CHAPTER V

Kinetics of Surfactin Production by *Bacillus subtilis* in a 5 L Stirred-tank Bioreactor

5.1. Introduction

Surfactants, either chemically or biologically produced, are defined as surface active agents with attractive wide-ranging properties (Al-Araji *et al.,* 2007). Surfactants produced by biological synthesis are known as biosurfactants and can be synthesized by a variety of bacteria, yeasts, and fungi through utilization of various carbon feedstocks (Chen *et al.,* 2007). Biosurfactants offer various advantages such as being less toxic, more biodegradable, and environmentally friendly compared to chemical surfactants, and unlike chemical surfactants, they do not lose their physiochemical properties at different temperatures and pH levels (Mulligan, 2005).

Among the many classes of biosurfactants, the lipopeptide group is attracting great interest because of its high surface activity and therapeutic potential (Nitschke *et al.* 2006). Surfactin is one of the most efficient biosurfactants known so far and belongs to the lipopeptide group (Wei *et al.,* 2007). Surfactin exhibits diverse biological activities such as antimicrobial (Fernandes *et al.,* 2007), hemolytic, antifungal, antiviral, and antimycoplasma properties (Singh *et al,.* 2004). *B. subtilis* is a sporulating rod bacterium that is one of the most studied Gram-positive bacteria (Driks, 2002). It is found in the soil and is known to be non-pathogenic in humans, and has a wide range of applications (Zweers *et al.,* 2008). The ability of *B. subtilis* strains to produce lipopeptide has been well documented over the last 50 years (Xiao *et al.* 2008) and has shown great potential for application in pharmaceutical and biotechnological fields in recent years (Mulligan 2005; Kowall *et al.,* 1998). Therefore, finding new strains of surfactin producers and knowledge of the kinetics of surfactin production can improve surfactin yield efficiency and can assist in reducing the total cost of surfactin production.

In all previous studies, analytical data were compared using the relationship between bacterial growth and surfactin concentration (Isa *et al.,* 2007). However, a kinetic model should be developed to explain substrate and product evolutions under operational fermentation conditions (Rodrigues *et al.,* 2006). This current study is aimed to elucidate the basic concept of kinetic model describing biomass cell growth, substrate (glucose) consumption, and surfactin production in the batch fermentation process by *B. subtilis* MSH1 and *B. subtilis* ATCC 21322 using a stirred submerged bioreactor.

5.2. Materials and Methods

5.2.1. Preparation of Culture Media

A defined mineral salts medium (MSM) described by Cooper *et al.* (1981) was used as fermentation media throughout this study. The media consisted of NH_4NO_3, 0.05 M; Na_2HPO_4, 0.04 M; KH_2PO_4, 0.03 M; $CaCl_2$, 7.0×10^{-6} M; $FeSO_4\cdot7H_2O$, 4.0×10^{-6} M; EDTA, 4.0×10^{-6} M; $MgSO_4$, 8.0×10^{-6} M; and 4% (w/v) glucose (Davis *et al.,* 2001; Isa *et al.,* 2008). All chemicals used were of analytical grade. Prepared medium was sterilized prior to fermentation.

5.2.2. Culture Conditons and Fermentation

B. subtilis MSH1 and *B. subtilis* ATCC 21332 were used in this study and were obtained from Microbiology Laboratory, Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM). *B. subtilis* MSH1 was isolated from oil contaminated soils collected in Kajang (Selangor), Malaysia and was identified by Shannaq and Isa (2013) as a surfactin-producer with the presence of 16s rDNA gene sequensing (accession no. JX080184.1), and *sfp* gene sequensing (accession no.CP002183.1), respectively (Shannaq and Isa, 2013). Meanwhile, *B. subtilis* ATCC 21332 is a commercial strain and a confirmed surfactin producer as according to previous studies (Shannaq and Isa, 2013). Stock culture was maintained on nutrient agar. Two loopfuls of grown bacterial cells from the nutrient agar were transferred to 25 mL of nutrient broth containing 40 g/L glucose, followed by the incubation of the culture broth on an orbital shaker at 200 rpm at 30°C for 24 h (Isa *et al.*, 2007). A

volume of 5 mL of culture broth was then transferred to a conical flask containing 45 mL of Cooper's medium (Cooper *et al.,* 1981). A total of five conical flasks were prepared. All flasks were incubated under the same conditions as previously described, for 16 h. A total volume of 250 mL was used to inoculate 4750 mL of Cooper's media (Isa *et al.,* 2007).

A submerged bioreactor (Sartorius Stedim, Germany) with a working volume of 5 L was employed for fermentation to produce surfactin. The bioreactor included an agitation system with two impellers on a single drive shaft connected to a motor. Agitation speed, dissolved oxygen, and pH were controlled by a fermentation control unit. The pH of the culture broth was maintained by automatic addition of 1.0 M NaOH and 1.0 M HCl (Isa *et al.,* 2007). The fermentation conditions were set at the temperature of 30°C, agitation speed of 100 rpm, air flow rate of 1 vvm^{-1}, and pH 7 for 55 h. *B. subtilis* MSH1 and *B. subtilis* ATCC 21332 were cultivated under the same conditions, in which batch fermentation mode was performed using a low level of dissolved oxygen (Isa *et al.,* 2007). Culture broth samples were withdrawn aseptically at regular time intervals for determination of bacterial growth, surfactin concentration, and glucose consumption.

5.2.3. Analytical Methods

5.2.3.1. Measurement of Bacterial Growth

Bacterial growth was measured by determining the biomass concentration (gram of dry cell weight per liter of culture medium) at different time intervals up to 55 h. Fixed volumes of the culture samples were withdrawn aseptically and transferred to centrifuge tubes for centrifugation at 10,000 rpm for 10 min. The supernatant was withdrawn and biomass left at the bottom of the tubes was dried using an oven at 105°C. The dry cell weight was then measured using a balance until constant reading achieved.

5.2.3.2. Measurement of Surfactin Concentration

Culture samples were withdrawn aseptically at various time intervals and centrifuged at 10,000 rpm for 10 min. The supernatant was then filtered through a 0.45 µm nylon filter membrane for surfactin and glucose analyses. Surfactin concentration was measured using high-performance liquid chromatography (HPLC; Agilent Technologies, 1200 Series, USA) equipped with Chromolith® high performance RP-18 (100 mm × 4.6 mm, 5 µm) and detected at 205 nm with a variable wavelength detector (VWD). Mixtures of mobile phase consisted of acetonitrile (ACN) and 3.8 mM trifluoroacetic acid (TFA) solution at the ratio of 80:20 were pumped with an isocratic mode at a flow rate of 2.2 mL/min. The sample injection was set at 30 µL and the duration of each analysis was within 8 min. Surfactin standard purchased from Sigma, with 98% purity, was used as a standard.

5.2.3.3. Measurement of Glucose Concentration

Glucose was measured by HPLC equipped with Chromolith® NH2 RP-18 (100 mm × 4.6 mm, 5 µm) and detected at 195 nm with a VWD. The mobile phase used was 3.8 mM TFA and was pumped with an isocratic mode at a flow rate of 0.5 mL/min. Total elution time for analysis was within 8 min.

5.2.3.4. Calculation of Kinetic Parameters

Substrate conversion was calculated according to equation as shown in Equation 3 (Eq. 3).

$$\Delta S(\%) = \frac{S_o - S}{S_o} \times 100 \qquad \text{(Eq. 3)}$$

where S_o is the initial glucose concentration and S is the glucose concentration in the samples at each time interval.

The volumetric productivity (P_p and P_x) was calculated as the ratio of maximum surfactin (P_{max}) or cell concentration (X_{max}) to the fermentation time when the maximum concentration of surfactin was achieved (t_{Pmax} and t_{Xmax}, respectively):

$$P_p = \frac{P_{max}}{t_{Pmax}} \qquad \text{(Eq. 4)}$$

$$P_x = \frac{X_{max}}{t_{Xmax}} \quad \text{(Eq. 5)}$$

The yield of surfactin on glucose ($Y_{P/S}$, g/g) was defined as:

$$Y_{p/s} = \frac{P_f - P_o}{S_f - S_o} \quad \text{(Eq. 6)}$$

The yield of cell mass on glucose ($Y_{X/S}$, g/g) was defined as:

$$Y_{x/s} = \frac{X_f - X_o}{S_f - S_o} \quad \text{(Eq. 7)}$$

The yield of surfactin on cell mass ($Y_{P/X}$, g/g) was defined as:

$$Y_{P/X} = \frac{P_f - P_o}{X_f - X_o} \quad \text{(Eq. 8)}$$

where P_o, X_o, S_o are the initial amount of surfactin concentration, biomass concentration, and glucose concentration, respectively. In addition, P_f, X_f, and S_f represent the amount of surfactin concentration, biomass concentration and glucose concentration in the samples at each time interval, respectively.

5.3. Results and Discussions

5.3.1. Bacterial Cell Growth in Cooper's Medium

Figure 9: Production of surfactin by *B. subtlis* ATCC 21332.

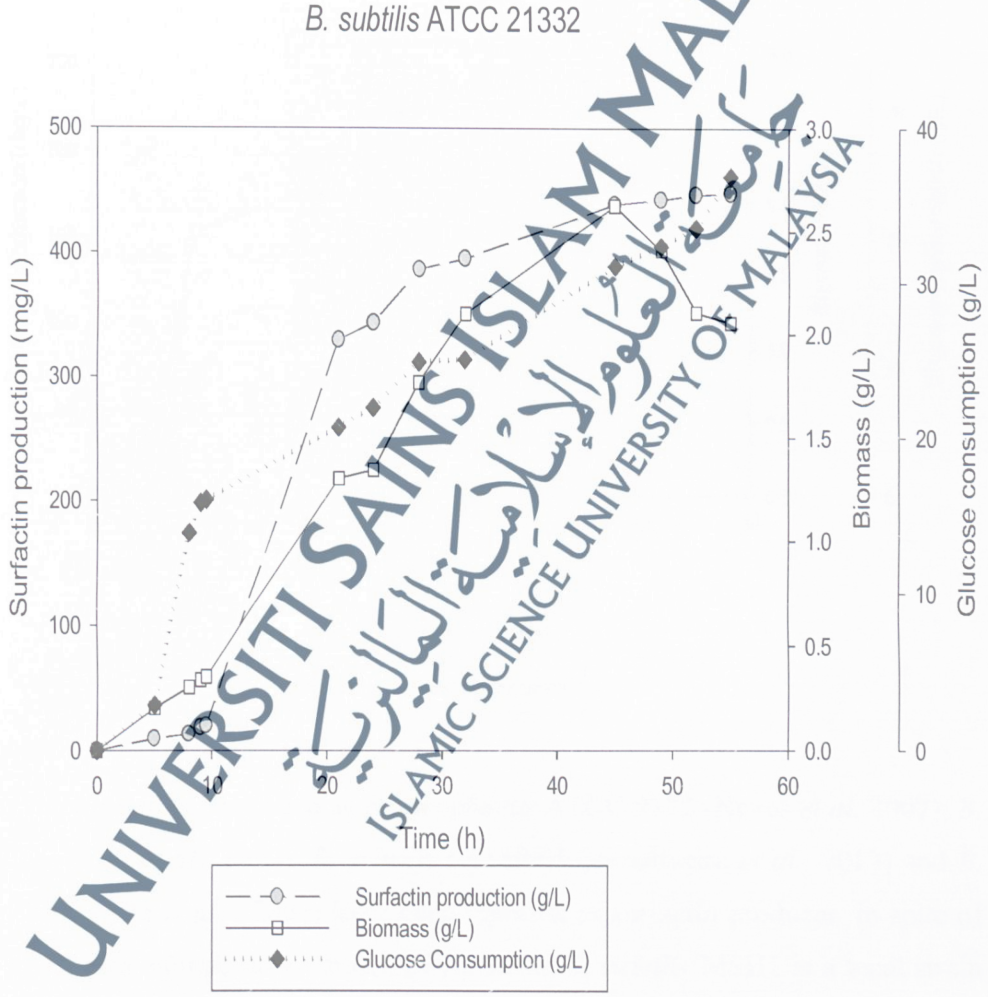
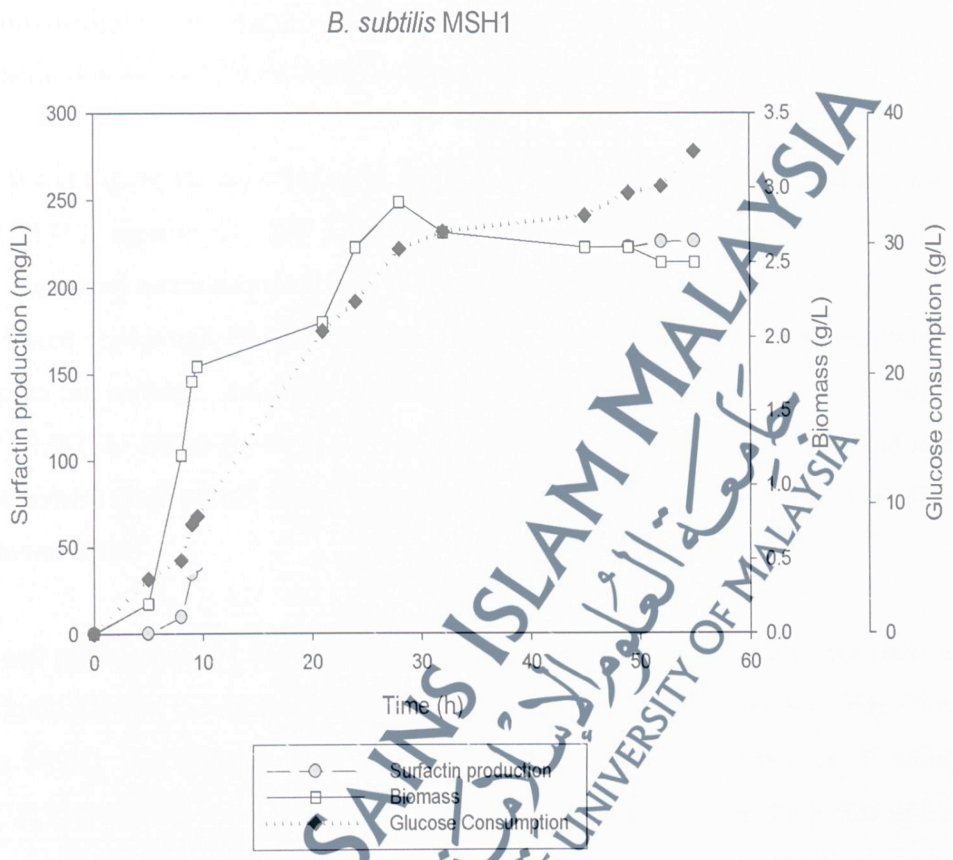


Figure 10: Production of surfactin by *B. subtlis* MSH1.



Various *Bacillus sp.* strains such as *B. atrophaeus* ATCC 9372 (Neves *et al.* 2007), *B. subtilis* C9 (Kim *et al.*, 1997), *B. subtilis* LAMI005 (de Oliveira *et al.,* 2013) and *B. subtilis* 20B (Joshi *et al.,* 2008) have been reported as surfactin producer. In spite of ability to produce competitive amount of surfactin, *B. subtilis* MSH1 is a local strain that has not been extensively studied and used for the production of surfactin (Shannaq *et al.,* 2013). In contrast, *B. subtilis* ATCC 21332 is commercially known as a surfactin producer and is able to produce surfactin in different types of substrate and media, such as potato substrate (Fox *et al.,* 2000), clarified cashew apple juice (de Oliveira *et al.* 2013), and Cooper's media (Isa *et al.,* 2007; Cooper *et al.,* 1981). Cooper's media with 4% (w/v) glucose was used because it has been designed to supply nutrients for bacterial cell growth and surfactin synthesis by *Bacillus* strains

strains (Isa *et al.,* 2007; Cooper *et al.,* 1981; Davis *et al.*, 2001; Yakimov *et al.,* 1997). The pH of fermentation broth was maintain at pH 7 to prevent the acidification of culture medium and reduction of pH level to less than pH 5 will cause precipitation of surfactin due to lost of its solubility (Wei *et al.,* 2003).

Figure 9 and Figure 10 show bacterial cell growth for *B. subtilis* MSH1 and *B. subtilis* ATCC 21332, repectively. The beginning of cell growth (lag phase) for *B. subtilis* MSH1 was short (approximately 5 h) compared with that for *B. subtilis* ATCC 21332, which lasted approximately 10 h, implying that *B. subtilis* MSH1 needed less than 5 h to adapt to the medium. An obvious pattern of the lag phase can be seen for *B. subtilis* ATCC 21332, in which the strain needed 10 h to adapt to the medium. This lag phase showed almost no apparent cell growth, due to adaptation of the microorganism to the new environment.

Later, cell growth entered the exponential phase, where the cell number increased in a logarithmic pattern. As shown in Figure 9 and Figure 10, the exponential phase for *B. subtilis* MSH1 was between 5 to 28 h, while the exponential phase for *B. subtilis* ATCC 21332 was between 10 to 45 h. In this phase, about 65% of the initial glucose (S_o) was consumed by both strains and the maximum glucose consumption occurred in this phase. This phase was shorter for *B. subtilis* MSH1 (23 h) than *B. subtilis* ATCC 21332 (35 h), indicating that *B. subtilis* MSH1 achieved the maximum cell growth 12 h earlier than *B. subtilis* ATCC 21332. As is evident in Figure 9 and Figure 10, the stationary growth phase for *B. subtilis* MSH1 began after 28 h of incubation, while the stationary phase for *B. subtilis* ATCC 21332 began after 45 h of incubation.

Cooper *et al.* (1981) and Kim *et al.* (1997) suggested biosurfactant production by *B. subtilis* strains was highly related to microbial cell growth, while Shepard and Mulligan (1987) stated that biosurfactant production mainly occurred at the end of the exponential phase or in the stationary phase of microbial growth. Based on Figure 9 and Figure 10, the production of surfactin was closely related to the growth of strains

where the maximum production occurred at the end of the exponential growth phase for both strains.

5.3.2. Relationship Between Surfactin Production, Cell Growth, and Glucose Consumption

Both strains were able to produce surfactin with approximately the same pattern, except in the log phase. Surfactin production by *B. subtilis* MSH1 and *B. subtilis* ATCC 21332 started in the early exponential growth phase (Figure 9 and Figure 10). Cell growth was slow and glucose consumption was also very low in the early exponential growth phase, where about 10% of S_o was consumed by both strains, considering the fact that surfactin is categorized as a secondary metabolite (Georgiou *et al.,* 1992). For *B. subtilis* ATCC 21332, surfactin was produced as cell growth started to enter the stationary phase, and the maximum concentration was attained at the end of the exponential phase, as the glucose concentration became lower due to consumption by the cells (Davis *et al.,* 1999). The maximum surfactin concentration (P_{max}) for both strains was attained in the stationary phase, with the values as high as 447.26 mg/L and 226.17 mg/L for *B. subtilis* ATCC 21332 and *B. subtilis* MSH1, respectively. The same pattern was observed for *B. subtilis* LB5a, where maximum surfactin production was attained in the stationary phase by using cassava waste as fermentation media (Nitschke *et al.,* 2006). Besides, a previous fermentative study using *B. subtilis* ATCC 21332 on various media showed that the highest surfactin concentration was attained in the stationary phase (Isa *et al.*, 2007; Isa *et al.,* 2008; Shanaq and Isa, 2013). In this phase, most of the glucose feedstock (S_o) had been consumed by the strains.

The P_{max} for *B. subtilis* ATCC 21332 was approximately two times higher than that for *B. subtilis* MSH1 under the same fermentation conditions. *B. subtilis* MSH1, which had a short lag phase, was able to quickly adapt to Cooper's media, which caused it to achieve P_{max} in a shorter time than *B. subtilis* ATCC 21332. A previous fermentative study using *B. subtilis* LAMI005 showed that the initial concentration of medium affected maximum cell concentration (de Oliveira *et al.,* 2013).

5.3.3. Kinetics of Surfactin Production by *B. subtilis* MSH1 and *B. subtilis* ATCC 21332

Table 9: Main kinetic results of surfactin production by *B. subtilis* MSH1 and *B. subtilis* ATCC 21332

B.subtilis strains	Surfactin Production				Biomass			Glucose Consumption			Yield		
	P_o (mg/L)	P_{max} (mg/L)	R_r (h^{-1})	r_p/x	X_o (g/L)	X_{max} (g/L)	μ_{max}	S_o (g/L)	S_f (g/L)	ΔS (%)	$Y_{p/s}$ (g/g)	$Y_{x/s}$ (g/g)	$Y_{p/x}$ (g/g)
ATCC 21332	9.51 ± 0.05	447.26 ± 11.87	0.125	9.105	0.21	2.62	0.224	39.19 ± 1.75	36.89 ± 0.02	94.13	0.015	0.085	0.178
MSH1	9.56 ± 0.40	226.17 ± 2.56	0.200	0.991	0.20	2.90	0.087	40.02 ± 0.70	37.06 ± 0.02	92.63	0.008	0.107	0.119

Table 9 shows the results obtained from kinetic studies of bacterial growth, glucose consumption, and surfactin production by *B. subtilis* MSH1 and *B. subtilis* ATCC 21332. It was found that the growth rate of *B. subtilis* MSH1 varied from 0.106 h^{-1} to 0.224 h^{-1}, while that of *B. subtilis* ATCC 21332 varied from 0.048 h^{-1} to 0.087 h^{-1}. The biomass specific growth rate (μ_{max}) for *B. subtilis* MSH1 was as high as 0.224 h^{-1}, which two times higher than that for *B. subtilis* ATCC 21332 (0.087 h^{-1}). This implied that *B. subtilis* MSH1 was better adapted to Cooper's medium. The highest biomass concentration (X_{max}) achieved by *B. subtilis* MSH1 was 2.90 g/L, while that achieved by *B. subtilis* ATCC 21332 was 2.62 g/L, confirming the higher μ_{max} for *B. subtilis* MSH1.

As shown in Table 9, the biomass yield ($Y_{x/s}$) produced by *B. subtilis* MSH1 (0.107 g/g) was 27% higher than that for *B. subtilis* ATCC 21332 (0.085 g/g), indicating that *B. subtilis* MSH1 showed higher growth kinetics compared with *B. subtilis* ATCC 21332, with higher values of μ_{max}, X_{max}, and $Y_{x/s}$. The factors responsible for this behavior, among which we should mention is the type of organism being used can contribute to this phenomena where *B. subtilis* MSH1 easily adapted to Cooper's medium compared with *B. subtilis* ATCC 21332.

The initial production of surfactin (P_o) for *B. subtilis* MSH1 and for *B. subtilis* ATCC 21332 were 9.56 mg/L and 9.51 mg/L respectively, when 33% of the total glucose had been consumed. The results showed that cell concentration (X) and volumetric biomass productivity (P_x) increased with increasing sugar consumption during the fermentation time, until the maximum biomass (X_{max}) was reached. As shown in Table 9, P_{max} attained by *B. subtilis* ATCC 21332 and for *B. subtilis* MSH1 were 438.64 ± 11.87 mg/L and 226.17 ± 5.62 mg/L respectively. Davis *et al.* (Davis *et al.,* 2001), who studied the production of surfactin by *B. subtilis* ATCC 21332 achieved a P_{max} of around 439 mg/L, which is similar to the results obtained in this work. Other authors were able to achieve the higher P_{max} of 583 mg/L under the same experimental conditions (Isa *et al.,* 2007). This set of fermentation data for the production of surfactin by *B. subtilis* ATCC 21332 showed similar trends to previously reported studies (Isa *et al.,* 2007; Davis *et al.,* 2001). It seems that *B. subtilis* MSH1 was unable

to compete with *B. subtilis* ATCC 21332, whose P_{max} was approximately two times greater than that of *B. subtilis* MSH1.

Glucose is a good carbon source for fermentative study of biosurfactant production by *Bacillus* strains and is widely used in previous studies (Isa *et al.,* 2007; Cooper *et al.,* 1981; Davis *et al.,* 2001; Isa *et al.,* 2008; de Oliveira *et al.,* 2013; Davis *et al.,* 1999). The carbon source supplied is able to assist the production of surfactin (de Oliveira *et al.,* 2013). It must be supplied in the medium up to the optimum level of 65.04 g/L. Considerable amount of glucose was consumed by both strains when the P_o increased to P_{max} during the course of fermentation process, when the μ_{max} for both strains was attained. Alternatively, surfactin production can be evaluated through the yield of biosurfactant on cell mass ($Y_{p/x}$) (de Oliveira *et al.,* 2013), which is a useful volume-independent parameter for scaling up the bioprocess (Neves *et al.,* 2007). The $Y_{p/x}$ obtained was 0.178 g/g and 0.119 g/g for *B. subtilis* MSH1 and *B. subtilis* ATCC 21332, respectively. The studies conducted by de Oliveira *et al.* (2013) and Davis *et al.* (1999) showed that the $Y_{p/x}$ value ranged from 0.0068 g/g to 0.075g/g when *B. subtilis* LAMI005 and *B. subtilis* ATCC 21332 were cultivated, depending on the intial substrate concentration in the culture medium.

The value of $Y_{p/s}$ shows the relationship between surfactin production and glucose consumption (Table 9). The $Y_{p/s}$ obtained was 0.008 g/g and 0.015 g/g for *B. subtilis* MSH1 and *B. subtilis* ATCC 21332, respectively. *B. subtilis* ATCC 21332 efficiently consumed a high amount of glucose (87 %) compared with *B. subtilis* MSH1. High substrate (glucose) consumption by bacterial cells (93%) was not limited by the carbon source in the culture medium, because only 66% of glucose had been consumed when the P_{max} was obtained. Davis *et al.* (1999) found that the cultivation of *B. subtilis* ATCC 21332 in medium with at least 30 g/L glucose was enough to avoid carbon limitation during fermentative activity. Since an increase in cell concentration with higher product formation was observed when sugar consumption was increased, it is possible that another nutrient in the culture medium was used by the bacteria cells.

The r_p/X reflects the activity of the microorganism in surfactin production (Rodrigues et al., 2006). As shown in Table 9, *B. subtilis* ATCC 21332 showed a high value of r_p/X (9.105 mg/Lh) compared with *B. subtilis* MSH1 (0.991 mg/Lh), implying the higher efficiency of *B. subtilis* ATCC 21332 in surfactin production (10 times) compared with *B. subtilis* MSH1. Surfactin production by both strains was closely related to bacterial growth (Cooper *et al.* 1981; Kim *et al.* 1997) and in addition, the type of organism and the culture medium are known to be the main factors for microbial growth pattern (de Oliveira *et al.* 2013). It can be observed in Figure 9 and Figure 10 that cell growth, surfactin production, and glucose consumption showed similar profiles for both strains, consistent with a previous study conducted using different types of *Bacillus* strains (Kim *et al.* 1997; de Oliveira *et al.* 2013). There was a strong correlation between surfactin production kinetics and biomass kinetics during bacterial growth. Therefore, in the conditions employed here, surfactin production by *B. subtilis* ATCC 21332 and *B. subtilis* MSH1 was associated with cell growth. There was relationship between surfactin production, cell growth, and glucose utilization (Kim *et al.,* 1997). Growth-associated production of biosurfactant has been reported for *Bacillus licheniformis* JF-2 (Lin *et al.,* 1994), *B. subtilis* C9 (Kim *et al.,* 1997), and *B. subtilis* LAMI005 (de Oliveira *et al.*, 2013). A direct relationship between biosurfactant production, cell growth, and carbohydrate utilization was observed during the production of biosurfactant by *B. subtilis* C9 (Kim *et al.,* 1997).

5.4. Conclusion

This model could be used to to assess productivity of any bacterial strain to produce surfactin through correlation of biomass concentration, surfactin concentration and glucose consumption at various stages in fermentation process. The results obtained showed *B. subtilis* MSH1 can be a good alternative for surfactin producer through utilization of Cooper's media formulation. *B. subtilis* MSH1 and *B. subtilis* ATCC 21332 were able to grow in Cooper's medium and produce surfactin in a stirred-tank bioreactor. In spite of the high consumption of glucose of approximately 93% by both bacterial strains studied, no carbon limitation was observed.

Throughout this study, *B. subtilis* MSH1 showed higher growth cell kinetics, exhibited higher values of μ_{max}, X_{max}, and $Y_{x/s}$ compared with *B. subtilis* ATCC 21332. The

biomass cell productivity of *B. subtilis* MSH1 was found to be approximately three times higher compared with *B. subtilis* ATCC 21332. While, *B. subtilis* ATCC 21332 showed higher surfactin production kinetics, with higher values of $r_{p/X}$, P_{max}, and $Y_{p/s}$ compared to *B. subtilis* MSH1. The maximum surfactin production of *B. subtilis* ATCC 21332 was found to be approximately two times higher compared with *B. subtilis* MSH1 under the same fermentation conditions. It was found that *B. subtilis* MSH1 had a short lag phase, was able to quickly adapt to Cooper's media and causing it to produce the P_{max} in a shorter time than *B. subtilis* ATCC 21332. In addition, *B. subtilis* MSH1 was unable to compete with *B. subtilis* ATCC 21332 in terms of yield efficiency of $Y_{p/s}$, $Y_{x/s}$ and $Y_{p/x}$.

A basic kinetic model had been suggested to analyze and describe the kinetics process of fermentation of surfactin producer strains of *B. subtilis* ATCC 21332 and *B. subtilis* MSH1. The kinetic model proposed using Eq.1 to Eq.16 and main value of kinetic shown in Table 9 can adequately explain the trends and interaction of all parameters involved in the fermentation course. Overall, this study provides some significant knowledge of important parameters and its correlation towards surfactin production. Findings of this study can be further extended to other surfactin producer strains of higher productivity, and should essentially follow the same kinetic trend.