an agitation speed of 400rpm.

Harvest time:

A - 0 hour
B - 2 hours
C - 4 hours
D - 8 hours
E - 12 hours
F - 16 hours
G - 20 hours
H - 24 hours

to the 24 hours in the Optimized medium in a 1.5L capacity Biolab fermenter. There was a gradual decrease in pH (Fig. 17) from an initial of 7.1 to 5.1 during the 24 hours of growth.

The growth of *B. licheniformis* JF-2 in the OM resulted in the synthesis of the lipopeptide which was measured by the drop in the surface tension of the medium. The quantification of the lipopeptide was carried out using the foam fractionation method. Figure 17 shows the surface tension profile of the cultivation medium in a time course study. The initial surface tension averaged 55.8 mN/m. These values decreased to 54.2 and 57.9 mN/m during the first and 4th hour of fermentation, respectively. Subsequently, there was a gradual increase and stabilized at 62.1 and 62.4 mN/m at the 12th and 24th hour, respectively.

The increase in surface tension of the cultivation medium was due to the removal of the lipopeptide through the foam fractionation method (Plate 8). This method removed up to 90% of the lipopeptide from the cultivation medium (Juwarkar, A., Personal communication, 1994). The surface tension of the collapsed foam averaged 28.8 mN/m and the maximum crude lipopeptide obtained from this averaged 2.96 g/L. The crude lipopeptide produced in the 1.5 litre Biolab fermenter showed an increase of 38.51% compared to the maximum yield obtained from shake flask cultures after 8 hours of fermentation.

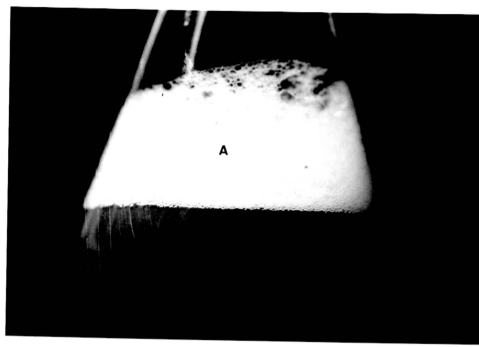


Plate 8: Foam collected by the foam fractionation method during the cultivation of *B. licheniformis* JF-2 using the optimized medium (OM) in a 1.5 litre Biolab fermenter. (A: denotes the foam and B: the collapsed foam, respectively).

4.5.2 Production of the biosurfactant by *B. licheniformis* JF-2 in Mineral salts medium compared to the Optimized medium in a 1.5 litre fermenter (B. Braun Biolab)

The mineral salts medium used by Lin *et al.* (1993) and Juwarkar *et al.* (1994) were designated MSM I (Appendix II) and MSM II (Appendix III), respectively. Fermentation trials which were conducted in a 1.5 litre Biolab fermenter were monoitored by measuring the absorbance (A_{480}) of the cultivation medium (Fig. 18). Growth was superior in the optimized medium (OM) compared to the mineral salts media. The lag phase in the OM lasted 2 hours, with an absorbance of 0.38, after which the log phase continued up to 24 hours, averaging 2.90. This was higher than the absorbance values of between 1.1 to 1.2 obtained by Jenneman *et al.* (1983) for growth in ME.

The lag phase in MSM-I constituted approximately 4 hours at 0.08 absorbance (Fig. 18) while the log phase continued to 0.29 at the 16th hour. Growth of *B. licheniformis* JF-2 was slightly better in MSM-II compared to MSM-I with a lag phase of 4 hours, reaching a maximum absorbance of 1.12 at the 24th hour of fermentation (Fig. 18).

The pH of MSM-I decreased from 6.9 to 6.8 after 24 hours whereas in MSM-II, it decreased gradually to 6.3 from 7.0. However, the optimized medium showed a steep decline in pH from an initial of 7.1 to 5.1 during 24 hours of fermentation (Fig. 19).

The application of the foam fractionation method for isolating the lipopeptide was effective in both the MSM-II and the OM. The initial decrease in surface tension from 55.8 mN/m to 54.4 mN/m in OM during the first 3 hours of

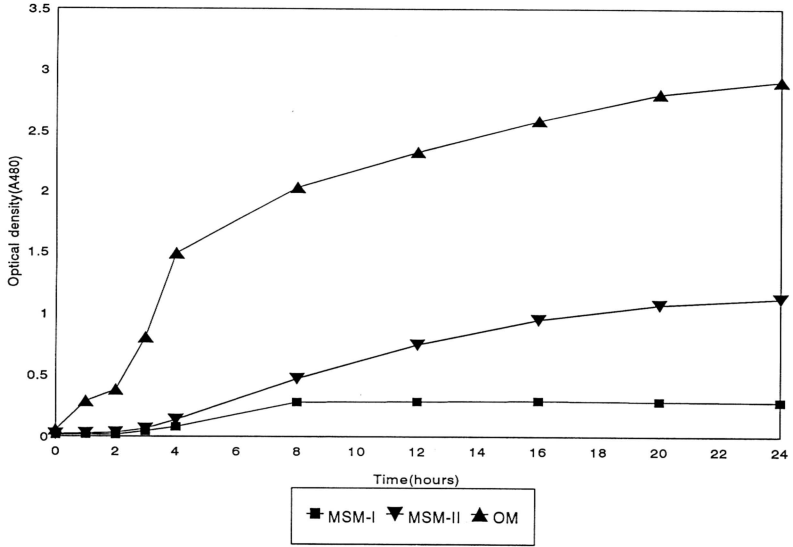


Fig. 18: Optical density profile of the growth of *B. licheniformis* JF-2 in the MSM-I and MSM-II compared to the Optimized Medium (OM) conducted in a 1.5L capacity Biolab fermenter at 40°C and at an agitation speed of 400 rpm.

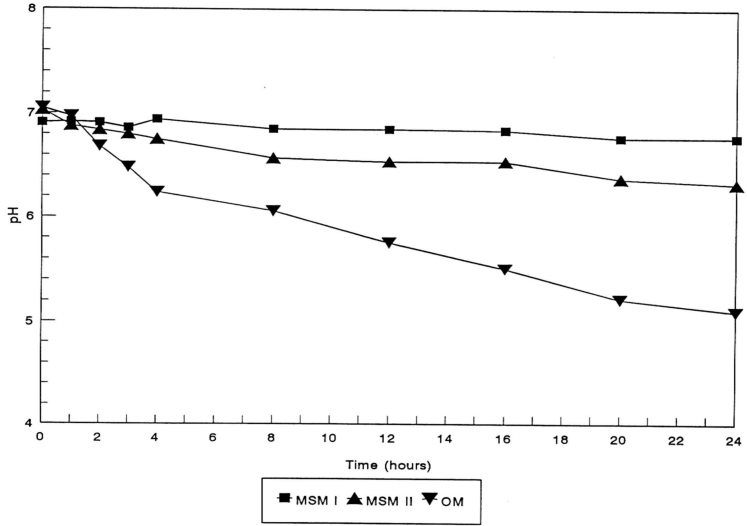


Fig. 19: pH profile of two mineral salts based medium compared to the Optimized Medium (OM) during the growth of *B. licheniformis* JF-2 in a 1.5L capacity Biolab fermenter at 40°C and at an agitation speed of 400 rpm.

fermentation was due to the accumulation of the lipopeptide biosurfactant in the medium, while the subsequent increase in surface tension from the 4th hour which reached an average of 62.2 mN/m (Fig. 20) at the 24th hour was due to the removal of the biosurfactant from the medium through the foam fractionation method. Similar findings were observed for the MSM-II while this was not the case in the MSM-I (Fig. 20). Our observation had shown that very little foam was produced during the growth of *B. licheniformis* JF-2 in MSM-I indicating that very little biosurfactant was present in the foam. This made the foam fractionation method ineffective as very little foam was collected during the fermentation process. The ineffective removal of the biosurfactant through the foam was detected by the decrease in the surface tension of the MSM-I in the fermentation vessel (Fig. 20). The surface tension of the MSM-I decreased from an average value of 71.6 to 35.9 mN/m after 8 hours of growth and thereafter increases gradually to 45.6 mN/m at the 24th hour.

The crude lipopeptide yield in the OM which averaged 2.96 g/L was higher than that in the MSM media (Table 5). This resulted in a lower surface tension measurement averaging 28.8 mN/m compared to 31.8 and 30.0 mN/m in MSM-I and MSM-II, respectively. The lipopeptide yield in the OM was higher to that in the MSM media. Also, the crude lipopeptide yield in the OM is the highest reported to date from literature search.

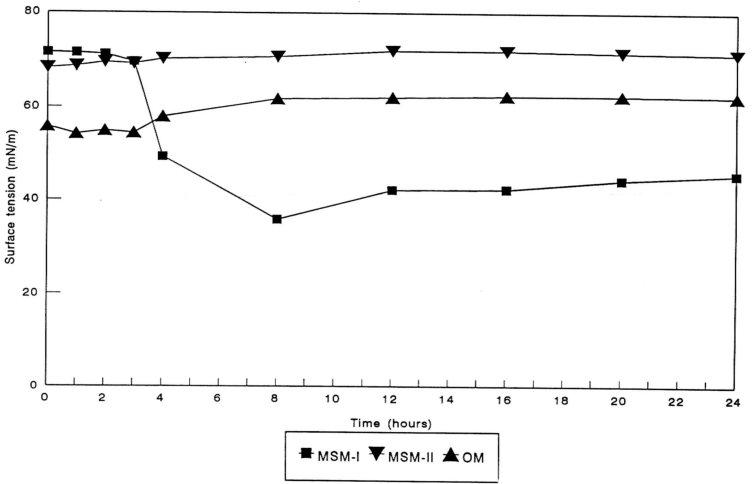


Fig. 20: Surface tension profile of two mineral salts based medium compared to the Optimized Medium (OM) during the growth of *B. licheniformis* JF-2 in a 1.5L capacity Biolab fermenter at 40 ^{0}C and at an agitation speed of 400 rpm. In both the Optimized Medium (OM) and MSM-II, there was no drop in the surface tension measured due to the effeicient removal of the lipopeptide biosurfactant by the foam fractionation method whereas for the MSM-I, less foam was produced and the foam fractionation method was ineffective.

Table 5: Surface tension measurements of the collapsed foam and the crude lipopeptide yield from the various media from the B. Braun Biolab fermenter studies.

Medium	Crude lipopeptide yield (g/L)	Surface tension (mN/m)
MSM-I	0.22	31.8
MSM-II	0.55	30.0
OM	2.96	28.8

4.6 Media formulation with locally available agro-industrial by-products for the cultivation of *B. licheniformis* JF-2

The annual output of agro-industrial by-products in the Asia-Pacific region is approximately 513 million tonnes (Davendra, 1990). Palm oil mill effluent (POME) is essentially a non-toxic by-product comprising 70% clarification sludge, 19% sterilizer condensate and 11% hydrocyclone washing. For each tonne of oil palm fruit processed, the estimated volume of POME produced is 0.8 m^3 (Lim & Chin, 1990).

In 1990, Malaysia contributed 25.9% of the total world production. Rubber Effluent (RE) is derived from process water and latex serum generated from latex concentrate, and skim rubber processing plants (Lim & Chin, 1990). Approximately, 100 million litres of effluent are discharged daily (Yap *et al.*, 1983).

Table 6 shows the characteristics of the various locally available wastewater for the cultivation of *B. licheniformis* JF-2. The utilization of locally available wastewater was aimed at reducing the cost of production of the biomass and the biosurfactant. This may be more attractive for commercial applications such as wastewater treatment, oil spill clean-up and microbial enhanced oil recovery.

Table 6: The characteristics of selected effluents used for the cultivation of *B. licheniformis* JF-2 in the shake flasks studies.

Parameters	POME	Rubber effluent	Dairy wastewater
COD (mg/L)	52,000	3520	2250
pH	4.5	5.2	5.6
Nitrogen (mg/L)	3.5	52.0	56.8
Phosphate (mg/L)	3.2	5.6	9.1
Total sugar (mg/L)	12.2	22.5	136.5
Reducing sugar (mg/L)	1.9	15.5	122.0

4.6.1 Palm oil mill effluent (POME) as a growth medium for *B. licheniformis* JF-2

Over the last 3 decades, the palm oil industry has attained a very important placed in Malaysian agriculture. Currently, there are more than 1.95 million hectares of land under oil palm or about one third of the total cultivated area in Malaysia (Ma & Hassan, 1990). Palm oil mill effluent is a liquid waste produced

during the extraction of palm oil (Jalaludin *et al.*, 1990). The COD of POME is between 15,500-106,360 mg/L; total solids between 11,450-164,950 mg/L, suspended solids averaging 30,385 mg/L and total nitrogen averaging 4.2 mg/L (Ma & Hassan, 1990). An attempt was made to utilize POME for the cultivation of *B. licheniformis* JF-2 and to determine its suitability for the production of the biosurfactant.

1 mL of the standardized inoculum was inoculated directly into 49 mL of sterilized palm oil mill effluent and incubated at 40°C for 8 hours. This study showed that palm oil mill effluent was apparently not a suitable medium for the cultivation of *B. licheniformis* JF-2. This was evident from the lack of increase in biomass yield (Fig. 21). The presence of suspended solids POME made it difficult to obtain homogenous samples for analyses. Table 7 describes the reducing sugar profile of the diluted (1:10) POME with *B. licheniformis* JF-2. There was a decrease in the reducing sugar concentration from an initial value of 1175.0 mg/L at the onset of fermentation to a value averaging 875.0 mg/L after 8 hours (Table 7). This amounted to a reduction of 25.53% in the reducing sugar concentration.

Table 7: The reducing sugar profile of POME during the growth of *B. licheniformis* JF-2 for 8 hours in shake flask cultures.

Fermentation (h)	Reducing sugar (mg/L)
0	1175.0
1	1100.0
2	1125.0
3	1100.0
4	1050.0
5	1025.0
6	975.0
7	950.0
8	875.0

The biomass studies showed that POME was a poor growth medium and as such there was very little change in pH and surface tension (Fig. 21) of the cultivation medium during this study.

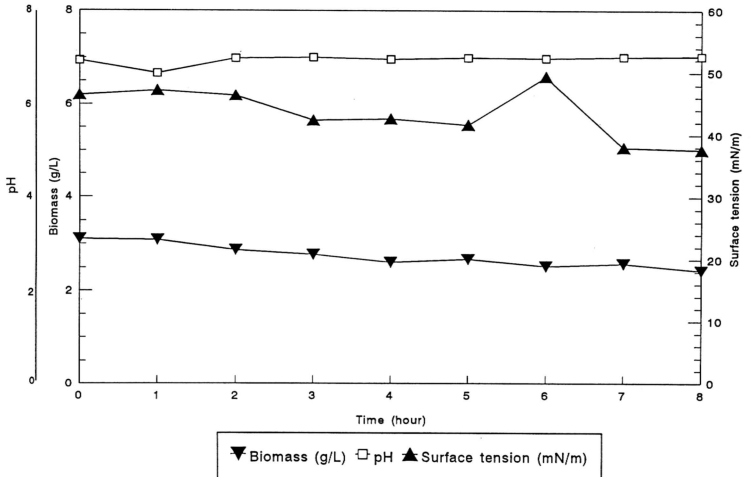


Fig. 21: The various paramenters measured during the growth of *B. licheniformis* JF-2 in palm oil mill effluent at 40C and shaken at 250 rpm in an orbital type incubator shaker.

4.6.2 Rubber effluent (RE) as a growth medium for *B. licheniformis* JF-2

The rubber processing industry has been identified as one of the major sources of organic pollution in Malaysia. 100 million litres of effluent containing 200 tonnes of BOD are discharged daily from this industry (John and Ong, 1979). Thus, an attempt was made to utilize rubber effluent as a substrate for the production of the biosurfactant. This could lead to a possible route for wastewater treatment with a potential of generating a product (Kosaric, 1992).

Rubber effluent at two dilutions; 1:100 (v/v) and 1:1000 (v/v) with distilled water was used for the cultivation of *B. licheniformis* JF-2. From this study, we found that rubber effluent was not a particularly suitable medium for the growth of *B. licheniformis* JF-2. The average values obtained for the maximum biomass yields were 0.07 g/L for 1:100 dilution at the 8th hour and 0.06 g/L for the 1:1000 dilution after 7 hours of fermentation, respectively (Fig. 22). Reducing sugar was utilized up to 40.0% in 1:100 dilution compared to 19.1% in the 1:1000 (Table 8 and Fig. 23) after 8 hours of growth.

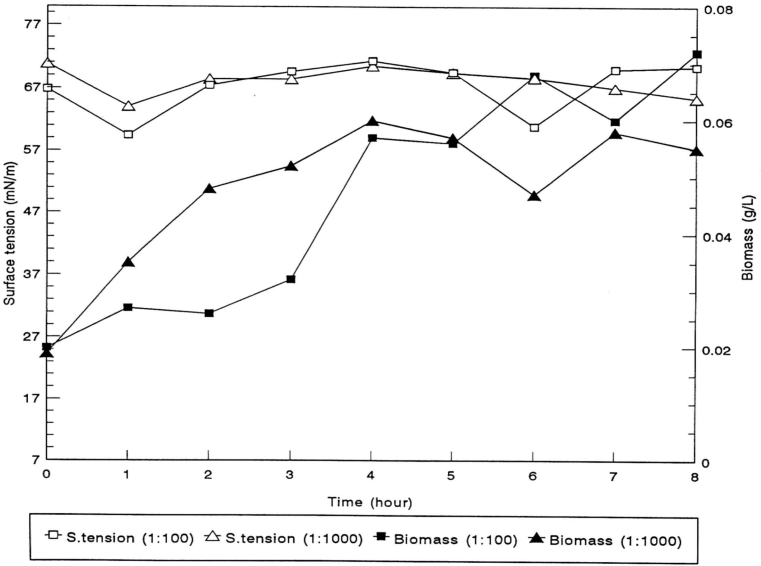


Fig. 22: Surface tension and Biomass profiles of *B. licheniformis* JF-2 during 8 hours of growth in rubber effluent diluted with distilled water in shake flasks at 40°C in an orbital type incubator shaker at 250 rpm.

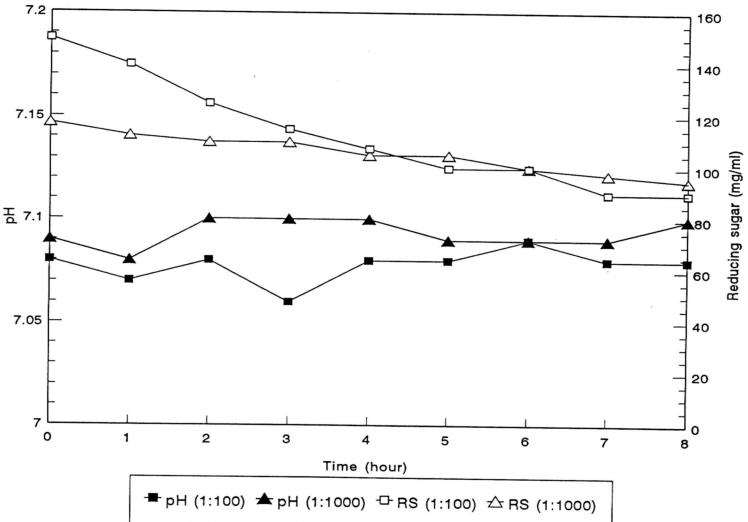


Fig. 23: pH and Reducing sugar (RS) profiles of the diluted rubber effluent during 8 hours of growth of *B. licheniformis* JF-2 in shake flasks at 40°C and shaken at 250 rpm.

Table 8: Percent reduction of the reducing sugar in Rubber effluent during the growth of *B. licheniformis* JF-2 in shake flasks after 8 hours of fermentation at 40°C and shaken at 250 rpm.

RE dilutions	% Reduction in reducing sugar
1:100	40.0
1:1000	19.1

There was very little change in pH in the cultivation medium during the 8 hours of growth of *B. licheniformis* JF-2. This varied between 7.0 and 7.1 for the 1:100 and 1:1000 dilution, respectively (Fig. 23).

The surface tension of the diluted rubber effluent (1:100) decreased from an intial average value of 66.8 mN/m to 60.8 mN/m at the 6th hour and subsequently increased to 70.4 mN/m at the 8th hour of fermentation. The surface tension profile of the 1:1000 diluted rubber tension profile of the 1:1000 diluted rubber effluent showed a similar trend (Fig. 22). The initial drop in the surface tension of the cultivation media occurred at the first hour of fermentation from an initial average value of 70.8 mN/m to 63.9 mN/m and from then onwards, the surface tension increased to 70.5 mN/m at the 4th hour of fermentation. Then, it gradually decreases to 65.3 mN/m at the 8th hour of fermentation.

4.6.3 Dairy wastewater as a cultivation medium for *B. licheniformis* JF-2

Locally available dairy wastewater obtained from Premier Milk (M) Sdn. Bhd., a factory producing sweetened condensed milk from powdered milk was used as a cheap source of raw material for the cultivation of *B. licheniformis* JF-

2. This wastewater performed better than rubber effluent and palm oil mill effluent. Dairy wastewater was used at 3 different concentrations to determine the growth and production of biosurfactant by *B. licheniformis* JF-2. The maximum biomass production averaged 0.71 g/L for the undiluted wastewater after 8 hours of fermentation compared to 0.25 g/L and 0.13 g/L for 1:1 and 1:10 dilutions, respectively (Fig. 24). The increase in biomass was concomitant with a decrease in the reducing sugar and pH during the course of the fermentation (Table 9 and Fig. 25). Reducing sugar was measured instead of lactose because of the short log phase (approximately 5 hours) during the growth of *B. licheniformis* JF-2 in the dairy wastewater. Reducing sugar was measured because sugar was added in the sweetened condensed milk process which will readily be utilized first before lactose from the milk during the short fermentation period of 8 hours.

Table 9: Reducing sugar concentrations in various dilutions of dairy wastewater during the growth of *B. licheniformis* JF-2 in shake flasks at 40°C and shaken at 250 rpm.

Time (h)	Reducing sugar (mg/ml)		
	Dilution 1:0	Dilution 1:1	Dilution 1:10
0	1485.0	692.5	135.0
1	1441.0	655.0	135.0
2	1300.0	627.5	132.5
3	1250.5	692.5	120.0
4	1302.5	747.5	117.5
5	1191.0	645.0	117.5
6	1227.5	635.0	122.5
7	1212.5	622.5	120.0
8	1190.0	455.0	105.0

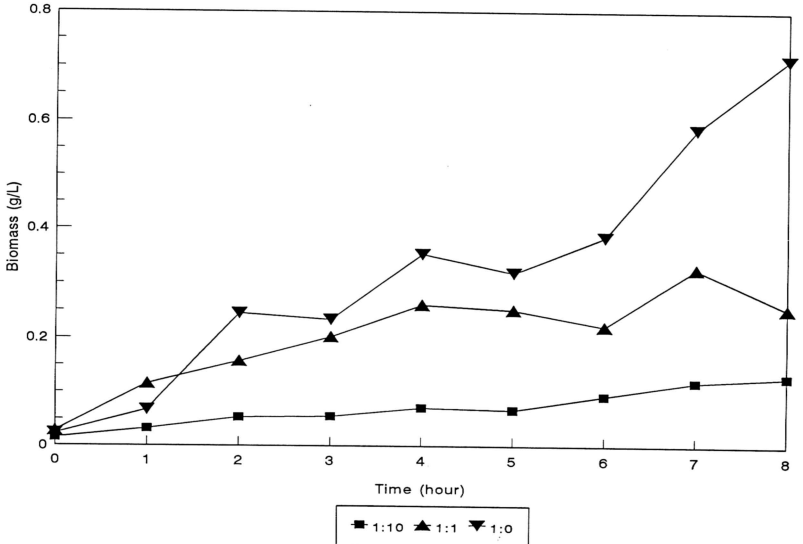


Fig. 24: Biomass of *B. licheniformis* JF-2 during the growth in various dilutions of dairy wastewater in shake flasks at 40^{0}C, initial pH of 7.0 and shaken at 250 rpm (diluted with distilled water to dilutions of 1:10, 1:1 and 1:0).

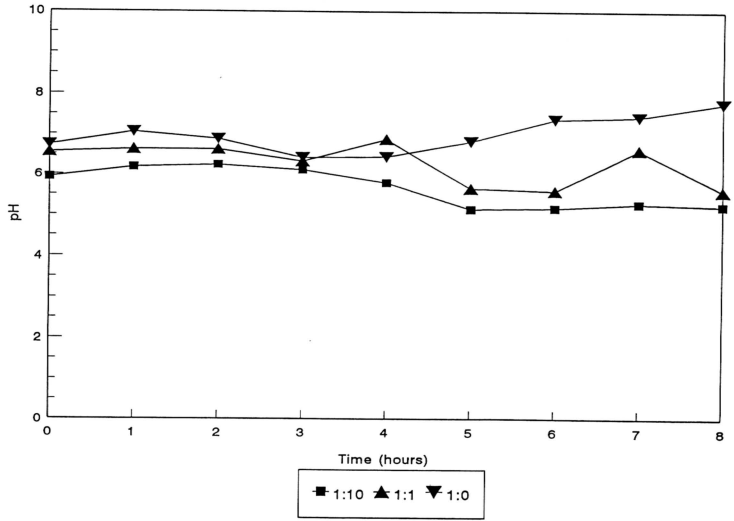


Fig. 25: pH profiles of the various dilutions of dairy wastewater supporting the growth of *B. licheniformis* JF-2 during 8 hours of fermentation at 40^{0}C, initial pH of 7.0 and shaken at 250 rpm.

In the 1:1 dilution medium reducing sugar utilized amounted to 34.32%. The sugar content decreased from an average value of 692.5 mg/L to 455.0 mg/L, whereas a decrease of 22.22% was observed for the 1:10 dilution and 19.86% for the undiluted (1:0) medium. The initial variation in the concentration of reducing sugar amounting to 1485.0 mg/L, 692.5 mg/L and 135.0 mg/L were due to the dilutions with distilled water for the preparation of the three different concentrations. Thus, the representation with percentages clarified the process characteristics. There was very little change in pH during the growth of *B. licheniformis* JF-2 in the dairy wastewater. The pH decreased gradually to about 5.0 and stabilized subsequently (Fig. 25). The lowest surface tension of 38.8 mN/m was recorded for the undiluted dairy wastewater at the 7th hour of fermentation whereas the 1:1 and 1:10 dilutions had the lowest surface tension values averaging 40.9 and 45.2 mN/m at the 7th and 5th hour of fermentation, respectively (Fig. 26). Subsequently, there was an increase to values averaging 39.4, 44.5 and 49.7 mN/m for the 1:0, 1:1 and 1:10 dilutions, respectively at the 8th hour of fermentation.

4.6.4 Use of sugar cane molasses as an additional carbon source in dairy wastewater for cultivation of *B. licheniformis* JF-2

Molasses, a by-product of the sugar cane industry was used as a cheap, additional carbon source to dairy wastewater to enhance the growth of *B. licheniformis* JF-2. Cane molasses consists of 3% (w/v) reducing sugar and 45.4% (w/v) sucrose (Sasidharan, 1995). At a concentration of 0.5% (v/v) in the cultivation medium, it enhanced the growth of the culture by increasing the number of colonies to 3.29×10^8 CFU/ml (Table 10). This was higher than the

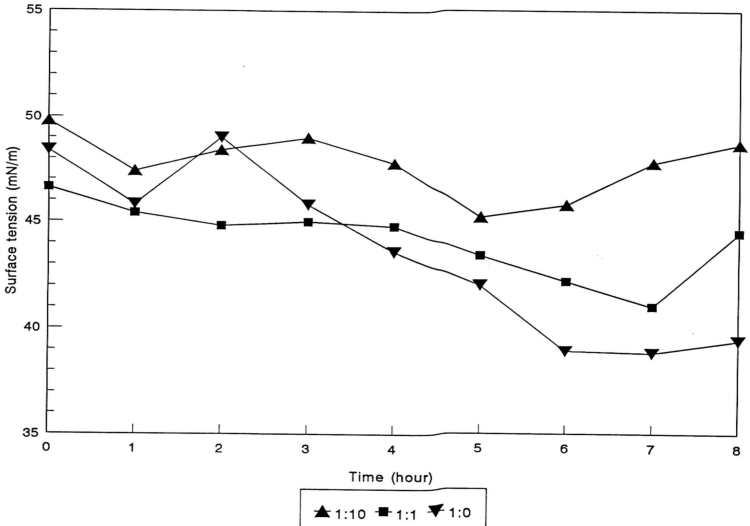


Fig. 26: Surface tension profiles of the various dilutions of dairy wastewater supporting the growth of *B. licheniformis* JF-2 during 8 hours of growth in shake flasks at 40°C, initial pH of 7.0 and shaken at 250 rpm.

control which managed at 5.00 x 10^5 CFU/ml after 8 hours of fermentation (Plate 9). Colony forming unit was used instead of dry cell weight (biomass) because of the presences of suspended particals in the cane molasses and dairy wastewater. This made it difficult to determine the actual cell biomss. Colony forming units was found to give a better indication of cell growth in the cultivation media. The increase in the CFU/ml was directly related to the reduction in surface tension of the growth medium (Table 11), measured at 34.6 which was lower than the control (37.0 mN/m).

Table 10: Colony Forming Units (CFU) of *B. licheniformis* JF-2 grown on dairy wastewater supplemented with molasses after 8 hours of fermentation in shake flask culture. 1mL of the cultivation medium is serially diluted in sterile distilled water and plated on Nutrient agar plates and left to incubate for 24 hours at 30°C.

% Molasses	Colony Forming Units/mL
0	5.00 x 10^5
0.1	5.40 x 10^5
0.2	1.56 x 10^6
0.3	6.00 x 10^5
0.4	1.23 x 10^6
0.5	3.29 x 10^8

Table 11: Surface tension of cultivation medium consisting of molasses after 8 hours of fermentation in shake flasks at 40°C and shaken at 250 rpm

% Molasses	Surface tension (mN/m)
0	37.0
0.1	36.1
0.2	35.6
0.3	35.8
0.4	35.8
0.5	34.6

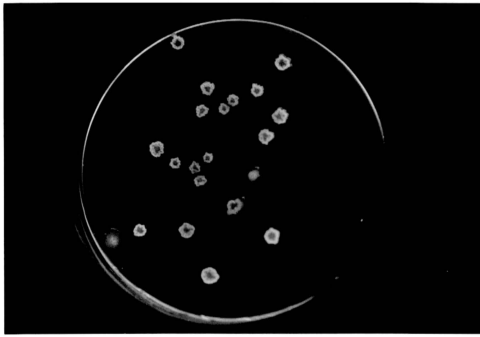


Plate 9: Colony Forming Units of *B. licheniformis* JF-2 per ml of dairy wastewater supplemented with molasses, grown on Nutrient agar plate after 24 hours of incubation at 30°C. A slight contamination is observed at the bottom left hand corner of the photograph.

The increase in the concentration of molasses resulted in a concomitant decrease in pH after 8 hours of fermentation. The maximum pH recorded was 6.88 in the control and the minimum was 6.7 at 0.5% (v/v) molasses (Table 12).

Table 12: pH of the dairy wastewater with molasses, recorded during the growth of *B. licheniformis* JF-2 after 8 hours of fermentation at 40°C, 250 rpm and at an initial pH of 7.0.

% Molasses	Final pH
0	6.88
0.1	6.82
0.2	6.79
0.3	6.76
0.4	6.74
0.5	6.71

Thus, dairy wastewater supplemented with 0.5% (v/v) molasses was found to be suitable for the growth of *B. licheniformis* JF-2. The highest number of CFU per ml averaged 3.29 x 10^8 with the lowest surface tension recorded at an average value of 34.6 mN/m.

4.6.5 Use of Fish Meal (FM) as a nitrogen source for the cultivation of *B. licheniformis* JF-2

An attempt was made to include Fish Meal (FM) as a cheap source of nitrogen in dairy wastewater for the cultivation of *B. licheniformis* JF-2. The addition of FM in the medium enhanced the growth which was measured by Colony Forming Units per ml.

The change in pH of the dairy wastewater supplemented with FM related to the growth of *B. licheniformis* JF-2 is shown in Table 13. The pH of the cultivation medium containing various concentrations of FM decreased to 6.6 after

8 hours of fermentation which was lower than the control which showed a final pH averaging 6.9.

Table 13: pH of the cultivation medium consisting of dairy wastewater and fish meal after 8 hours of fermentation with *B. licheniformis* JF-2 at 40°C and shaken at 250 rpm.

Percentage Fish Meal	Final pH
0	6.9
1	6.6
2	6.6
3	6.6
4	6.6
5	6.6

An increase in the concentration of FM resulted in an increase in the growth of *B. licheniformis* JF-2 which was measured as colony forming units (CFU) per ml. Fish meal at 5% (w/v) registered a colony count averaging 3.09 x 10^8 which was much higher than the control which achieved a colony count averaging 5.00 x 10^4 (CFU/ml) (Table 14).

Table 14: Colony Forming Units (per ml) of *B. licheniformis* JF-2 obtained from the cultivation medium consisting of dairy wastewater supplemented with FM after 8 hours of incubation at 40°C and shaken at 250 rpm.

Percentage Fish Meal	Colony Forming Units / mL (CFU/mL)
0	5.00×10^4
1	8.60×10^5
2	1.56×10^6
3	7.00×10^6
4	1.77×10^7
5	3.09×10^8

Increase in the colony count with fish meal resulted in a reduction in the surface tension of the dairy wastewater (Table 15). The maximum reduction achieved at 5% (w/v) FM was to a value averaging 30.9 mN/m which was lower than the control which registered an average value of 38.3 mN/m.

Table 15: Surface tension of the cultivation medium supplemented with FM, after 8 hours of fermentation in shake flask culture at 40°C and shaken at 250 rpm.

Percentage Fish Meal	Surface tension (mN/m)
0	38.3
1	34.2
2	33.2
3	32.6
4	32.8
5	30.9

Locally available wastewater was used for the cultivation of *B. licheniformis* JF-2. This was aimed at reducing the cost of the biomass production. Undiluted dairy wastewater obtained from the processing of sweetened condensed milk from powdered milk was found to be suitable for the cultivation of *B. licheniformis* JF-2. The maximum biomass obtained after 8 hours of fermentation at 40°C averaged 0.71 g/L (Fig. 24). The production of the lipopeptide biosurfactant was detected in the broth by the reduction of the surface tension of the cultivation medium to an average value of 38.8 mN/m by the 7th hour of fermentation. Growth was very poor in both rubber effluent and palm oil mill effluent. Further studies were than conducted with only undiluted dairy wastewater.

The addition of sugar cane molasses and fish meal were to supplement the

dairy wastewater with a carbon and nitrogen source, respectively. Due to the presence of particals in sugar cane molasses and fish meal, biomass was not accurately determined. Therefore, Colony forming unit was used to determine the growth of *B. licheniformis* JF-2. Sugar cane molasses and fish meal were found to enhance the growth of *B. licheniformis* JF-2. The addition of 5% (v/v) of cane molasses in the dairy wastewater resulted in the number of colonies to increase to an average value of 3.29 x10^8 CFU/mL as compared to the control which showed a colony count of 5.00 x10^5 CFU/mL (no cane molasses was added). The surface tension of the 5% (v/v) containing cane molasses medium showed an average value of 34.6 mN/m as compared to the control which measured 37.0 mN/m.

Dairy wastewater supplemented with fish meal as a nitrogen source showed similar results. Increasing concentration of fish meal in the medium resulted in the increase in the number of colony count. Dairy wastewater at 5% (w/v) concentration of fish meal showed a colony count of 3.09 x10^8 CFU/mL as compared to the control (no fish meal added) which showed a colony count of only 5.00 x10^4 CFU/mL. The medium with the highest concentration of fish meal at 5% (w/v) recorded the lowest surface tension averaging 30.9 mN/m as compared to the control which measured 38.3 mN/m. The lowering of the surface tension of the cultivation medium clearly showed the presence of the lipopeptide biosurfactant from *B. licheniformis* JF-2.

4.7 Application of the biosurfactant in the removal of crude oil from solid surfaces.

An attempt was made to determine the ability of the lipopeptide biosurfactant to disperse crude oil (Vietnam crude from Petronas, Malaysia) from solid glass surfaces. A set of beakers of 250 ml capacity were each lined with a

mixture containing 30 ml of crude oil to which was added 30 ml of an 8 hour culture of *B. licheniformis* JF-2 grown in the optimized medium (OM). This was compared to another set of beakers which contained 30 ml of crude oil mixed with 30 ml of uninoculated sterilized OM (Plate 10).

Emulsification occurred due to the presence of the lipopeptide biosurfactant which resulted in the settling of oil from the surface of beakers in set B to the bottom compared to the control (Set A) which did not show any settling of the oil.

4.8 *In-situ* application of *B. licheniformis* JF-2 compared to a bioaugmentation culture (JAD 969P) for the treatment of edible palm oil processing wastewater.

Wastewater containing 10ml of waste palm oil from an edible palm oil processing factory (Lam Soon, Petaling Jaya, Selangor) was treated aerobically with *B. licheniformis* JF-2 for a period of 15 days. The initial COD of the wastewater was between 2120-2125 mg/L. Initially, the COD in the *B. licheniformis* JF-2 treated wastewater increased to a maximum value averaging 4540 mg/L (Fig. 27) by the 3rd day of treatment compared to the control which achieved a maximum value averaging 3960 mg/L on the 5th day of treatment. This increase may be attributed to the emulsification of the oil which was dispersed in the medium. Subsequently, there was a decrease to an average value of 750 mg/L in the JF-2 treated wastewater and 1420 mg/L in the control. This featured a reduction of 65.3% in COD for the JF-2 treated wastewater and 33.0% for the control (C1). The growth of *B. licheniformis* JF-2 in the palm oil

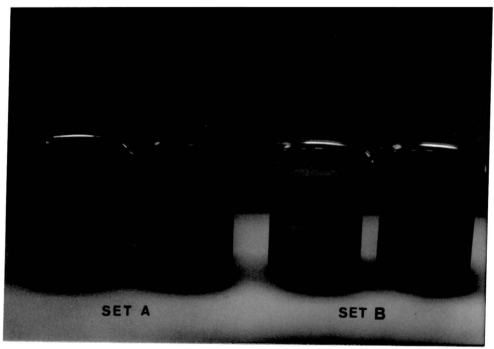


Plate10: Emulsification of crude oil (Vietnam crude) by the culture of *B. licheniformis* JF-2 grown in OM. Set A (duplicate) consisted of 30 ml of sterile OM (control) and 30 ml of crude oil while set B contined 30 ml of a 8 hour old culture of *B. licheniformis* JF-2 with 30 ml of crude oil. The contents were shaken for 1 hour in an orbital incubator shaker at 250 rpm at 40°C.

processing wastewater produced biosurfactant which was monitored by measuring the surface tension of the wastewater which decreased by 49.3% compared to 17.9% in the control (C1) (Fig. 28).

The JAD 969P which consisted of a commercially available booster culture complex (containing *B.subtilis*, *B.licheniformis*, *Pseudomonas aeruginosa*, *P.stutzeri*, *P.fluorescens* and *Escherichia coli*) was inoculated into the palm oil processing wastewater which contained 10ml of waste palm oil which was acclimatized with activated sludge. The control (C2) consisted of the wastewater with 10ml of the waste palm oil acclimatized with the activated sludge. An 8 day treatment period was sufficient to reduce 85.7% of the COD from 2720 to 390 mg/L for the control (C2) whereas the wastewater with the JAD 969P culture complex showed a 94.0% reduction from 2760 to 166 mg/L (Fig. 27). The monitoring on the reduction of the COD for the JAD 969P bioaugmentation culture complex was stopped by the 8th day as it was found that the rate of the COD removal from the palm oil processing wastewater was declining gradually when compared to the *B.licheniformis* JF-2. The increased efficiency of the JAD 969P culture complex in reducing the COD of the wastewater lies in the amount or concentration of waste palm oil present which was 10 times less than the wastewater treated by *B.licheniformis* JF-2. The commercially available bioaugmentation culture complex with the activated sludge was found to treat the palm oil processing wastewater more efficiently than the *B. licheniformis* JF-2 culture. The interesting finding here is that *B. licheniformis* JF-2 can be used for treating palm oil processing wastewater containing higher concentrations of waste palm oil with a COD removal efficiency of 65.3% for a treatment period of 15 days.

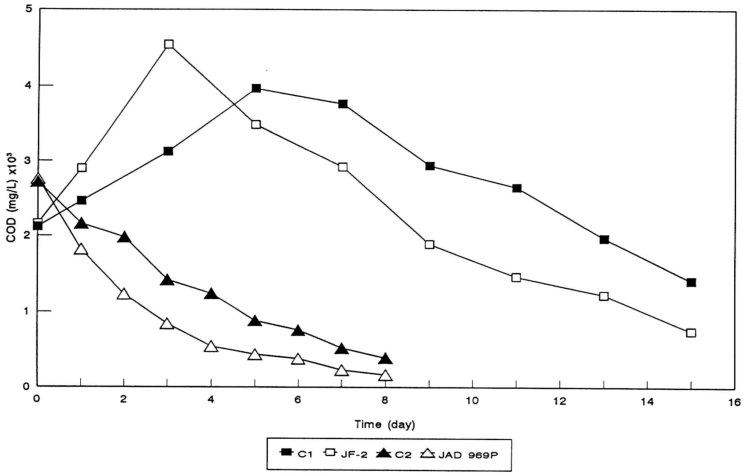


Fig. 27: COD profiles of the edible palm oil processing wastewater treated with *B. licheniformis* JF-2 and JAD 969P bioaugmentation culture complex at room temperature and with an air flow rate of 1000 cm^3/min (C1 - control for the JF-2 treated wastewater; C2 - control for JAD 969P bioaugmentation culture complex). [C1 and JF-2 contains 100ml of waste palm oil; C2 and JAD 969P 10ml of waste palm oil].