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# Molecular Characterization of *Bacillus subtilis* Surfactin Producing Strain and the Factors Affecting its Production

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## ABSTRACT

The PCR amplification of 16S rRNA gene and surfactin gene from the 14 *B. subtilis* isolates capable of producing a lipopeptide biosurfactant (surfactin) was performed. Only two isolates producing a high yield of surfactin were selected and subjected to molecular characterization, identification and optimization of their surfactin production. These two isolates were identified as *B. subtilis* BBk1 strain AB01335-1 and strain AB02238-1 by nucleotide sequence of 16S rRNA and surfactin gene was determined. A high yield of surfactin was obtained from a culture of *B. subtilis* using carbohydrate substrate as a carbon source; among carbohydrates glucose enhanced the best surfactin production. The optimum glucose concentration was 40 g/L. $NH_4NO_3$ was the best nitrogen source for surfactin production at a concentration of 4.5 g/L at initial pH 6.5- 7, incubation temperature 30$^o$C, 90% volumetric $O_2$ and agitation speed 200 rpm. The optimum incubation time was 72 h and inoculum size 1.5-2 % (v/v). A high yield of surfactin was elucidated upon addition of 40 mg/L $MnSO_4$ and 6 mM $FeSO_4$.

**Key Words:** *Bacillus subtilis;* Surfactin gene; 16srRNA gene; PCR; Sequencing

## INTRODUCTION

Biosurfactant(s) spontaneous release and function are often related to hydrocarbon uptake; therefore they are predominantly synthesized by hydrocarbon-degrading microorganisms (Banat, 1995). Some biosurfactants, however, have been reported to be produced on water-soluble compounds, such as glucose, sucrose, glycerol or ethanol (Passeri, 1992; Hommel & Huse, 1993). Furthermore, they can be produced using relatively simple and inexpensive procedures and substrates (Lang & Wullbrandt, 1999; Makkar & Cameotra, 1999).

Different strategies must be devised and explored to reduce production costs, include high yields and product accumulation, economical engineering processes, and use of cost-free or cost-credit feed stocks for microbial growth and surfactant production (Makkar & Cameotra, 1999).

The foci for reduction of biosurfactant production costs are the microbes (selected, adapted, or engineered for high yields of product) (Makkar & Cameotra, 1999).

Environmental factors and growth conditions such as temperature, agitation, and oxygen availability also affect biosurfactant production through their effect on cellular growth or activity (Cameotra & Makkar, 1998).

Production under growth limiting conditions is characterized by a sharp increase in the biosurfactant level as a result of limitation of one or more of medium components. A number of investigations have demonstrated an over production of biosurfactants by *Pseudomonas* sp. when the culture reaches the stationary phase of growth, due to limitation of nitrogen and iron (Guerra-Santos *et al.,* 1986; Mulligan & Gibbs, 1989).

Iron concentration has a dramatic effect on rhamnolipid production by *Pseudomonas aeruginosa*, resulting in a three-fold increase in production when cells were shifted from medium containing 36 µM iron to medium containing 18 µM iron (Guerra-Santos *et al.,* 1984; 1986).

Iron limitation stimulates biosurfactant production in *Pseudomonas fluorescens* (Persson *et al.,* 1990 a,b), and *Pseudomonas aeruginosa* (Guerra-Santos *et al.,* 1984, 1986). Whereas addition of iron and manganese salts stimulates biosurfactant production in both *B. subtilis* (Cooper *et al.,* 1981), and *Rhodococcus* sp. (Abu-Ruwaida *et al.,* 1991).

The pH of the medium plays an important role in sophorolipid production by *T. bombicola* (Gobbert *et al.,* 1984). Rhamnolipids production by *Pseudomonas* sp. was at its maximum in a pH range of 6.0 - 6.5 and decreased sharply above pH 7.0 (Guerra-Santos *et al.,* 1984). Powalla *et al.* (1989) in their study on penta- and disaccharide lipids production by *Nocardia corynebacteroides* found it to be unaffected by the pH in the range of 6.5-8.0.

The *Bacillus subtilis* strain is the bacterial species most often studied for the production of surfactin (Rongswang *et al*., 2002). Three genetic loci, srfA operon, com A and sfp are essential for surfactin production in *B. subtilis*. The srfA operon encode protein which forms a non-ribosomal peptide synthetase complex (surfactin synthetase) (Cosmina *et al*., 1993). However, com A, encodes a transcription activator of the srf A gene (Roggiani & Dubnau, 1993) and sfp encodes 4\ -phosphopantetheinyl transferase (sfp/pptase) an

activating enzyme of srf A multienzyme complex (Nakano *et al*., 1992; Lambalot *et al*., 1996). sfp / pptase converts the inactive protein which form surfactin synthetase to active forms (Pfeifer *et al*., 2001). Thus pptase play an important role for the production of biosurfactants, antibiotics and polyketide (Rongswang *et al*., 2002).

The present study deals with molecular characterization of *B. subtilis* strains capable of producing surfactin and optimizing the cultural conditions to maximize the surfactin yield and analyzing the individual organism and interactive effects of these critical parameters on surfactin production.

## MATERIALS AND METHODS

**Isolation of DNA of *B. subtilis*.** Genomic DNA of the studied isolates was extracted by pure Gene (DNA purification Kit, USA).

**PCR amplification of 16S rRNA and surfactin gene from *B. subtilis*.** The PCR amplification was performed using a PCR machine (Gene Amp 2400, Perkin Elmer Co, Norwalk, CT). The 16S rRNA gene in *B. subtilis* was amplified by PCR using the following universal primers, Forward: 5`- AAGAGTTTGATCATGGCTCAG-3` and reverse: 5` AGGAGGTGATCCAACCGCA-3` and the PCR program was set to denaturation at 94°C for 1 min annealing at 55°C for 1 min and extension at 72°C for 1 min for a total of 35 cycles. Also, the surfactin gene in *B. subtilis* was amplified by PCR using the following primers, forward: 5`-CTAGAATTCAGATTTACGGAATTTATATG-3` and reverse:5`-GGGGAATTCAGGGTGTGCGGCGCATAC-3` and the PCR program was set to denaturation at 95°C for 1 min. annealing at 55°C for 1 min. and extension at 72°C for 1 min for a total of 30 cycles. The PCR primers were synthesized at Finan Specialized Gene Technology Center, Egypt on Abi (Applied biosystem 394 DNA/RNA synthesizer). The PCR product was determined by comparing them with 100 bp ladder marker (Amersham Pharmacia Biotech, USA).

**Extraction of crude biosurfactant.** The crude biosurfactant was isolated from the cell free broth of 72 h grown culture. The bacterial cells were removed from surfactant containing culture broth by centrifugation at 10.000 rpm at 4°C for 20 min. The supernatant was precipitated overnight at 4°C by adding concentrated HCl to achieve a final pH of 2.0, to precipitate lipids and proteins. Grey white pellets formed by precipitation were collected by centrifugation at 10.000 rpm at 4°C for 20 min. The pellets were left to dry and surfactin was extracted.

**Extraction of surfactin.** The dried biosurfactant was resuspended in dichloromethane in a separating funnel and shaken vigorously; surfactin was recovered in the organic layer at the top. The extraction was performed twice, and the organic layers were pooled and evaporated. The residue was re-dissolved in water.

**Effect of different carbon sources.** Different carbon sources were added to the production medium replacing glucose. Carbohydrates such as galactose, lactose, maltose, mannitol, sucrose, starch, fructose or vegetable oils including sun flower oil, corn oil, castor oil or olive oil. Also, hydrocarbons involving paraffin oil, n- hexadecane or glycerol were tested. To 50 mL mineral salt medium, the mentioned carbon sources were added individually and the medium was inoculated with an over night broth culture and incubated with shaking at 150 rpm at 30°C for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of glucose concentration**. From an over night broth culture, 50 mL sterile glucose mineral salt medium were inoculated. Final glucose concentration reaches between 5 - 60 g/L in 10 g interval. Cultures were incubated with shaking at 150 rpm at 30°C for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of different nitrogen sources.** Different organic nitrogen sources such as urea, beef extract, yeast extract or casein hydrolysate and inorganic nitrogen sources including $NH_4Cl$, $NH_4NO_3$, $(NH_4)_2SO_4$, $(NH_4)_2S_2O_8$, $NH_4Mo$, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, $NH_4HCO_3$ and $NaNO_3$ were added to the production medium replacing ammonium nitrate in 50 mL mineral salt medium. The medium was inoculated with an over night broth culture and incubated with shaking at 150 rpm at 30°C for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of different ammonium sulfate concentrations.** From an over night broth culture, 50 mL sterile mineral salt medium were inoculated. The medium contain from 2- 10 g/L ammonium nitrate in 2 g interval. Cultures were incubated with shaking at 150 rpm at 30°C for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of pH.** Fifty ml sterile glucose mineral salt medium in 250 mL Erlenmeyer flasks were adjusted at different pH values from 5- 9 in 0.5 interval were inoculated with an over night culture of the strains under study and incubated for 72 h at 30°C with shaking at 150 rpm. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of incubation time.** In 250 mL Erlenmeyer flasks, 50 mL sterile glucose mineral salt medium were inoculated with an over night culture of the strains under study and incubated at 30°C with shaking at 150 rpm for 24 - 144 h in 24 h interval. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of agitation speed.** In 250 mL Erlenmeyer flasks, 50 mL sterile glucose mineral salt medium were inoculated with an over night culture. The medium incubated at 30°C for 72 h with shaking at different agitation speed from 0-200 rpm in 50 rpm interval. The cells were removed by centrifugation and supernatant was used for estimation of

surfactin concentration.

**Effect of aeration.** To in 250 mL Erlenmeyer flasks, containing sterile glucose mineral salt medium in different volumes ranging from 25, 50, 75, 100, 125, 150 and 175 mL corresponding to volumetric oxygen percentage of 90, 80, 70, 60, 50, 40 and 30% respectively. The medium was inoculated with an over night culture of the strains under study and incubated at 30°C for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of inoculum size.** From an over night broth culture, 50 mL sterile glucose mineral salt medium were inoculated with different inoculum size from an over night broth culture of $10^6$ cfu/mL in volumes from 0.25- 3.0 mL in 0.25 mL interval. Cultures were incubated at 30°C with shaking at 150 rpm for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of incubation temperature.** From an over night broth culture, 50 mL sterile glucose mineral salt medium were inoculated with an over night broth culture. Cultures were incubated with shaking at 150 rpm at different temperatures ranging from 25- 60°C in 5°C interval for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of ferrous sulfate concentration.** Different ferrous sulfate concentrations were added to 50 mL sterile glucose mineral salt medium, in 250 mL Erlenmeyer flasks, to reach final concentration of ferrous sulfate as 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 mM/L. the medium was inoculated with an over night culture of the strains under study and incubated at 30°C with shaking at 150 rpm for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Effect of manganese sulfate concentration.** Different manganese sulfate concentrations were added to 50 mL sterile glucose mineral salt medium, in 250 mL Erlenmeyer flasks, to reach final concentration of manganese sulfate as 10, 20, 30, 40, 50, and 60 mg/L. the medium was inoculated with an over night culture of the strains under study and incubated at 30°C with shaking at 150 rpm for 72 h. The cells were removed by centrifugation and supernatant was used for estimation of surfactin concentration.

**Nucleotide sequence analysis.** The nucleotide sequence of *B. subtilis* 16S rRNA and surfactin gene was determined by dideoxy- chain termination method (Sanger *et al*., 1977) using an autosequencer (Alfexpress DNA sequencer) from Pharmacia Biotech. DNA sequencing data was analyzed by using DNASIS software (Hitach Software, Tokyo, Japan) and BLAST program (NCBI, Bethesda, USA).

## RESULTS AND DISCUSSION

Structural and biochemical characterization of a biosurfactant produced by 14 bacterial isolates belonging to *B. subtilis* isolated from soil was performed in a previous work (Bashandy *et al*., 2004) the isolates were selected by measuring the oil film collapsing activity.

The PCR amplification of 16S rRNA gene (Fig. 1) and surfactin gene (Fig. 2) from the 14 *B. subtilis* isolates capable of producing a lipopeptide biosurfactant (surfactin) was performed.

Among these 14 isolates, two of them were superior in surfactin production. These two isolates were subjected to sequencing.

The bases of 16S rRNA gene were sequenced. Comparative sequences of analysis suggest that the two isolates belong to the Bacillus group, with highest identity to *B. subtilis* (BBk1) AB01335-1 and AB02238-1.

The alteration of variables such as nitrogen source, carbon source and agitation speed may influence surfactin production. Some studies had been undertaken with the objective of optimizing surfactin production by the alteration of salient variables such as medium composition, agitation speed and aeration rate.

It was demonstrated that different carbon sources in the medium affected the composition of bisurcatant production (Syldatrk & Wagner, 1987). When the production medium was supplemented with different carbohydrates as sole carbon sources (Fig. 3), there was a significant increase of surfactin yield in *B. subtilis* (BBk1) AB01335-1 and AB02238-1 compared with other used carbon sources. This indicated that glucose, sucrose and mannose enhanced the production of high yield of surfactin when added to the production medium of *B. subtilis* (BBk1) AB01335-1 and AB02238-1.

The addition of hydrocarbons or vegetable oils to the production medium resulted in a low level of surfactin production compared with carbohydrates especially glucose. Unlike the microorganisms producing glycolipid, polymeric or fatty acid type biosurfactants, such as *Candida* sp (Kitamoto *et al.*, 1993; Hommel *et al.*, 1994), *Pseudomonas* sp. (Zhang *et al*., 1992), *Acenirobacter* sp. (Navon-venezia *et al*., 1995). *B. subtilis* require only carbohydrates to produce lipopeptide type biosurfactants like other *Bacillus* sp. (Horowitz *et al*., 1990; Banat, 1993; Lin *et al*., 1994).

Since the production medium was optimized with glucose as sole carbon source, different concentrations of glucose were examined for the best yield of surfactin from the studied strains. Glucose was added to the production medium in concentrations, 5; 10; 20; 30; 40; 50 and 60 g/L, the results obtained (Fig. 4) elucidated that there was a significant increase in surfactin production upon addition of glucose up to 30 g/L, although there was a linear increase in surfactin concentration with increasing the initial glucose concentration up to 30 g/L.

Medium constituents other than carbon sources also affect the production of surfactin. The selected *B. subtilis* strains were cultivated in mineral salt medium containing, individually, different organic or inorganic nitrogen sources. The results obtained (Fig. 5) showed that inorganic nitrogen

**Fig. 1. Electrophoresis analysis of 16s rRNA gene of the selected isolates. M, DNA marker (100 bp ladder, Amersham Pharmacia-Biotech USA); lane 1, isolate 210; lane 2, isolate 13; lane 3, isolate 266; lane 4, isolate 5; lane 5, isolate 7; lane 6, isolate 186; lane 7, isolate 267; lane 8, isolate 262; lane 9, isolate 157; lane 10, isolate 192; lane 11, isolate 11; lane 12, isolate 8; lane 13, isolate 15; lane 14, isolate 205**

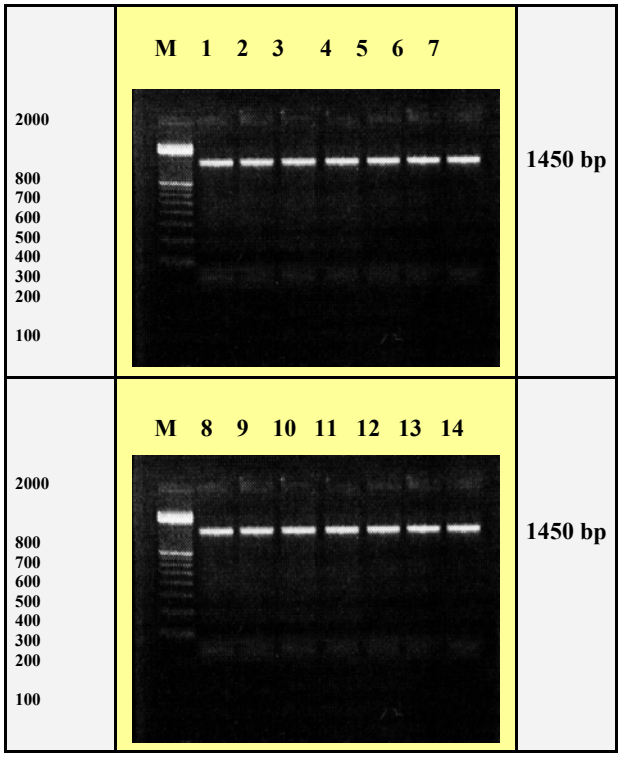



**Fig. 2. Electrophoresis analysis of s*fp* gene of the selected isolates. M, DNA marker (100 bp ladder, Amersham Pharmacia-Biotech USA); lane 1, isolate 210; lane 2, isolate 13; lane 3, isolate 266; lane 4, isolate 5; lane 5, isolate 7; lane 6, isolate 186; lane 7, isolate 267; lane 8, isolate 262; lane 9, isolate 157; lane 10, isolate 192; lane 11, isolate 11;lane 12, isolate 8; lane 13, isolate 15; lane 14, isolate 205**

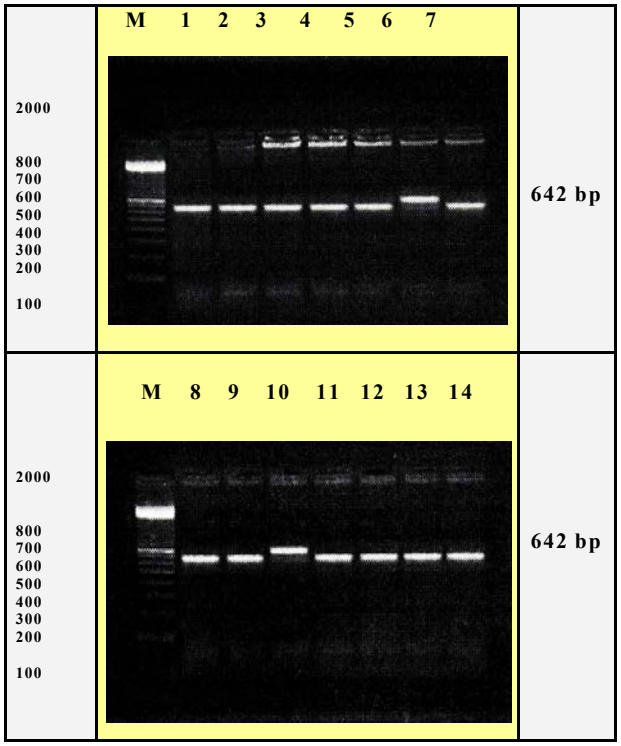



**Fig. 3. Influence of carbon sources on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1**

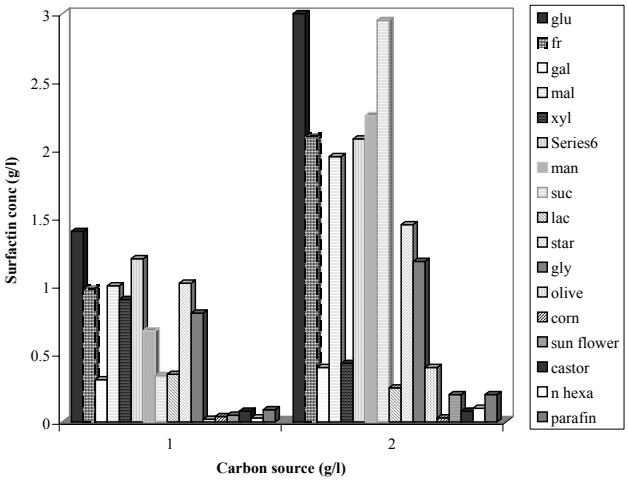



**Fig. 4. Influence of glucose concentration on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-**

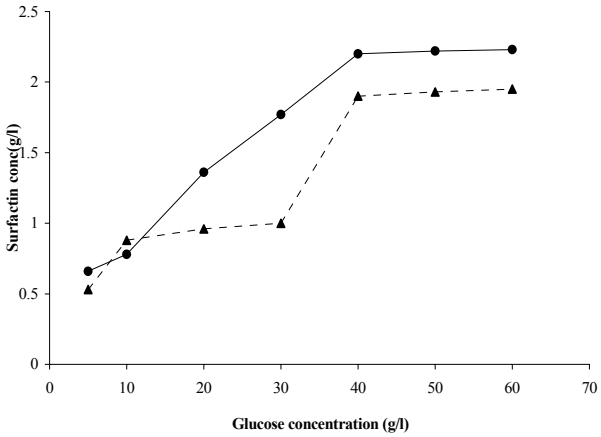


**Fig. 5. Influence of nitrogen sources on surfactin production from selected B. subtilis (BBk1). ▲, AB01335-1; ●, AB02238-1**

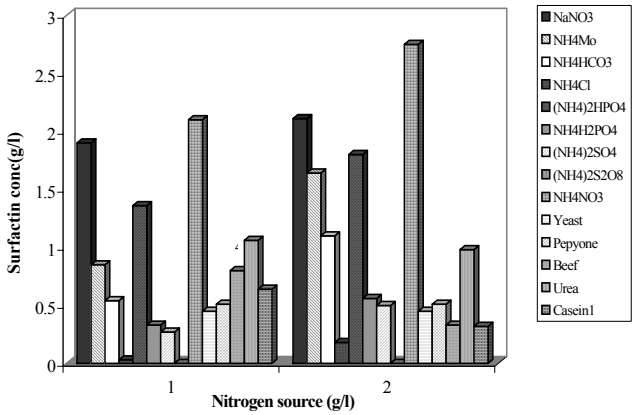



**Fig. 6. Influence of $(NH_4)_2NO_3$ concentration on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1**

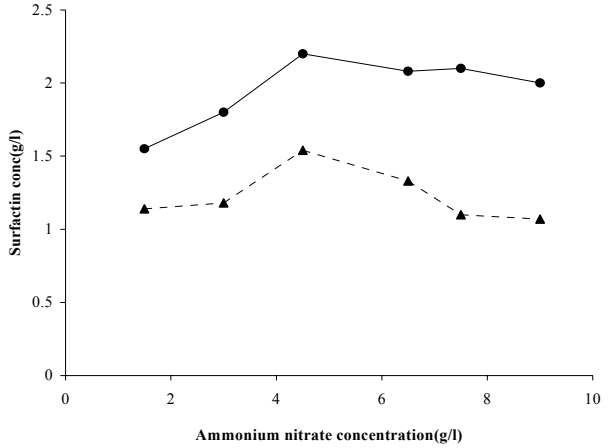



**Fig. 7. Influence of initial pH on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1**

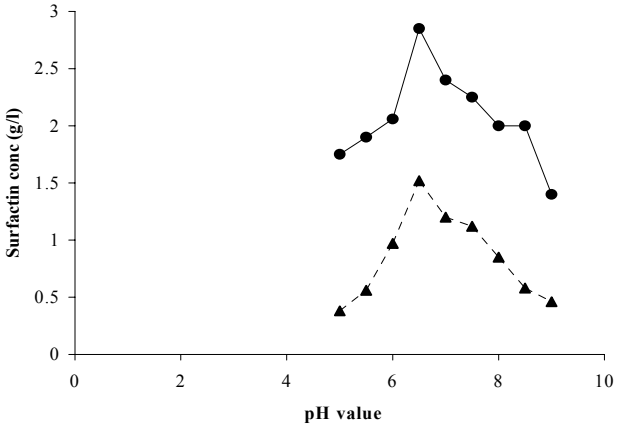



**Fig. 8. Influence of incubation time on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1**

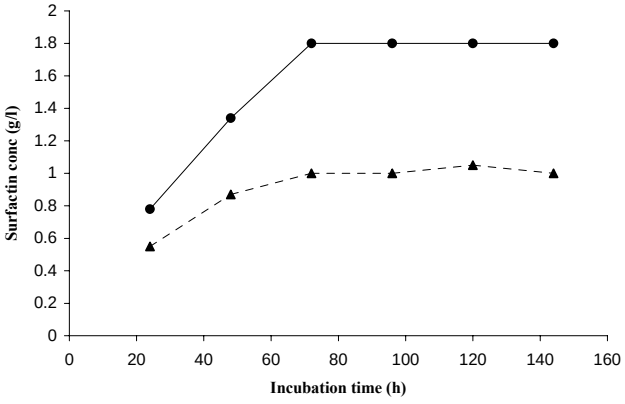



**Fig. 9. Influence of agitation speed on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1**

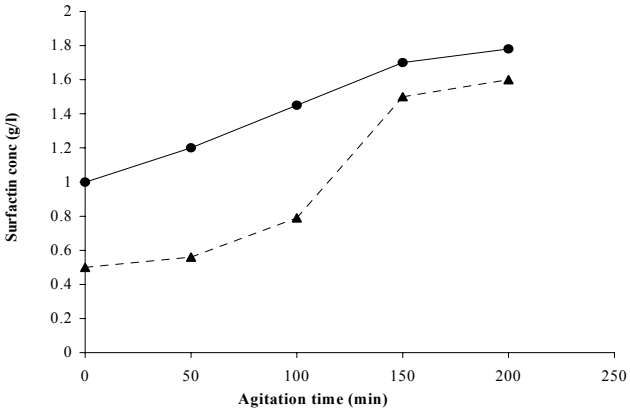
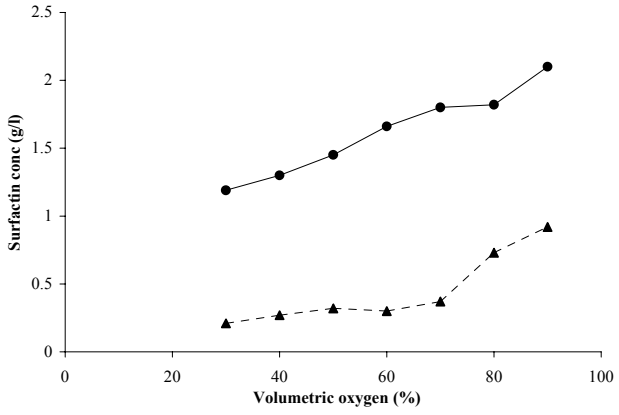



**Fig. 10. Influence of volumetric $O_2$ on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1**



sources are efficiently enhanced the strains for production of surfactin than organic nitrogen sources. Also, it was observed that among the inorganic nitrogen sources, $NH_4NO_3$, and $NaNO_3$ gave a high yield of surfactin concentration compared with the other inorganic nitrogen sources used in this study. It has been demonstrated that ammonium salts and urea among the inorganic nitrogen salts preferred for biosurfactant production in *Arthobacter sp.*, whereas nitrate supported maximum surfactin production in *Ps. Aeruginosa* (Guerra-Santos *et al.,* 1984; Robert, 1989; MacElwee *et al*., 1990).

The strains under study were examined for surfactin production after addition of different concentrations of $(NH_4)_2NO_3$ to the production medium. It was observed that (Fig. 6), ammonium nitrate concentrations below or above 4.6 g/L resulted in low level of surfactin concentration in all the studied strains.

Environmental factors and growth conditions such as temperature, agitation, and oxygen availability also affect biosurfactant production through their effect on cellular growth or activity (Cameotra & Makkar, 1998).

The pH of the medium and incubation temperature plays an important role in biosurfactant production. As shown in Fig. 7, there were very low yield of surfactin when the production medium was adjusted at pH values lower than or above 6.5 and the maximum yield of surfactin concentration was achieved at pH 6.5- 7.

The pH of the medium plays an important role in

**Fig. 11. Influence of inoculum size on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1**

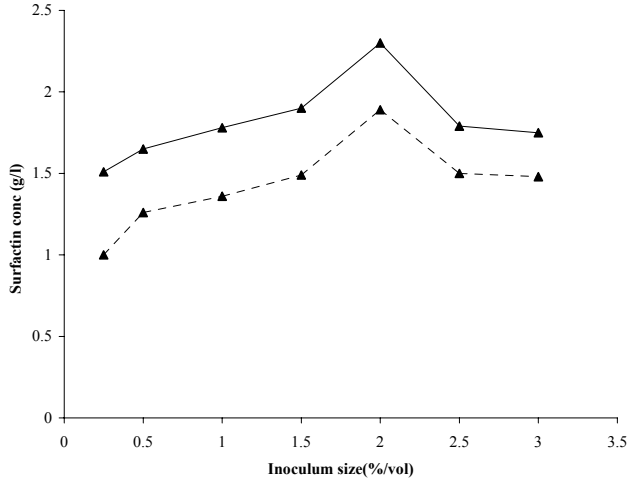



**Fig. 12. Influence of incubation temperature on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1**

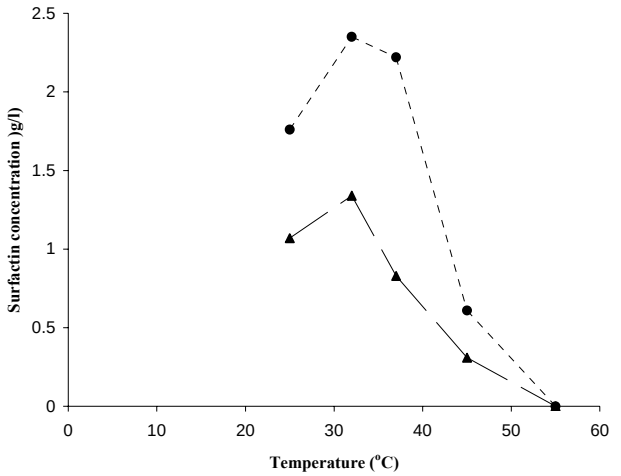



**Fig. 13. Influence of $FeSO_4$ on surfactin production from selected *B. subtilis* (BBk1). ▲, AB01335-1; ●, AB02238-1.**

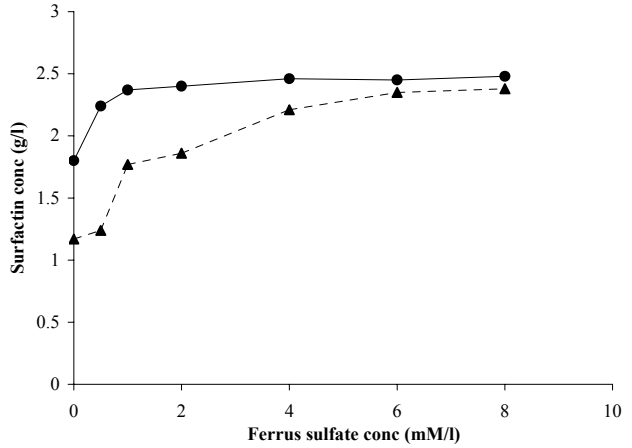
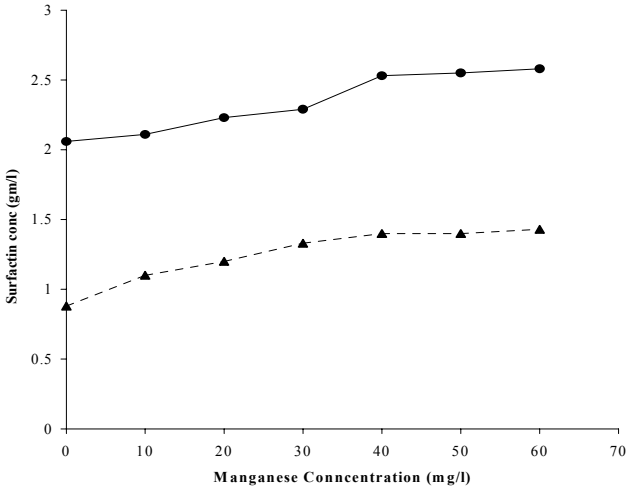



**Fig. 14: Influence of $MnSO_4$ on surfactin production from selected *B. subtilis* (BBk1) ▲,AB01335-1; ●,AB02238-1**



sophorolipid production by *Torulopsis bombicola* (Gobbert *et al.,* 1984). Rhamnolipids production by *Pseudomonas* sp. (Guerra-Santos *et al.,* 1984) was at its maximum in a pH range of 6.0-6.5 and decreased sharply above pH 7**.** Powalla *et al.* (1989) in their study on penta- and disaccharide lipids production by *Nocardia corynebacteroides* found it to be unaffected by the pH in the range of 6.5 - 8.0.

An indispensable but variable input to a fermentation process is inoculum or seed culture. The development and management of the inoculum through various stages has a definite effect on the subsequent performance and economics of the process, it is well known that the age and density of inoculum used directly influences the duration of lag phase, specific growth rate, biomass yield and quality of the final product.

Therefore, when the surfactin production medium was inoculated with the studied strains and incubated for different incubation times from 24- 144 h, it was observed that (Fig. 8) surfactin concentration increases with the increase in incubation period up to 72 h but no increase in surfactin concentration was observed above this level on further increase in incubation period.

The agitation speed was also investigated in this study (Fig. 9), the inoculated cultures were incubated at 30°C at different agitation speed from 0- 200 rpm, it was demonstrated that an increase in agitation speed up to 150 rpm results in an increase in surfactin production but not more. This may be due to the effect of shear on the studied *Bacillus* sp above 150 rpm. In this respect biosurfactant production in yeast was increased with increasing the agitation speed (Shepperd & Cooper, 1990). They concluded that oxygen transfer is one of the key parameters for the process, optimization and scale up of surfactin production in *B. subtilis*.

The influence of the percentage of volumetric oxygen on surfactin production was studied (Fig. 10). The selected *B. subtilis* strains were inoculated in mineral salt medium in different volumes ranging from 25, 50, 75, 100, 125, 150 and 175 mL corresponding to volumetric oxygen percentage of 90, 80, 70, 60, 50, 40 and 30% respectively. The obtained results showed that there was an increase in surfactin production with increasing the percentage of volumetric oxygen up to 90%.

Sheppered and Cooper (1990) found that volumetric oxygen transfer is a key parameter in surfactin production from *B. subtilis*.

When the strains were inoculated into the production medium, the inoculum size was adjusted from 0.5 to 3.5 % (v/v). The data provided (Fig. 11) showed that the inoculum size less or more than 2 % (v/v) resulted in a low level of surfactin yield, indicating that the optimum size of the inoculum that should be added to the production medium was 2 % (v/v).

The culture of the selected *B. subtilis* strains was incubated at different temperatures from 25- 60°C. As illustrated in Fig. 12, the incubation temperature was found to have a great effect on surfactin production from the studied strains. The temperature values below or above 30°C caused a low yield of surfactin production, the biosurfactant concentration reached its maximum at temperature 30°C.

In *Arthobacter* sp, (Morikawa *et al*., 1993) and *Pseudomonas* sp. (Syldatrk *et al*., 1985) temperature causes alteration in the composition of the produced biosurfactant.

Surfactin production was performed with mineral salt medium containing different initial concentration of $FeSO_4$ (0, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 mM. It was observed (Fig. 13) that there was an increase in the surfactin concentration with increasing $FeSO_4$ concentration in the production medium, this may be due to that high concentrations of iron enhances surfactin production in these strains.

This finding is in consistent with Yu *et al*. (2003) who demonstrated that a *B. subtilis* strain was used to produce surfactin and they reported that the addition of iron at an appropriate amount significantly improved the biosurfactant production.

This could be explained in view that iron is a common cofactor for microbial enzymes and proteins and thus it is an essential mineral nutrient to microorganisms, which have developed a variety of strategies for acquiring iron, while simultaneously protecting them from the potential toxicity of iron.

The effects of some inorganic salts on the biosurfactant production were investigated. The addition of $MnSO_4$ was resulted in significant enhancement of biosurfactant production. The results obtained (Fig. 14) showed that there was an increase of surfactin production with the increase in $MnSO_4$ concentration and the highest biosurfactant concentration was attained at a concentration of 50 mg/L of $MnSO_4.4H_2O$. It was observed that the highest biosurfactant concentration was attained at a concentration of 50 mg/L of $MnSO_4$ $.4H_2O$ in *B. subtilis* (Cooper *et al*., 1981; Kim *et al*., 1997).

It could be concluded that the surfactin production medium from *B. subtilis* strains as mineral salt medium should contain glucose as sole carbon source at concentration of 30 g/L and $(NH4)_2NO_3$ as a nitrogen source at concentration of 4.5 g/L. the optimum incubation temperature is 30°C, with 90% volumetric oxygen, at initial pH 6.5- 7. The optimum incubation time is 72 h with shaking at 150 rpm and inoculum size, 1.5- 2 % (v/v). A high yield of surfactin was obtained upon addition of 40 mg/L $MnSO_4$ and 6 mM $FeSO_4$.

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