5.0 DISCUSSION

5.1 Process Optimization

The objective of the process optimization study was to increase the biomass and biosurfactant yield of *B. licheniformis* JF-2. This was achieved by modifying the cultivation medium (Nutrient broth) until the maximum yield of the biosurfactant and biomass was obtained. The initial temperature and pH studies in shake flasks using Nutrient broth showed that the maximum growth in terms of biomass and biosurfactant yield was achieved at the 8th hour of fermentation at 40^{0}C, pH of 7.0 and shaken at 250rpm. Media optimization studies were then carried out to increase the yields of biosurfactant and biomass.

It has been well established that the physiology of a microorganism is not only determined by its genetic information but is also a function of the environmental conditions (Harder & Dijkuizen, 1983; Meyer *et al.*, 1985). Medium composition plays an important role in the production of surface active compounds by many microorganisms. Minor differences in the amount of critical nutrients may change the limiting nutrient and therefore influence the performance of the cells (Jenny & Deltrieu, 1993).

The intial study on the growth of *B. licheniformis* JF-2 was carried out using Medium E, with an initial pH of 7.0 and a temperature of 40^{0}C. Table 16 shows the maximum absorbance (A_{480}) measured during the shake flask fermentation.

Table 16 : The absorbance of Medium E and Nutrient broth supporting the growth of *B. licheniformis* JF-2, with an initial pH of 7.0, at 40^{0}C and shaken at 250 rpm in an orbital type incubator shaker.

Cultivation Medium	Maximum Absorbance (A_{480})	Fermentation Period (hours)
Medium E	1.02	20
Nutrient broth	1.14	8

Our study showed that Medium E was a poor growth medium for *B. licheniformis* JF-2. 20 hours is required to achieve maximum growth in Medium E (Fig. 15) as compared to only 8 hours in Nutrient broth (Fig. 7). The absorbance measured in Medium E at the 8th hour of fermentation was 0.58 (Fig. 15) and 1.02 at the 20th hour whereas in Nutrient broth, it was measured at 1.14 (Fig. 7) at the 8th hour of fermentation. The use of Nutrient broth as the cultivation medium of *B. licheniformis* JF-2 had shortened the fermentation period by 12 hours, from 20 hours (Medium E) to 8 hours (Nutrient broth), to acheive maximum biomass, measured in terms of absorbance (A_{480}). Therefore, Nutrient broth was selected over Medium E for media optimization studies.

Table 17 shows the biomass and crude lipopeptide yield after 8 hours of growth in the various modified Nutrient broth and finally to arrive at an optimized cultivation medium termed as the Optimized Medium.

Table 17: The biomass and crude biosurfactant production after 8 hours of growth in various modified Nutrient broth media at 40°C, initial pH of 7.0 and shaken at 250 rpm in shake flasks.

Cultivation Medium	Biomass (g/L)	Crude lipopeptide yield (g/L)
NB	1.14	0.22
NB + 3% NaCl	1.40	0.31
NB + 5% glucose + 3% NaCl	0.97	1.73
NB + 0.3% YE	1.24	0.89
Optimized Medium: NB + 0.3% YE + 3% NaCl + 5% glucose	1.61	1.82

Studies by Jenneman *et al.* (1983) on the growth and biosurfactant production by *B. licheniformis* JF-2 in a sucrose minimal medium under the influence of high NaCl concentrations at different pH did not show any significant improvement in biosurfactant formation. Javaheri *et al.* (1985) investigated the biosurfactant production of *B. licheniformis* JF-2 under anaerobic conditions with glucose as the carbon source. Yeast extract and sodium nitrate were also found to be essential for anaerobic growth. However, no significant differences to aerobic biosurfactant production was observed.

Nutrient broth used as a basal medium during the temperature and pH optimization studies showed a yield averaging 1.14g/L and 0.22g/L for biomass and crude lipopeptide yield, respectively (Table 17). The addition of 3% NaCl into the cultivation medium (Nutrient broth) brought about a marginal increase in the biomass and crude lipopeptide yield which averaged 1.40 g/L and 0.31 g/L, respectively. A marginal increase of 22.8% for biomass and 40.9% for the crude

lipopeptide yield was achieved when compared to the yields obtained when *B. licheniformis* JF-2 was grown in Nutrient broth (Table 17). When 5% (w/v) glucose was added to the cultviation medium containing Nutrient broth and 3% NaCl, the biomass and crude lipopeptide yields was averaging 0.97 g/L and 1.73 g/L,respectively.

Yeast extract has been commonly used as a nitrogen source (Hommel *et al.*, 1987). Jenneman *et al.* (1985) showed that the presence of yeast extract is essential for the growth of *B. licheniformis* JF-2 in modified Medium E. Therefore, the next stage in the media optimization process, yeast extrat was added to the Nutrient broth. 0.3% (w/v) yeast extract was found to be the critical concentration which showed a biomass yield of 1.24 g/L and a crude lipopeptide yield of 0.89 g/L. The crude lipopeptide yield increased by 0.67 g/L when compared to the yield of 0.22 g/L obtained from the growth of *B. licheniformis* JF-2 in Nutrient broth.

From our optimization studies, the formulated Optimized Medium consisted of Nutrient broth, 0.3% (w/v) yeast extract, 3% (w/v) NaCl and 5% (w/v) glucose, produced the highest yields in terms of biomass and crude lipopeptide averaging 1.61 g/L and 1.82 g/L, respectively. When compared to the yields of the Optimized Medium with Nutrient broth, the biomass increased by 0.47 g/L and crude lipopeptide by 1.6 g/L. The combination of the critical concentrations of yeast extract, sodium chloride and glucose could have triggered the production of the lipopeptide biosurfactant. This study showed that the biomass and the crude lipopeptide yield are the highest reported thus far in the literature.

5.2 Scale-up to a 1.5L capacity Biolab fermenter.

Scale-up to a 1.5L capacity B. Braun Biolab fermenter studies were carried

out to enhance the biomass production measured in terms of absorbance and crude biosurfactant yield using the Optimized Medium. This was then compared to the growth of *B. licheniformis* JF-2 in Mineral Salt Medium I (MSM-I) and Mineral Salt Medium II (MSM-II). The results of the experiments are summarized in Table 18.

Many microorganisms release the biosurfactants into the culture medium either at the stationary phase or throughout the exponential phase (Lin, 1996). In our shake flask studies using the Optimized Medium, the production of biosurfactant begins at the mid exponential phase which was detected by the drop in the surface tension of the culltivation medium and quantification by obtaining the crude lipopeptide biosurfactant through the acid precipitation method.

Table 18 depicts the maximum absorbance and crude lipopeptide yield obtained from 3 different mediums. In this study, the Optimised Medium proved to be a superior medium compared to MSM-I and MSM-II in terms of biomass (measured as absorbance) and crude lipopeptide yield.

Table 18: The scale-up to a 1.5L capacity Biolab fermenter using various cultivation medium at 40°C, an initial pH of 7.0 and with an impeller speed of 400 rpm.

Medium	Absorbance (A_{480})	Crude lipopeptide yield (g/L)
OM	2.90	2.96
MSM-I	0.29	0.22
MSM-II	1.12	0.55

OM : Optimized Medium
MSM-I : Mineral Salt Medium I
MSM-II : Mineral Salt Medium II

The maximum biomass achieved averaged 3.15 g/L (Section 4.5.1) and a crude lipopeptide yield of 2.96 g/L (Table 18) after 24 hours of growth in the Optimized Medium. The scale-up to the 1.5L capacity Biolab fermenter resulted in an increase in the biomass of *B. licheniformis* JF-2 by 1.96 times and the crude lipopeptide yield by 1.63 times when compared to the yield obtained from the shake flask studies (Section 4.2.4) using the Optimized Medium.

The improved aeration and mixing in the fermenter increased the mass transfer in the cultivation medium which resulted in the higher yields of biomass and crude lipopeptide yield. Another factor that has to be considered here is that the biosurfactant that was synthesized and excreted into the cultivation medium was continuously removed through the foam by the foam fraction method. During the fermentation period of 24 hours, no significant drop in the surface tension of the Optimized Medium was observed which indicated that all the lipopeptide biosurfactant present in the cultivation medium was efficiently removed by foam fractionation. Acid precipitation of the sampled cultivation medium did not yield any crude lipopeptide. Cooper *et al.* (1981) had showed that the continuous removal by foam fractionation was employed to enhance the production of surfactin by *Bacillus subtilis*. Other fermentation techniques by scale-up models to enhance the biosurfactant production include the application of an air lift fermenter and aqueous two phase fermentation (Jenny & Deltrieu, 1993).

When the growth of *B. licheniformis* JF-2 in Optimized Medium was compared to the growth in the Mineral Salt Medium I and II, it was found to be far superior in the Optimized Medium. After 8 hours of growth in the Optimized Medium, the maximum absorbance achieved averaged 2.90 with a crude lipopeptide yield averaging **2.96 g/L.** The absorbance measured in the MSM-I averaged 0.29 with a crude lipopeptide yield averaging 0.22 g/L, whereas for

MSM-II, the maximum absorbance averaged 1.12 with a crude lipopeptide yield averaging 0.55 g/L. Studies conducted by Lin *et al.* (1994b) on the growth of *B. licheniformis* JF-2 using a Mineral Salt Medium supplemented with 1% glucose at 42°C in a 2L fermenter, resulted in a biomass yield averaging between 1.2 - 1.3 g/L at the 11th hour of fermentation. The maximum purified biosurfactant yield averaged 34.64 mg/L, was achieved at the 5th hour of fermentation with a 30% dissolved oxygen concentration in the cultivation medium. The biomass obtained from the growth of *B. licheniformis* JF-2 in the Optimized Medium in this study was higher (3.15 g/L) than the value reported by Lin *et al.* (1994b) which was approximately 1.3 g/L.

Studies conducted by Lin *et al.* (1994a) showed that 250 mg of the crude lipopeptide (acid precipitate) was obtained in one litre of the cultivation medium after lyophilization and further purification of the crude lipopeptide biosurfactant. This could be worked out to show that for every 1 mg of crude lipopeptide biosurfactant produced, 0.136 mg or 13.6% was the pure lipopeptide biosurfactant based on the total lyophilized weight of the crude lipopeptide.

With regards to the above computation, our crude lipopeptide yield could be quantified.The maximum crude lipopeptide yield obtained from our study was 2.96 g/L.The pure lipopeptide concentration in the crude lipopeptide biosurfactant at 13.6% would yield 0.402 g/L or 402.5 mg/L. This would mean that for every litre of the cultivation medium, 402.5 mg/L of the pure lipopeptide would be obtained. In terms of pure lipopetide yield per gram of biomass would be 12.78 mg of pure lipopeptide per gram of dry cell weight. The CMC of the crude lipopeptide at 13.6% purity was determined to be 165.9 mg/L. Therefore, the lipopeptide biosurfactant at 100% purity would give a CMC value of 22.56 mg/L. This value is 2.26 times higher than the value of 10 mg/L reported by Lin *et al.* (1994a).

5.3 Application of agro-industrial by-products in the cultivation media for the growth of *B. licheniformis* JF-2

Organic materials found in wastewaters could be used as substrates for the production of biosurfactants (Kosaric, 1992). Locally available agro-industrial by-products were tested for the production of biomass and biosurfactant from *B. lichenifomis* JF-2. This was done with a view to reduce the cost of biomass and lipopeptide biosurfactant production. Molasses was used extensively by Kosaric (1992) for the production of biosurfactant at Ringgit Malaysia 1.85/kg to 3.00/kg (equivalent to Canadian $1.00/kg to $3.00/kg).

In this study, 3 types of wastewater; rubber effluent, dairy wastewater and palm oil mill effluent were used. These wastewaters were diluted to various concentrations with distilled water and sterilized. These were then used to cultivate *B. licheniformis* JF-2 in shake flask studies. From these studies, we found that undiluted dairy wastewater was able to support the growth of *B. licheniformis* JF-2.

The maximum biomass obtained from the undiluted wastewater after 8 hours of fermentation averaged 0.71 g/L. After 7 hours of fermentation, the surface tension was reduced to 38.8 mN/m from an initial value of 48.4 mN/m, a reduction of 19.8% was achieved. The reduction in the surface tension of the dairy wastewater during the growth of *B. licheniformis* JF-2 clearly indicates the presence of the extracellular lipopeptide biosurfactant in the dairy wastewater. Apart from growing in the dairy wastewater, *B. licheniformis* JF-2 was able to produce the extracellular lipopeptide biosurfactant which **resulted** in the drop of the surface tension measured. Cultivation medium plated on Nutrient agar plates showed that *B. licheniformis* JF-2 was able to retain its volcanic shaped colonies which is ideal for biosurfactant production.

The growth of *B. licheniformis* JF-2 was also measured by colony forming units on agar medium. Colony Forming Units per ml (CFU/ml) was used instead of biomass (dry cell weight) because molasses and fish meal contained particles which made it difficult to accurately determine the biomass or measure the absorbance. Therefore, Colony Forming Units per ml (CFU/mL) was used to gauge the growth of *B. licheniformis* JF-2 after 8 hours of fermentation. Undiluted dairy wastewater produced 5.00 x10^5 colony forming units per ml (CFU/ml) after 8 hours of fermentation. When sugar cane molasses was added at 0.5% (v/v) to the medium, the number of colonies increased to 3.29 x 10^8 CFU/ml. Likewise, when fish meal was added at 5% (w/v), the count increased to 3.09 x 10^8 CFU/ml. The control which consisted of 0% (w/v) fish meal had only a colony count of 5.00 x 10^4 CFU/ml. This amounted to an increase of 99.98%. The dairy wastewater containing 0.5% (v/v) cane molasses and 5% (w/v) fish meal which showed the highest colony counts each had lowered the surface tension of the cultivation medium to values averaging 34.6 and 30.9 mN/m, respectively compared to an initial value of 48.4 mN/m. The growth of *B. licheniformis* JF-2 in dairy wastewater indicates that this microorganism could be used for treating the wastewater. For effective treatment, suspended solids from the wastewater should be removed and treatment by the aerobic process should commence.

5.4 Wastewater treatment

Malaysia's domestic water supply comes mainly from surface waters. The threat of inheriting a future where clean water is a rare commodity has compelled the law makers to enforce stringent measures designed to protect and manage our

water resources from being polluted by industries (Phang, 1987).

A locally available wastewater from Lam Soon, Petaling Jaya consisting of wastewater containing 100 ml of waste palm oil from the processing of edible palm oil was treated by using *B. licheniformis* JF-2 in an aerobic treatment for a period of 15 days. Changes in the COD of the treated effluent and the decrease in the surface tension were monitored every alternate days for 15 days. *Bacillus licheniformis* JF-2 was found to have the ability to remove COD of the edible palm oil processing wastewater and at the same time produce the lipopeptide biosurfactant which was measured by the reduction of the surface tension of the wastewater. The wastewater containing *B. licheniformis* JF-2 showed an increase in the COD from an initial value of between 2120.0 - 2125.0 mg/L to 4540.0 mg/L by the 3rd day of treatment. This increase in the COD was due to the emulsification of the waste palm oil present in the wastewater. After this peak, the COD of the wastewater decreases to 750.0 mg/L by the 15th day of treatment. This featured a reduction of 65.3% in COD for the *B. licheniformis* JF-2 treated wastewater and 33.0% for the control. The treatment period of 15 days is termed as the hydraulic retention time. The increased hydraulic tension time of 15 days can be reduced by reducing the amount of waste palm oil present in the wastewater. This is clearly demonstrated in the study with the JAD 969P culture complex. The volume of the waste palm oil is ten times less (10 ml) than that present in the *B. licheniformis* JF-2 treated edible palm oil processing wastewater. No increase in the COD of the wastewater (Fig. 27) was observed indictaing very little emulsification of oil.

In many studies, oil has been found to be detrimental in biological wastewater treatment systems. The smaller volume of oil in the JAD 969P culture complex treated wastewater had reduced the hydraulic retentiontime to 8 days

with a COD removal efficiency of 94.0%. For this process to be economically viable, the cost and hydraulic retention time needs to be reduced. In the food processing industries, the successful operation of their wastewater treatment plant depends on the complete removal of oil from the raw effluent or wastewater prior to treatment. When the hydraulic retention time is reduced, the cost of the treating the effluent becomes lower.