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A novel approach for surfactin production by locally isolated *Bacillus subtilis* with commercial potentials

G.A. Amin and O. Al-Zahrani

Abstract— Surfactin produced by *B.subtilis* BDCC-TUSA-3 from Maldex-15 as a growth associated product in conventional batch process. Maldex-15 is a cheap industrial by-product recovered during manufacturing of high fructose syrup from corn starch. Surfactin production was greatly improved in exponential fed-batch fermentation. Maldex-15 and other nutrients were exponentially fed into the culture based on the specific growth rate of the bacterium. In order to maximize surfactin yield and productivity, conversion of different quantities of Maldex-15 into surfactin was investigated in five different fermentation runs. In all runs, most of Maldex-15 was consumed and converted into surfactin and cell biomass with appreciable efficiencies. The best results were obtained with fermentation run supplied with 204 g Maldex-15. Up to 36.1 $g.l^{-1}$ of surfactin and cell biomass of 31.8 $g.l^{-1}$ were achieved in 12 hrs. Also, markedly substrate yield of 0.272 g/g and volumetric reactor productivity of 2.58 $g.1^{-1}.h^{-1}$ were obtained confirming the establishment of a cost effective commercial surfactin production.

Keywords Surfactin; *Bacillus subtilis*; Fed-batch cultivation

INTRODUCTION

With current advances in petroleum biotechnology, attention has been given to microbial enhancing oil recovery (MEOR) processes. Biosurfactants produced by certain microorganisms is one of the most promising MEOR agents [1, 2, 3]. They enhanced the recovery of oil by reducing the interfacial mediating changes in the wettability index of the system [4, 5]. Biosurfactants have several advantages over chemical surfactants such as lower toxicity, higher biodegradability and effectiveness at extreme temperatures or pH values [6]. Many attempts have been made to develop fermentation process for the cost-effective production of biosurfactants with a little success. These include conventional batch fermentation [7], batch fermentation with addition of solid carriers [8], solid state batch fermentation [9], fed-batch fermentation [10] and continuous fermentation using airlift reactor [11]. In all trials, the ultimate goal was hindered by uncompetitive considerably low volumetric reactor productivity due to incomplete utilization of fermentative substrate. In fact, both overfeeding and underfeeding of fermentative substrate were found to be detrimental to cell growth and product formation in many fermentation processes [12].

In previous investigation, *B. subtilis* BDCC-TUSA-3 was isolated from solid wastes at Jeddah Refinery and shown to produce high concentrations of surfactin in conventional batch fermentation [13]. However, volumetric productivity was still low from economical point of view. The objective of this study was to investigate possibilities of using the exponential fed-batch strategy, in order to adequately supply fermentative substrate and other nutrients to the bacterium *B. subtilis* and hopefully maximize cell growth and surfactin production.

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METHODS

Microorganisms and media

The bacterium *B. subtilis* BDCC-TUSA-3 was isolated from solid wastes at Jeddah refinery and designated as BDCC-TUSA-3 [13]. The mineral salt medium (MSM) with the following composition: NaCl (0.001%), $MgSO_4$ (0.06%), $CaCl_2$ (0.004%), $FeSO_4$ (0.002%) and 0.1 ml. of trace element solution containing ($g.l^{-1}$) 2.32 $ZnSO_4.7H_2O$, 1.78

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$MnSO_4.4H_2O$, 0.56 H_3BO_3, 1.0 $CuSO_4.5H_2O$, 0.39 $NaMoO_4.2H_2O$, 0.42 $CoCl_2.6H_2O$, 1.0 EDTA, 0.004 $NiCl_2.6H_2O$ and 0.66 Kl was used through this investigation.

Time course for cell growth and surfactin production

In a conventional batch cultivation, a fermentation vessel of 3-l was charged with 1700 ml of MSM containing 40 $g.^{l-}1$ of Maldex-15 was conducted. Maldex-15 is an α-amylase dextrin produced during the commercial production of high fructose syrup from corn starch. It contains 1% glucose, 3% maltose, 5% maltotriose and 91% of oligo- and megalosaccharides [13]. Fermentation temperature and pH of 30 ^{0}C and 7$\pm$ 0.2 were used respectively. Sterile air stream was introduced into the bioreactor at a rate of 1.0 volume of air/bioreactor working volume/min (v/v/min). Agitation was set at 500 rpm and dissolved oxygen concentration was monitored and maintained above 50% saturation. Samples were taken at regular intervals and analyzed for biomass and surfactin.

Fermentation procedure

The exponential-fed strategy, normally used for the industrial production of baker's yeast [14] and more recently adapted [15], was used in order to supply Maldex-15 and other nutrients to the bacterium *B. subtilis* BDCC-TUSA-3 for surfactin production. Cultivation conditions were set as described above. Five different fermentation runs were conducted in order to test conversion efficiency of different Maldex-15 amounts into cell biomass and surfactin. In each fermentation run, the bioreactor was charged with 500 ml of water and sterilized. After cooling, it was inoculated with the calculated initial biomass concentration and allowed to work for almost one minute in order to disperse bacterial cells. Then, fermentation process was initiated and different small volumes of concentrated medium containing the calculated amounts of Maldex-15 and other nutrients were exponentially-fed into the bioreactor on hourly basis and over 12 hours to reach a final volume of 1500 ml. Cultivation conditions were set as described above.

Biomass (dry weight, DW)

Bacterial biomass was determined by centrifuging a known volume of fermentation broth at 1000g for 15 min. The biomass was washed twice with distilled water and dried overnight at 90°C to constant weight.

Analysis

The total carbohydrate in the culture broth was estimated by the phenol-sulfuric acid method using mannose as a standard [16]. The growth rate was determined according to [17]. The following equation was used.

$$\mu = (\log X_2 - \log X_1)/\log e.t$$

Where μ = specific growth rate, loge = natural Logarithmic, X_1 and X_2 = Two values for biomass (DW) concentrations at mid-Logarithmic phase of cell growth and t= time between measurements of X_1 and X_2.

Emulsification Stability (E_{24}) Test

E_{24} of culture samples was determined by adding 2 ml of oil to the same amount of culture, mixing with a vortex for 2 min and leaving to stand for 24 hours. The E_{24} index is given as percentage of height of emulsified layer divided by total height of the liquid column (mm) [18].

Surface tension measurements

They were made with a Fisher Autotensiomat (Fisher Scientific Co., Pittsburgh, Pa.). Relative surfactin concentrations were determined by diluting the broth until the critical micelle concentration (CMC) was attained [19].

Surfactin isolation

The method described by [7] was used. Crude surfactin was isolated by adding concentrated hydrochloric acid to culture broth after removing biomass by centrifugation. A precipitate formed by decreasing the pH to 2 which could be collected, dried, and extracted with dichloromethane. The solvent was removed under reduced pressure to give an off-white solid. Further purification was achieved by recrystallization. The

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dichloromethane extract was dissolved in distilled water containing sufficient NaOH to give pH 7. This solution was filtered through Whatman no. 4 paper and reduced to pH 2 with concentrated HCl. The white solid was collected as a pellet after centrifugation.

RESULTS AND DISCUSSION

Cell growth and surfactin production in batch fermentation

Fig. 1 shows growth pattern and surfactin production from Maldex-15 by the *B. subtilis* BDCC-TUSA-3 grown in batch culture. As evidenced Madex-15 supported bacterial growth and multiplication with almost no lag phase (Fig. 1A). Maximum biomass concentration of 4.47 $g.l^{-1}$ was achieved after 20 h with simultaneous production of the maximum production of surfactin of 4.25 $g.l^{-1}$. This result was in good agreement with the data previously obtained [13]. Moreover, both pattern of cell growth and surfactin synthesis confirmed a typical process for growth associated product (Fig. 1). Similarly, [20] reported a maximum surfactin production in mid log phase. In addition, a growth associated production of biosurfactant has been reported for *B. subtilis* by [7] and [21] and for *Bacillus licheniformis* by [22]. The specific growth rate of the bacterium was calculated based on the plot of log (cell dry weight at mid log phase) versus time (Fig. 1A) as described in Materials and Methods section [17] using the following equation:

$$\mu = (\log X_2 - \log X_1) / \log e.t \qquad (1)$$

The bacterium achieved a specific growth rate of 0.435h^{-1}. Similarly, [11] developed a process for continuous surfactin production using airlift bioreactor utilizing *B.subtilis* and recording specific growth rates, reported in literature, 0.44 h^{-1}.

Exponential fed-batch strategy for surfactin production

The possibility of utilizing exponentially fed-batch process for production of surfactin by *B. subtilis* BDCC-TUSA-3 was

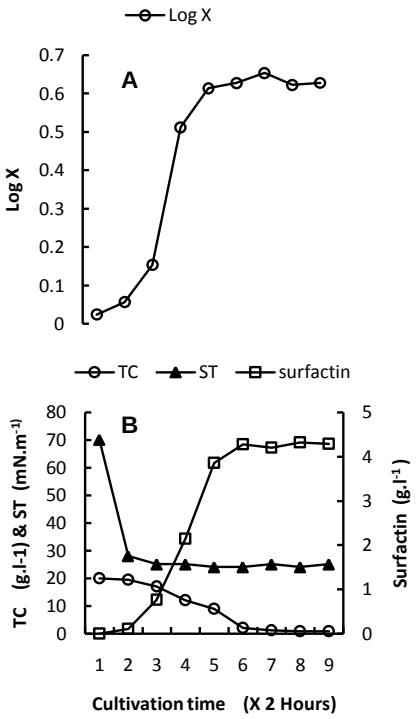

Figure 1. Patern of cell growth and surfactin formation by *B. subtilis* in batch culture. TC, total carbohydrates and ST, surface tension.

investigated. Supply of predetermined fermentative substrate (Maldex-15) and other nutrients on hourly basis was tested. In order to accomplish this, the specific growth rate of the bacterium had to be determined.

The equation previously developed by [14] and more recently adapted by [15] was used in order to satisfy Maldex-15 required for growth and surfactant production on hourly basis as follows:

$$S = 0.563\ X_0\ (e^{\mu t} - 1) \qquad (2)$$

Since S, is the hourly added Maldex-15; X_0, is the initial bacterial biomass (seed cells); μ, the specific growth rate; t, is the time.

Using the above-estimated specific growth rate (0.435 h^{-1}), the initial bacterial biomass (X_0) required to convert certain amount of Maldex-15 into biomass in 12 hours was calculated.

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Table 1. Initial amounts of biomass (seed cells) used during bacterial surfactin production and the required amounts of Maldex-15 in five different fermentation runs.

Fermentation runs	Run 1	Run 2	Run 3	Run 4	Run 5
Initial amounts of biomass (g)	0.657	0.986	1.314	1.643	1.971
Total amounts of added Maldex-15 (g)	68.0	102.0	136.0	170.0	204.0

Table 2. The hourly added Maldex-15 to *B. subtilis* grown in an exponential fed-batch fermentation for surfactin production.

Cultivation time (hr)	Hourly added Maldex-15 (g) in fermentation runs				
	Run 1	Run 2	Run 3	Run 4	Run 5
1	0.2016	0.3025	0.4031	0.5041	0.6047
2	0.3114	0.4674	0.6229	0.7788	0.9343
3	0.4812	0.7222	0.9623	1.2032	1.4435
4	0.7433	1.1155	1.4865	1.8590	2.2298
5	1.1485	1.7236	2.2969	2.8720	3.4453
6	1.7744	2.6629	3.5486	4.4370	5.3228
7	2.7413	4.1141	5.4824	6.8550	8.2235
8	4.2352	6.3560	8.4699	10.5906	12.7049
9	6.5434	9.8201	13.0858	16.3622	19.6287
10	10.1091	15.1713	20.2168	25.2787	30.3252
11	15.6182	23.4392	31.2343	39.0548	46.8516
12	24.1297	36.2129	48.2560	60.3384	72.3840
Total Maldex-15 added (g)	68	102	136	170	204

For instance, in order to calculate the initial amount of biomass required to convert 68 g of Maldex-15 into bacterial biomass and surfactin, equation (2), was used.

$$X_0 = 68/ [0.563 (e^{0.435x\ 12} – 1)]$$

$$(X_0) = 68/ [0.563 (e^{5.22} -1)]$$

$$= 68/ [0.563 (184.93 -1)]$$

$$= 68/103.55$$

$$= 0.657 \text{ gm cells}$$

Similarly, the initial biomass amounts for the other three fermentation runs were calculated. The produced data are tabulated in Table 1. Then, the obtained value for initial bacterial cells (X_0) was used to calculate the hourly added portions of Maldex-15 (S) over the first stage of fermentation run 1, using equation (2) as follows:

$$S = 0.563 \times 0.657 (e^{0.435t} – 1)$$

Where, t was substituted by 1 for the first hour, by 2 for the second hour and so on. Similarly, quantities of the hourly added Maldex-15 for the other four fermentation runs were calculated and tabulated in Table 2. Fermentation rums were conducted as described above and samples were taken, on hourly basis, and surface tension measurements for cell-free supernatants were determined. Other parameters such cell biomass, surfactin total carbohydrates were determined at end of each run.

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Table 3. Emulsification index (E24) of cell-free supernatant of five exponentially fed-batch runs with various petroleum hydrocarbons.

Cultivation Runs	Emulsification index (E_{24})		
	Motor oil	Crude oil	Kerosene
1	87	93	80
2	88	96	81
3	87	95	85
4	88	96	85
5	89	97	86

bacterial growth was recorded as the result of exponential feed regime utilized and biomass concentration increased with the increase of added glucose and the highest concentration (31.78 g.$^{l-}$1) was obtained with fermentation run supplied with the highest amount of Maldex-15 (204 g). This coincided with a similar increase in surfactin synthesis that led to a decrease in surface tension of fermentation broth for all runs (Fig. 2 A&B). The highest surfactin production of 36.07 g .l^{-1} was recorded for run 5, which is certainly a concentration favorable for further a cost effective downstream process. No residual carbohydrates were detected in fermentation runs 1, 2and 3 but not with fermentation runs 4 and 5 where 0.70 and 3.63 g .l^{-1} of total carbohydrates were detected. In previous study utilizing conventional batch fermentation (Amin 2010), only 5.96 and 4.65 g .l^{-1} of cell biomass and surfactin were obtained with residual carbohydrates of 1.73% of initial concentration. This represents more than 5 and 7 folds increase respectively, in favor of present study. The hourly added Maldex-15 was most probably assimilated rapidly during each respective hour to form new cells shutting off most of other metabolic pathways with a minimum loss of sugar to by-product synthesis similar to the findings of Pirt (1975) and Amin (2010).

With respect to kinetics of cell growth and surfactin production and compared to conventional batch fermentation [13], the proposed exponential feeding regime supported much higher growth yield, surfactin yield from Maldex-15 and cell biomass and volumetric reactor productivity of 0.240 $g_{c/s}$, 1.13 $g_{p/c}$0.272 $g_{p/s}$ and 2.775 g.l^{-1}.h^{-1} respectively (Table 3).

The effect of several feeding strategies on microbial growth and formation of biosurfactants have been reported by other investigators. A constant feeding flow rate to the bacterium *B. subtilis* BDCC-TUSA-3 did not support cell growth and surfactin production [9,21]. Fed-batch cultivation of *Pseudomonas aerugenosa* with pulse feeding strategy using multiple substrates (diesel and yeast extract), gave comparable rhamnolipid and biomass production of 13.4 and 24.1 g.l^{-1} respectively. However, much lower volumetric reactor productivity of 0.054 g.l^{-1}.h^{-1} was recorded due to extension of working time.

The proposed approach of exponential fed-batch fermentation process is highly satisfactory. Moreover, the catabolic repression that regulates formation of certain metabolic products at high glucose concentrations [12] must have been successfully avoided during the present study.

Maldex-15 and other nutrients were fed adequately to the growing cells at their specific growth rate maximizing cell growth and surfactin formation, yield and productivity.

Compared with other fermentation processes (Table 5), the proposed process exhibits superiority with respect to cell growth and surfactin production. While the highest biosurfactant concentration of 95 g .l^{-1} [23] is intriguing, the volumetric productivity for the present study is > 5 folds compared to this reported by the authors. Certainly, this represents a tremendous reduction in production costs when such process is to be considered for implementation on industrial scale.

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Table 4. Performance of *B. subtilis* in exponentially fed-batch cultivation process for surfactin production.

Total Maldex-15 added (g)	Maldex-15 conversion		Cell growth		Surfactin production			
	% *	Residual Carbohydrates ($g.l^{-1}$)	Biomass ($g.l^{-1}$)	Biomass yield (g_c/g_s)**	Final conc. Surfactin ($g.l^{-1}$)	Surfactin yield (Y)		Productivity ($g.l^{-1}.h^{-1}$)
						(g_p/g_c) **	(g_p/g_s)	
68	100	-	11.11	0.245	12.11	1.09	0.267	0.865
102	100	-	16.80	0.247	18.14	1.08	0.267	1.296
136	100	-	22.32	0.246	25.00	1.12	0.276	1.786
170	99.4	0.70	27.38	0.243	27.27	1.00	0.242	1.948
204	97.3	3.63	31.78	0.240	36.07	1.13	0.272	2.576

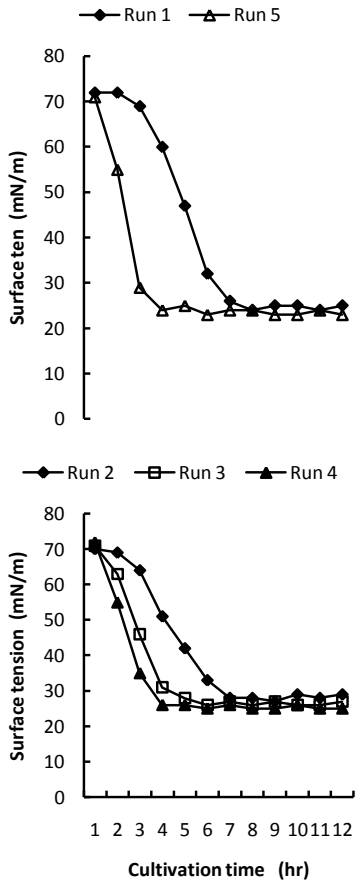

Figure 2. Pattern of surface tension of cell-free culture broth of exponentially fed-batch fermentation runs.

While the emulsification indices, ranged between 81-97%, recorded for the three hydrocarbons, Kerosine, crude oil and motor oil (Table 3), there were slight differences in emulsification activities of *B.subtilis* BDCC-TUSA-3 produced from the five fermentation runs. The emulsification indices obtained are very much comparable with those obtained for surfactin produced by *B. subtilis* [6], rhamnolipid [10] and for commercial chemical surfactants [21]. The highest emulsification index of 97% was achieved with crude oil, compared with 89% for motor oil and 86% for kerosene (Table 3). These finding shows that high quality surfactin produced under experimental conditions could be considered as a potential candidate to be used in bioremediation of petroleum contaminated sites or in microbial enhanced oil recovery applications.

CONCLUSION

Time course profiles showed that surfactin produced by *B . subtilis* BDCC-TUSA-3 was growth-associated and exhibited excellent emulsification properties. *B. subtilis* BDCC-TUSA-3 also showed a preferential emulsification of crude oil to motor oil and kerosene. With the proposed exponential feeding strategy, maximum concentrations of crude surfactin and cell biomass of 36.1 and 31.8 $g.l^{-1}$ respectively and volumetric reactor productivity of 2.78 $g.l^{-1}.h^{-1}$ were favorable compared to those reported in literature for both biologically produced and chemically synthesized surfactants in terms of quantity

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Table 5.Comparison of biosurfactant productivities in different cultivation systems.

References	Bio-surfactant	Cultivation system	Micro-organism	Fermen-tative substrate	Cell biomass ($g.l^{-1}$)	biosurfactant			
						Final conc. ($g.l^{-1}$)	Yield (g_p/g_c)	Yield (g_p/gs)	Pro-ductivity ($g.l^{-1}.h^{-1}$)
Amin (2011)	Surfactin	Batch culture	*B. subtilis*	Maldex-15	5.96	4.65	0.780	0.118	0.115
This work	Surfactin	Exponential fed-batch culture	*B. Subtilis*	Maldex-15	31.78	36.07	1.13	0.272	2.576
Kim et al., (2006)	Mannosyl-erythritol lipid	Conventional fed-batch culture	*Candida sp.*SY16	Glucose	-	95.0	-	-	0.479
Nu et al., 2012	Rhamnolipid	Conventional fed-batch culture	*Ps. Aeruginosa*	Diesel	24.0	13.4	-	-	0.065
Makkarod and Cameotra (2001)	Surfactin	Batch culture with foam fractionation	*B. subtilis*	Sucrose	-	4.50	-	-	0.500
Gong et al., 2009	Surfactin	Batch culture	*B. subtilis* E8	Starch	6.50	12.0	0.894	-	0.375

and quality. Thus, the proposed process of surfactin production from Maldex-15,a by-product recovered during manufacturing of high fructose syrup from corn starch, seemed to achieve the requirements for commercial applications. Compared to a conventional batch reactor of 100 m^3 producing from 4% (w/v) Maldex-15 about 465 kg of surfactin in 30 h (Amin 2010), the exponentially fed-batch reactor with only 5 m^3 would produce 180 kg surfactin from 20.4% (w/v) Maldex-15 in 30 h.

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About Author

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