# MODIFIED FERMENTATION MEDIA FOR ENHANCED PRODUCTION OF SURFACTIN BY LOCAL ISOLATED BACILLUS SUBTILIS

**[1]TENGKU AB RAHMAN TENGKU NAWAWI, [2]MOHD HAFEZ MOHD ISA**

[1,2]Faculty of Science and Technology, Universiti Sains Islam Malaysia, Bandar Baru Nilai, 71800 Nilai, Negeri Sembilan
E-mail: kumeynawawi@gmail.com, m.hafez@usim.edu.my

**Abstract-** The effect of media components that are required in fermentation process to yield microbial product is investigated. In this experiment, the production of surfactin, produced from local isolated Bacillus subtilis 3M was enhanced through modification of Cooper's media formulation. Three important components selected for enhancement of surfactin yield were glucose, ammonium nitrate, ferrous sulfate and manganese sulfate concentrations. High pressure liquid chromatography (HPLC) was used to determine surfactin concentration contain in the fermentation broth. Modification of glucose, ammonium nitrate, ferrous sulfate and manganese sulfate concentration in Cooper's media formulation resulted to significant increase in surfactin yield. Application of 30 g/L, 0.05 M, 120 µM and 20 mM of glucose, ammonium nitrate, ferrous sulfate and manganese sulfate, respectively in modified Cooper's media formulation resulted to 181.1 mg/L of surfactin production compared to 90 mg/L of surfactin using unmodified Cooper's media. Results obtained in this study provided key parameters for response surface methodology study for further optimization of surfactin using local isolated Bacillus Subtilis.

**Keywords-** OFAT, Optimization, Media Condition, Surfactin

## I. INTRODUCTION

A lipopeptide biosurfactant, surfactin is mainly produced by Bacillus sp. and is characterized by an amino acid chain (peptide) which is linked to a fatty acid. In the last decade, biosurfactant such as surfactin have received increasing attention as they show potential candidates for many commercial applications in the petroleum, pharmaceutical, cosmetic and food processing industries. The properties of surfactin that makes them applicable for industrial use include pH stability, thermal stability, solubility and surface activity.

Despite possessing such advantages, the production of surfactin has not been thoroughly commercialized because of their low yields and production costs. In order to get maximum production, one of the possible methods is by development of optimization process such as culture conditions and media components. Hence, at the right level of conditions and nutrients, certain formulation can provide an ideal microenvironment for supporting growth and metabolite production.

The old-fashioned strategy of medium optimization involves varying one variable at a time, with all other variables held constant. This approach usually termed as one-factor-at-a-time (OFAT) technique. Currently, most researchers favor multivariate optimization which offers efficient technique for experimentation that conclusively solves complex problem with many variables. Furthermore, in the situation limited by time and resource pressure, free experimentation like OFAT, is possible. The aim of this research was to find out the effect of different concentration of medium components towards production of surfactin and biomass by B. subtilis strain. OFAT technique was used as preliminary screening experiment to determine the critical medium components before subjected obtained result to statistical multivariate optimization in the future.

## II. MATERIAL AND METHOD

A. Culture Maintenance and Seed Culture Preparation
The organism local isolated Bacillus subtilis 3M was isolated from UKM Laboratory. The latter was cultured on nutrient agar at 37 °C for 24 h and maintained at 4 °C. For the seed cultures, Bacillus subtilis 3M from an agar slant was inoculated into 50 ml of sterile brain heart infusion (BHI) broth medium containing (g/l): brain infusion solids (12.5), beef heart infusion solids (5.0), proteose peptone (10.0), glucose (2.0), sodium chloride (5.0), di-sodium phosphate (2.5) and incubated in 100 ml conical flasks at 30 °C, 200 rpm for 24 h on rotary shaker.

B. Inoculum Preparation
For surfactin production, freshly grown culture from BHI broth culture was inoculated into 250 ml Erlenmeyer flask containing 100 ml of sterile inoculation Cooper's medium reported by which is composed of 4% glucose (40 g/L), $KH_2PO_4$ = 30 mM, $Na_2HPO_4$ = 40 mM, $NH_4NO_3$ = 50 mM, $MgSO_4$ = 0.8 mM, $FeSO_4$ = 4 µM, $CaCl_2$ = 7 µM and EDTA = 4 µM and incubated on rotary shaker at 200 rpm for 24 h at 30 °C.

C. Shake Flask Fermentation and Optimization Study
One-factor-at-a-time (OFAT) optimization is used in order to determine the central value for future work on statistical optimization experiments. This is carried out by changing only one variable at a time while

fixing others variables. The parameters subjected to OFAT optimization are glucose, ammonium nitrate, ferrous sulfate and manganese sulfate. The shake flask fermentation was carried out using Cooper's medium. Concentration of media components selected was varied according to the experimental design. In all cases a 250 ml containing 50 ml of media was inoculated with 5% (v/v) inoculums and incubated at 30 °C in a rotary shaker with 200 rpm for 96 h. All experiments were carried out in triplicate.

D. Analysis of Surfactin

20 ml of the sample broth was centrifuged (10,000g, 20 min, 4 °C) to remove the biomass. Afterwards, the cell-free supernatant was subjected to an acid precipitation by adjusting the pH to 2.0 with 6 M HCl and left overnight at 4 °C. Finally, the precipitate was collected by centrifugation (10,000g, 20 min, 4 °C) and dissolved in two milliliters of methanol. Surfactin concentration was determined by reverse phase HPLC. The system used was a Agilent 1260 Infinity Quaternary LC System (USA), with a C18 column (5 µm, Merck). The mobile phase consisted of 20% of 3.8 mM trifluoroacetic acid and 80% acetonitrile; all solvents were HPLC grade. Sample size was 10 µl and the elution rate was 1 ml $min^{-1}$. The absorbance of the eluent was monitored at 205 nm. Surfactin from Sigma (St Louis, MO) served as standard.

## III. RESULTS AND DISCUSSION

a) Effect of glucose

Effect of different glucose concentrations on surfactin and biomass concentration produce from Bacillus subtilis 3M was investigated. Glucose was selected as an energy source for the bacteria to use since it is a simple sugar which is a monosaccharide. Glucose gave better biosurfactant yield rather than sucrose and glycerol. Fig. 1 shows the effect of glucose concentration on production of surfactin and cell dry weight while other variables fixed at following concentration: 0.05 M $NH_4NO_3$, 4 µM $FeSO_4$ and 1.5 mM $MnSO_4$. Different levels of glucose concentration from 20 g/L to 60 g/L were tested to determine the optimal concentration for surfactin production. Highest production of surfactin was observed at 30 g/L glucose concentration. However, the production of surfactin decreased significantly with concentration more than 40 g/L indicating possible inhibitory effect of glucose at high concentration. The biomass concentration is proportional to the production of surfactin.

b) Effect of $NH_4NO_3$

Fig. 2 shows the effect of $NH_4NO_3$ on surfactin and biomass concentration while glucose concentration was fixed according to their optimal concentration of 30 g/L obtained from previous OFAT experiment. Five $NH_4NO_3$ levels ranging from 0.03 M to 0.07 M were used to evaluate the repercussion of surfactin and biomass concentration of fermentation broth after 96 h. Glucose concentration was fixed at optimal value of 30 g/L according to previous OFAT result, while concentration of $FeSO_4$ and $MnSO_4$ were fixed at 4 µM and 1.5 mM respectively.

Based on Fig. 2, highest measurements of surfactin and biomass concentration were peaking at 0.05 M $NH_4NO_3$ with 95.6 mg/L and 4.1 mg/L respectively. The evidence that nitrogen plays an important role in the production of biosurfactant was observed in various literatures. Abouseoud et al reported an investigation on the best sources of nitrogen for growth and biosurfactant synthesis which were sodium nitrate and ammonium nitrate. Meanwhile, the organisms growing in a medium lacking a nitrogen source tend to have the least reduction in surface tension.

c) Effect of $MnSO_4$

In order to study the effect of surfactin and biomass production from fermention broth of B. subtilis 3M, five $MnSO_4$ levels from 0.5 to 2.5 mM were used while glucose and $NH_4NO_3$ concentration were fixed according to their optimal concentration of 30 g/L and 0.05 M respectively. Concentration of $FeSO_4$ was fixed at 120 µM. Cooper's media as proposed by Cooper et al were supplemented with manganese salts but the concentration was indefinite. Hence, five different levels ranging from 0.5 mM to 2.5 mM are used to determine optimal concentration needed for surfactin and biomass production. All other variables were fixed at their respective optimal value obtained from previous OFAT experiments. Based on Fig. 3, highest concentration of surfactin (179.4 mg/L) and biomass (3.0 mg/L) were obtained at 2.0 M of $MnSO_4$. In the presence of inorganic cations such as manganese, nitrogen utilization and $K^+$ uptake were affected, hence increasing the production of surfatin. However, further work is needed to explain the role of $Mn^{2+}$ in the growth of organism, surfactin production and the physiology that leads to enhancing surfactin production.

d) Effect of $FeSO_4$

Fig. 4 illustrates that the surfactin and biomass production from fermention broth of B. subtilis 3M were influenced by varying ferrous sulfate concentration. Five $FeSO_4$ levels from 40 to 200 µM were used while glucose, $NH_4NO_3$ and manganese nitrate concentration were fixed according to their optimal concentration of 30 g/L, 0.05 M and 2.0 mM respectively. The Cooper's media proposed by Cooper et al. suggested the appropriate amount of $FeSO_4$ was 4 µM but addition of iron up to a level of 4 mM can induce overproduction of surfactin as well as biomass growth as reported by Based on Fig. 4, surfactin and biomass concentration at first increased steadily parallel to addition of $FeSO_4$ and reached its highest value (181.1 mg/L) at 120 µM but then again

decreased beyond 160 µM concentration. This was parallel as reported by where excessive amounts of iron were needed for heavy growth, but the additional iron does not improve the yield of surfactin per cell. Moreover, rapid acidification of broth caused by iron addition induced disappearance of surfactin as pH drop below 5.0

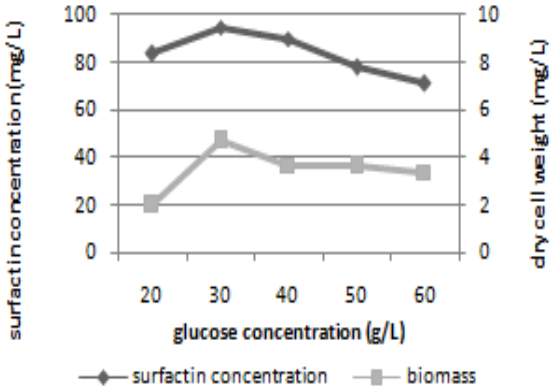



**Fig. 1: Effect of glucose concentration on surfactin and biomass production**

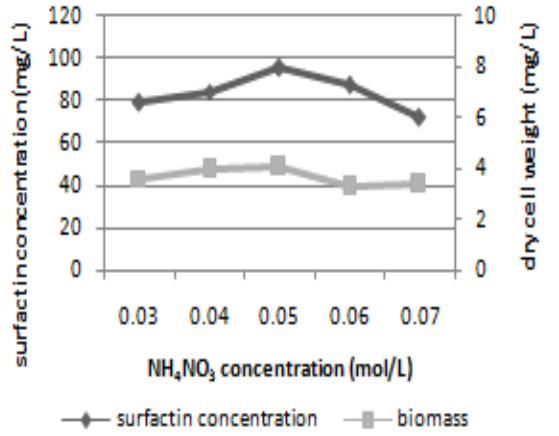



**Fig. 2: Effect of ammonium nitrate concentration on surfactin and biomass production**

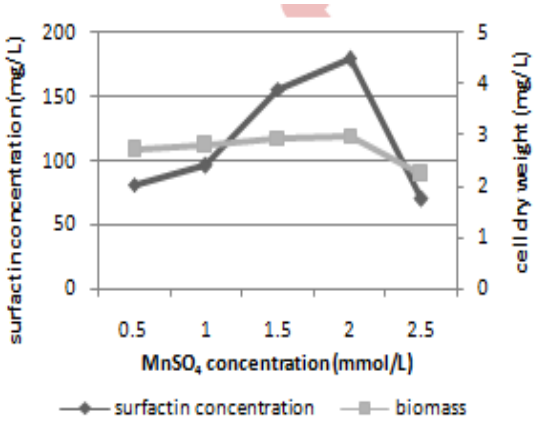



**Fig. 3: Effect of manganese sulfate concentration on surfactin and biomass production**

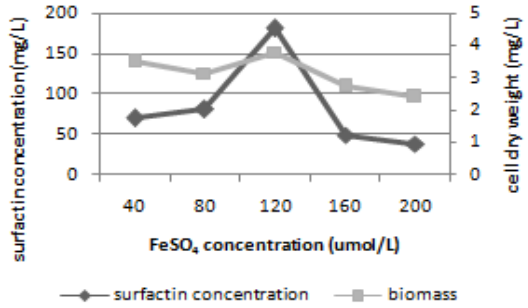



**Fig. 4: Effect of ferrous sulfate concentration on surfactin and biomass production**

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