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Optimization of Surfactin Production by *Bacillus subtilis* Isolate BS5

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Abstract *Bacillus subtilis* BS5 is a soil isolate that produces promising yield of surfactin biosurfactant in mineral salts medium (MSM). It was found that cellular growth and surfactin production in MSM were greatly affected by the environmental fermentation conditions and the medium components (carbon and nitrogen sources and minerals). Optimum environmental conditions for high surfactin production on the shake flask level were found to be a slightly acidic initial pH (6.5–6.8), an incubation temperature of 30°C, a 90% volumetric aeration percentage, and an inoculum size of 2% *v/v*. For media components, it was found that the optimum carbon source was molasses (160 ml/l), whereas the optimum nitrogen source was $NaNO_3$ (5 g/l) and the optimum trace elements were $ZnSO_4 \cdot 7H_2O$ (0.16 g/l), $FeCl_3 \cdot 6H_2O$ (0.27 g/l), and $MnSO_4 \cdot H_2O$ (0.017 g/l). A modified MSM (molasses MSM), combining the optimum medium components, was formulated and resulted in threefold increase in surfactin productivity that reached 1.12 g/l. No plasmid could be detected in the tested isolate, revealing that biosurfactant production by *B. subtilis* isolate BS5 is chromosomally mediated but not plasmid-mediated.

Keywords Surfactin · *Bacillus* · Production · Optimization

Introduction

Naturally occurring surface-active compounds derived from microorganisms have gained attention in the past few decades because of their biodegradability, low toxicity, ecologic acceptability, and ability to be produced from renewable and cheaper substrates [1, 2]. Biosurfactants find applications in the cosmetic, pharmaceutical, and food industries as emulsifiers, humectants, dispersants, and detergents [3, 4]. Moreover, they are suited for

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environmental applications such as bioremediation, dispersion of oil spills, and waste treatment [3].

Among the many classes of biosurfactants, lipopeptides are of great interest because of their high surface activities and therapeutic potential [5]. Surfactin is one of the most efficient biosurfactants so far known which belongs to the lipopeptide family excreted by *Bacillus subtilis* spp. [6]. The increasing interest in surfactin is because of its amphiphilic character, which is responsible for its excellent surface-active properties as it reduces the surface tension of water from 72 to 27 mN/m at a concentration as low as 0.005% [7]. In addition, surfactins exhibit diverse biological activities such as antiviral and antimycoplasma [8, 9], antitumoral [10], inhibition of fibrin clot, and antibacterial properties [7].

Although promising, biosurfactants, in general, compete with difficulty against the chemically synthesized compounds on the surfactant market because of their high production costs (at least 50 times more expensive, depending on the biosurfactant and its purity) [11, 12]. Production cost is considered the bottleneck of many biotechnological processes [5]. The success of biosurfactant production depends, in one of its strategies, on the development of cheaper processes and the use of low-cost raw materials, which account for 10–30% of the overall cost [5]. Molasses [13], peat hydrolysate [14], and potato process effluents [15] are examples of alternative substrates that have been suggested for biosurfactant production by *B. subtilis*. Other strategies include optimizing the different environmental and media components which affect production [16]. Many authors reported the effect of environmental factors, pH, temperature, and aeration on the bacterial cell growth and biosurfactant production [17]. The influence of metal ions on biosurfactant production has been reported by some other authors [18, 6]. Others reported the pronounced effect of carbon source used in bacterial culture on biosurfactant production [19, 20]. Some authors also reported the effect and the role of nitrogen sources on the production of surface-active compounds by microorganisms [21]. Therefore, in this study, the effect of these factors on surfactin production by *B. subtilis* isolate BS5 was studied. This isolate was recovered from Egyptian soil through an extensive screening program and showed potential surfactin productivity [22]. The study aimed at improving surfactin production, taking into account the economic considerations of the production process; this was accomplished through optimizing the environmental and nutritional production conditions. Plasmid extraction procedure was also performed in the *B. subtilis* isolate BS5 to know whether production is chromosomally or plasmid-mediated.

Materials and Methods

Microorganisms

B. subtilis isolate BS5 is a promising surfactin producer obtained through an extensive screening program [22]. *Escherichia coli* DH5α/pUC18 (Hanahan 1983; Vieira and Messing 1982) was kindly provided by Dr. Khaled Abou-Shanab (Faculty of Pharmacy, Ain Shams University, Cairo, Egypt).

Culture Media

The basal mineral salts medium (MSM) and the modified MSM (M1, M2, and M3) were used in this study and their compositions are listed in Table 1.

Table 1 Composition of the culture media used for surfactin production by *B. subtilis* isolate BS5.

Nature of ingredients	Basal medium	Modified media		
	MSM	M1	M2	M3[a]
C-source (amount/l)	Glucose (20 g)	Glucose (20 g)	Glucose (20 g)	Molasses[b] (160 ml)
N-source (amount/l)	$NaNO_3$ (2.5 g)	$NaNO_3$ (2.5 g)	$NaNO_3$ (5 g)	$NaNO_3$ (5 g)
Minerals (amount/l)	$ZnSO_4 \cdot 7H_2O$ (0.0015 g) $FeSO_4 \cdot 7H_2O$ (0.0005 g) $MnSO_4 \cdot H_2O$ (0.0015 g) H_3BO_3 (0.0003 g) $CuSO_4 \cdot 5H_2O$ (0.00015 g) $Na_2MoO_4 \cdot 2H_2O$ (0.0001 g) $CaCl_2 \cdot 2H_2O$ (0.05 g)	$ZnSO_4 \cdot 7H_2O$ (0.16 g) $FeCl_3 \cdot 6H_2O$ (0.27 g) $MnSO_4 \cdot H_2O$ (0.017 g)		
Other common ingredients (amount/l)	$MgSO_4 \cdot 7H_2O$ NaCl KCl H_3PO_4 (85%) dH_2O		0.4 g 1 g 1 g 10 ml To 1,000 ml	
KOH pellets (for pH adjustment)			about 16 g (to give pH 7.2)	

[a] M3 is the optimized medium.

[b] Stock molasses solution was prepared by suspending an amount of local grade molasses (Sugars and Complementary Industries, Hawamdeya, Egypt) in an equal amount of dH_2O and then exposed to the steam of boiling water bath for 1 h in a measuring cylinder. The supernatant was then collected and used for medium preparation.

Fermentative Production of Surfactin

The seed culture was prepared by transferring a loopful from a fresh culture grown onto nutrient agar into 25 ml MSM contained in 250 ml Erlenmeyer flask. The flask was incubated at 250 rpm and 30°C for 36 h.

The production process was carried out, unless otherwise mentioned, in Erlenmeyer flasks (250 ml) containing 50 ml aliquots of the fermentation medium under test. The flasks were inoculated with the seed culture at 2% *v*/*v* and incubated in a shaking incubator (250 rpm) at 30°C for 7 days (for time course experiments) and 3 days (other experiments). At different time intervals (time course experiments) or at the end the incubation period (other experiments), the fermentation broth was sampled for determination of biomass, biosurfactant concentration, and biosurfactant activity.

Analytical Methods

Biomass Determination

Cellular growth was expressed in terms of dry cell weight which was calculated from the equation of a calibration curve constructed between optical density ($OD_{578\ nm}$) and dry cell weight of the tested isolate *B. subtilis* BS5 [23].

$$\text{Dry weight (g\%)} = OD_{578\,nm}/65.648$$

Surfactin Concentration

The gravimetric method described by Nitschke and Pastore [5] was used for the determination of surfactin concentration with minor modification. The culture broth was centrifuged at 10,000×*g* for 10 min to prepare the cell free supernatant (CFS). An aliquot of the CFS was acidified to pH 2.0 using 1 N HCl in preweighed plastic tubes, left overnight at 4°C, and then centrifuged at 10,000×*g* for 20 min. The resulting supernatant was discarded and the remaining pellet was dried in an incubator at 37°C for 24–48 h (till reaching a constant weight). The net weight of the crude precipitate was determined, and the crude surfactin concentration (in g/l) was calculated.

Surfactin Activity

This was carried out using the following methods:

Oil spreading test The oil spreading test (OST) was conducted as developed by Morikawa et al. [24] and recommended by Youssef et al. [25]. The diameter of the visually detectable clear halo was measured.

Measuring surface tension stalagmometrically The biosurfactant activity was monitored by measuring surface tension using Traube's stalagmometer (drop weight method) at 25°C. The surface tension of the test sample was measured using the following equation:

$$\gamma_{test} = \gamma_0/m_0 \times m_{test}$$

where γ_0 is the surface tension of distilled water, m_0 is the weight of distilled water per one drop, and m_{test} is the weight of the sample (CFS) per one drop [26]. The percentage of surface tension (ST) reduction was determined from the following equation:

$$\%\ \text{ST reduction} = [(ST_{initial} - ST_{final})/ST_{initial}] \times 100.$$

Different Factors Affecting Surfactin Production

The time course of surfactin production in MSM was studied to determine the time required for maximum surfactin production. The effect of different environmental and media components on surfactin production was studied. In all cases, the monitored fermentation parameters included biomass, biosurfactant concentration, and biosurfactant activity.

Effect of Different Environmental Fermentation Conditions

The factors studied included: effect of aeration (studied by varying the volume of headspace in 250 ml conical flasks created by variation of the volume of the medium in the flask which will be reflected in variation of the level of aeration), initial pH, incubation temperature, inoculum size.

Effect of Different Media Components on Surfactin Production

The effect of different carbon sources, nitrogen sources, and minerals was studied. For studying the effect of the addition of different carbon sources on surfactin production by the tested isolate, the basal C-source (glucose) present in MSM was replaced with other carbon sources. The tested carbon source was added at a concentration equivalent in its carbon content to that of glucose (20 g/l) except for oils and hydrocarbons where they were used at 2% *v*/*v*. The tested carbon sources were carbohydrate sources (D-glucose, D-fructose, sucrose, maltose, lactose, galactose, D(+)-mannose, D-sorbitol, glycerol, glucose syrup, molasses, and malt extract), vegetable oils (soybean oil and olive oil), and hydrocarbons (hexadecane and paraffin oil).

The effect of the addition of different nitrogen sources on surfactin production was studied similarly. The studied nitrogen sources were classified into organic (ammonium oxalate, urea, yeast extract, peptone, tryptone, and corn steep liquor) and inorganic sources (sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium bromide, ammonium carbonate, and ammonium sulfate).

The amino acids (leucine, aspartic acid, valine, and glutamic acid) that constitute the peptide moiety of surfactin were incorporated in the culture medium for studying their effect on surfactin production. The amino acids D,L-leucine, L-aspartic acid, L-valine were added to MSM at 0.1 mM, whereas glutamic acid was added in replacement of sodium nitrate in MSM (at a concentration equivalent in nitrogen content).

The effect of the addition of different multivalent cations on surfactin production was studied by adding them to the glucose–magnesium medium at a concentration of 0.1 mM. The results were compared to the control flask which received no additional multivalent cations (glucose–magnesium medium), i.e., it was devoid of trace elements and calcium.

Glucose–magnesium medium was derived from basal MSM by stepwise removal of the multivalent cations and the study of the effect of removal on growth. The divalent cations already present in the basal medium were calcium chloride, magnesium sulfate, and those present in the trace elements solution ($FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, K_3BO_3, $CuSO_4 \cdot 5H_2O$, and $Na_2MoO_4 \cdot 2H_2O$). Growth under four different conditions was studied as follows: growth in MSM that was devoid of divalent cations and trace elements solution (TES), growth in MSM devoid of TES and Mg^{++} but containing Ca^{++} , growth in MSM devoid of TES and Ca^{++} but containing Mg^{++}, and finally, MSM devoid of TES only but containing both Mg^{++} and Ca^{++}.

Biosurfactant Production by B. subtilis Isolate BS5 in Different Modified Mineral Salts Media

Based on the results accumulated from the study on media components affecting surfactin production, three modified media (M1, M2, and M3) were formulated. These media combined the optimum elements selected from each nutritive category, i.e., from carbon and nitrogen sources as well as trace elements (Table 1; "Materials and Methods" section).

Both growth and surfactin production were tested in such media. The results of the modified media (M1, M2, and M3) were compared with those of MSM.

Isolation and Detection of Plasmids in *B. subtilis* Isolate BS5

Plasmid Extraction

Plasmid extraction was performed using the alkaline lysis method of Birnboim and Doly [27]. *E. coli* DH5α/pUC18 was used as a standard *E. coli* strain bearing the pUC18 plasmid to act as a positive control. Agarose gel electrophoresis was carried out essentially as described by Sambrouk and Russell [28] at an agarose concentration of 0.7%.

Results

Different Factors Affecting Surfactin Production

Before studying the effect of environmental factors and the different medium components affecting biosurfactant production, the incubation period at which maximum biosurfactant production occurs was determined.

Time Course of Surfactin Production in MSM

The results (Fig. 1) showed that surfactin production started early in the exponential phase and the production kinetics paralleled the biomass kinetics to a large extent during logarithmic growth. The production profile was biphasic, the first phase extended up to 72 h during which production was increasing at a high rate, whereas in the second phase, production was increasing at a slower rate and a slight increase in surfactin production was obtained. For growth, maximum biomass was obtained after 72 h of incubation followed by a plateau for 2 days, then a decline till the end of the incubation (Fig. 1). Therefore, the results of subsequent experiments were taken after 72 h of incubation.

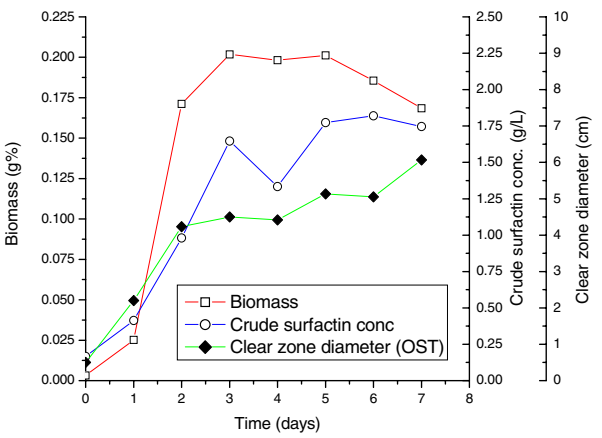

Fig. 1 Time course of growth and surfactin production by *B. subtilis* isolate BS5 in MSM. Surfactin activity was measured using the clear zone diameter as determined by the OST

Effect of Different Environmental Fermentation Conditions

Effect of Aeration

The results in Fig. 2 showed that the highest bacterial growth was attained at an aeration percentage of 80% (volume medium is 20%). However, maximum surfactin production was obtained at an aeration percentage of 90% (volume of medium is 10%) and there was a sharp decline in surfactin production upon decrease of aeration.

Effect of Inoculum Size

There was a gradual and slow increase in surfactin production upon increasing the inoculum size up to 6% *v/v*. However, bacterial growth decreased upon increasing inoculum size (Fig. 3).

Effect of Medium Initial pH

From the results presented in Fig. 4, it appears that *B. subtilis* isolate BS5 grew at pH 6 to 9.0 with the highest levels of cell growth recorded at pH values ranging from 6.5 to 9.0. However, the highest levels of surfactin production were obtained at pH 6.8. Other pH values were accompanied by decreased surfactin production.

Effect of Incubation Temperature

The results displayed in Fig. 5 show that *B. subtilis* isolate BS5 could grow at all tested temperatures. However, relatively high cell growth and surfactin production was obtained at incubation temperatures of 25 and 30°C.

Effect of Different Media Components on Surfactin Production

Effect of Carbon Sources

The results in Fig. 6 showed that the best carbon source for surfactin production and growth is molasses. None of the other carbon sources was found to be superior to glucose;

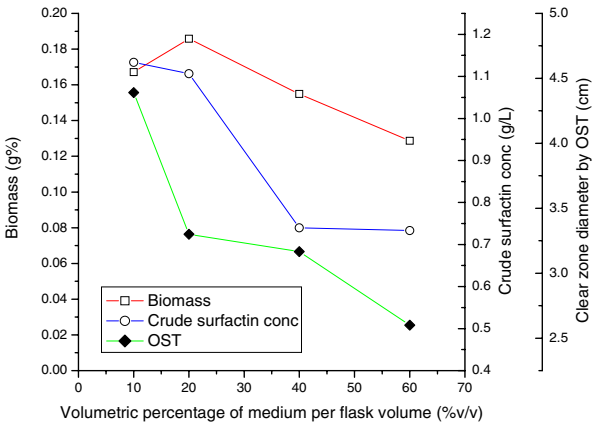

Fig. 2 Effect of aeration (different volumes of MSM in 250 ml flasks) on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM. Surfactin activity was measured using the clear zone diameter as determined by the OST

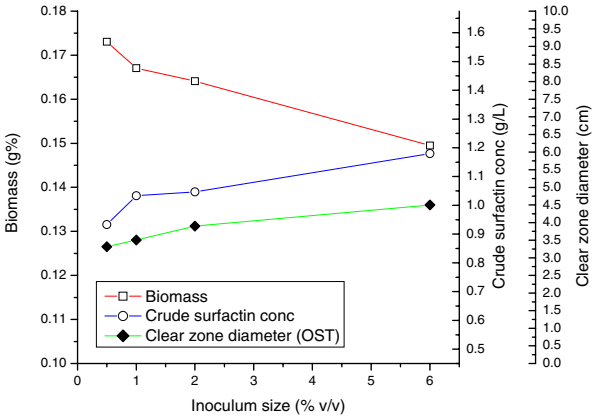

Fig. 3 Effect of inoculum size on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM. Surfactin activity was measured using the clear zone diameter as determined by the OST

however, sucrose, maltose, D-sorbitol, and malt extract achieved levels of surfactin production and cell growth that were comparable to those of glucose. The sugars, lactose and galactose, as well as the tested oils and hydrocarbon (HC) sharply inhibited bacterial growth and resulted in lower surfactin productivities compared to glucose. Glucose, malt extract, and molasses were further studied at different concentrations (Fig. 7a–c).

From Fig. 7a and b, it was found that the optimum concentrations of glucose and malt extract for biosurfactant production and growth were 2 to 4 g% for glucose and 4 g% for malt extract. In the case of molasses, both surfactin production and growth increased steadily by increasing molasses concentration up to 16% v/v (Fig. 7c).

Effect of Nitrogen Sources

As shown in Fig. 8, sodium nitrate was the best nitrogen source for surfactin production. However, other tested nitrogen sources decreased surfactin production with different degrees and such decrease was more pronounced in case of protein nitrogen sources. It was observed that surfactin productivity in terms of surfactin activity may or may not coherently correlate with that in terms of surfactin concentration. Regarding bacterial growth, the effect of nitrogen sources was either comparable to that of sodium nitrate or caused some degrees of inhibition.

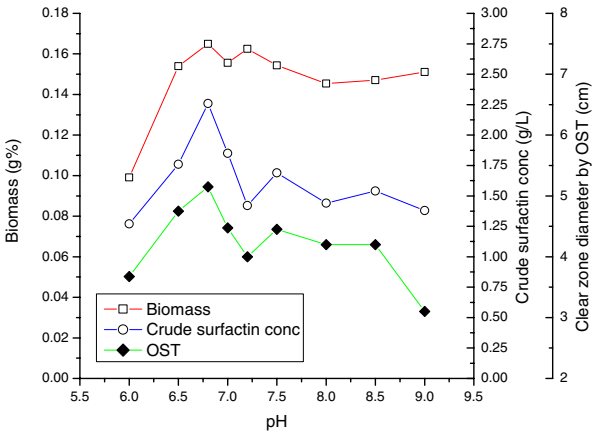

Fig. 4 Effect of initial pH on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM

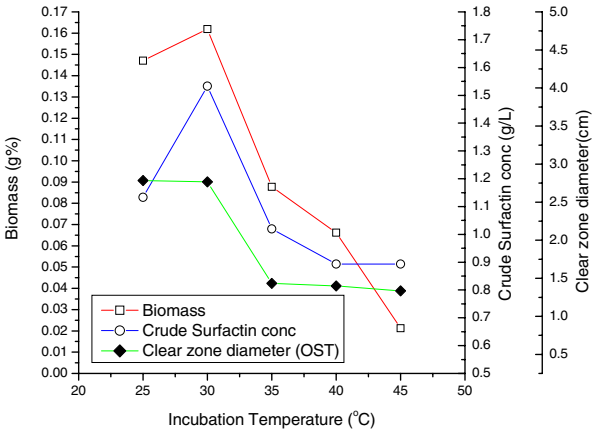

Fig. 5 Effect of incubation temperature on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM. Surfactin activity was measured using the clear zone diameter as determined by the OST

As sodium nitrate proved to be the best nitrogen source for surfactin production, its effect was further studied at different concentrations (Fig. 9a). In addition, the two other nitrogen sources (ammonium nitrate and ammonium oxalate), which showed comparable surfactin productivity and/or surfactin concentration, were similarly studied (Fig. 9b and c).

The results in Fig. 9a revealed that the highest surfactin productivities were achieved at 0.5–1 g% sodium nitrate concentration, however, the highest biomass values were obtained at 0.25–2.5 g%. Figure 9b and c also shows that the optimum concentrations of ammonium nitrate and ammonium oxalate that produced the highest growth and surfactin production were 0.25–1 g% for ammonium nitrate and 1 g% for ammonium oxalate.

Effect of Some Structural Amino Acids on Surfactin Production in MSM

The results shown in Fig. 10 revealed that no enhancement of surfactin production was gained by the use of amino acids, either those added to MSM at 0.1 M (D,L-leucine, L-

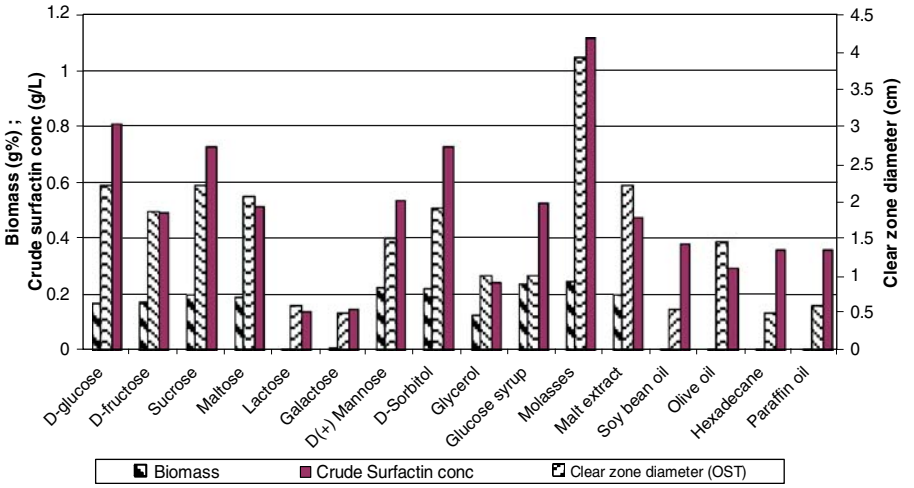

Fig. 6 Effect of different carbon sources on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM after 72 h incubation. Surfactin activity was measured using the clear zone diameter as determined by the OST

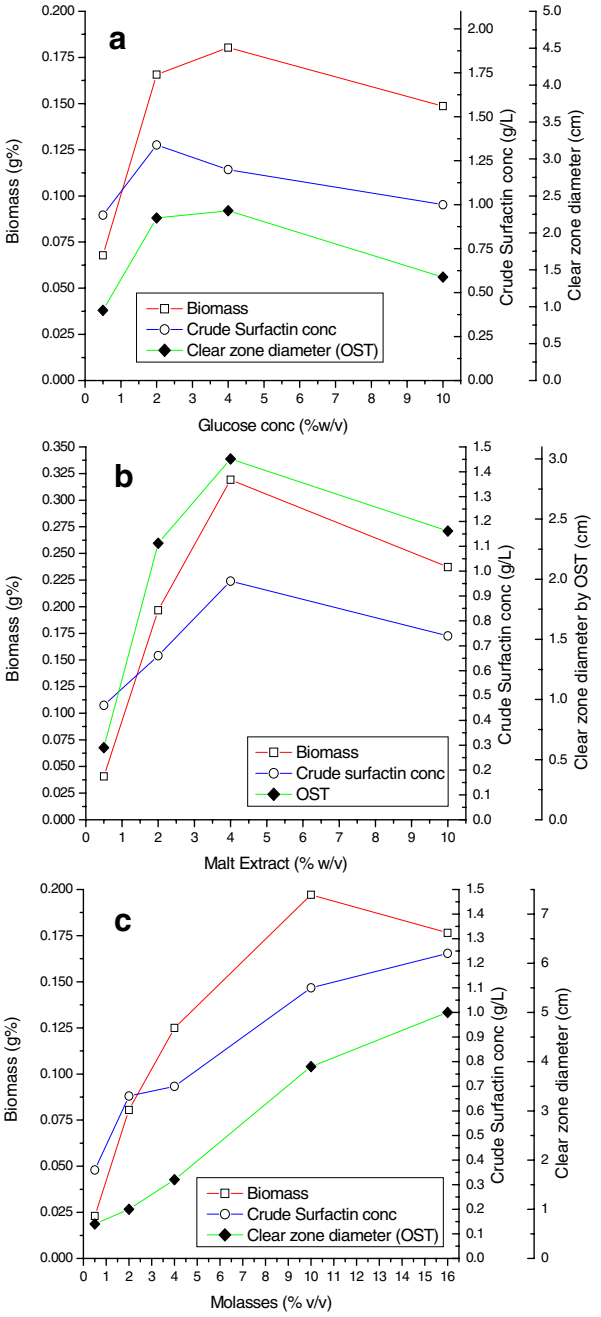

Fig. 7 Effect of different concentrations of **a** glucose, **b** malt extract, **c** molasses on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM after 72 h incubation. Surfactin activity was measured using the clear zone diameter as determined by the OST

aspartic acid, L-valine) or that used in replacement of sodium nitrate in MSM (glutamic acid at 0.432 g%) although the latter was relatively less inhibitory better than other tested amino acids. However, when the effect of glutamic acid on surfactin production was further studied at different concentrations, maximum surfactin production and bacterial growth were obtained

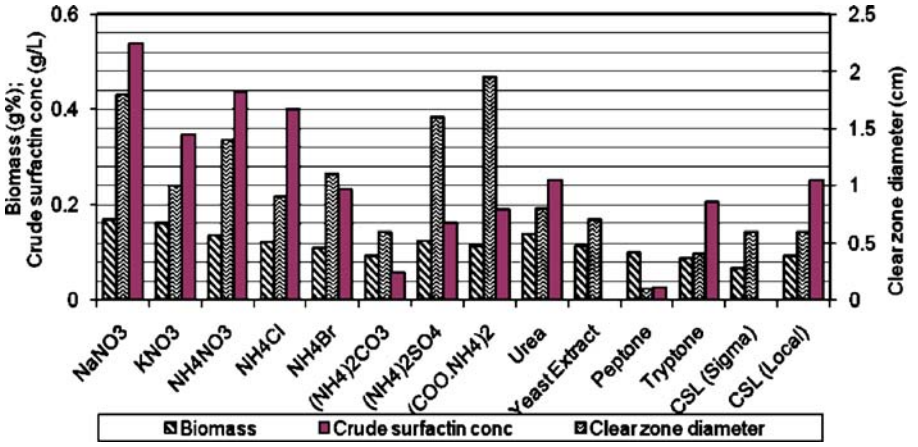

Fig. 8 Effect of different nitrogen sources on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM after 72 h incubation. Surfactin activity was measured using the clear zone diameter as determined by the OST

at 1 g% (Fig. 11). The level of surfactin production (in terms of activity) at this concentration (1 g% glutamic acid) was comparable to that produced by sodium nitrate at 0.5 g% (Fig. 9a).

Effect of Multivalent Cations As shown in Fig. 12, it is clear that Mg^{++} is essential for growth as its removal was accompanied by a substantial reduction in microbial growth. However, Ca^{++} was not as essential as Mg^{++} because its removal was not accompanied by a significant change in bacterial growth. Therefore, a modified MSM (glucose–magnesium medium) that was devoid of the TES and Ca^{++} was used for studying of the effect of the addition of different multivalent cations.

Effect of the Addition of Multivalent Cations to the Glucose–Magnesium Medium

As shown in Fig. 13, growth and surfactin production by *B. subtilis* isolate BS5 were greatly enhanced in the presence of the following multivalent cations: zinc, iron (II), iron (III), and manganese (II) at 0.0001 M concentrations. Regarding surfactin production, iron (III) showed better results than iron (II). Other multivalent cations caused either comparable results to that of the control or resulted in different degrees of inhibition of both growth and surfactin production. These cations (Zn, iron III, Mn) together with magnesium (which proved to be essential for bacterial growth) were further evaluated at different concentrations to find out the optimum concentrations of each (Fig. 14a–d).

The results plotted in Fig. 14a–d show that the optimum concentration of zinc sulfate, ferric chloride, and magnesium sulfate for maximum biosurfactant production is 1 mM, however, that of manganese sulfate is 0.1 mM.

Biosurfactant Production by *B. subtilis* Isolate BS5 in Different Modified Mineral Salts Media

From Fig. 15, it is clear that the medium containing the optimum minerals (M1) increased surfactin productivity over that in MSM. The medium (M2) that combined both optimum

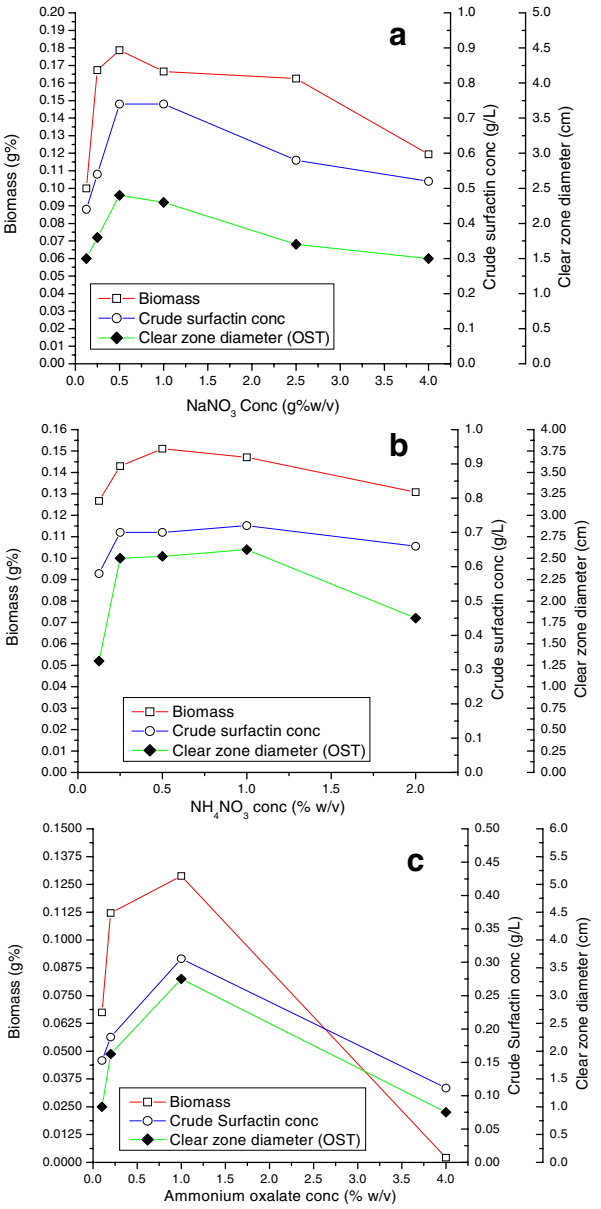

Fig. 9 Effect of different concentrations of **a** sodium nitrate, **b** ammonium nitrate, **c** ammonium oxalate on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM after 72 h incubation. Surfactin activity was measured using the clear zone diameter as determined by the OST

trace elements and optimum nitrogen source together did not significantly increase surfactin activity and bacterial growth. It is interesting to note that upon the application of optimum trace elements, nitrogen source, and carbon source (M3), their effects together were so dramatic. An increase in surfactin production (in terms of surfactin concentration or surfactin activity) of about threefold was reached (if compared with MSM). This optimum M3 medium, which contained optimum carbon source (molasses 160 ml/l), optimum nitrogen source ($NaNO_3$ 5 g/l), and optimum trace elements, was designated as molasses MSM (MMSM).

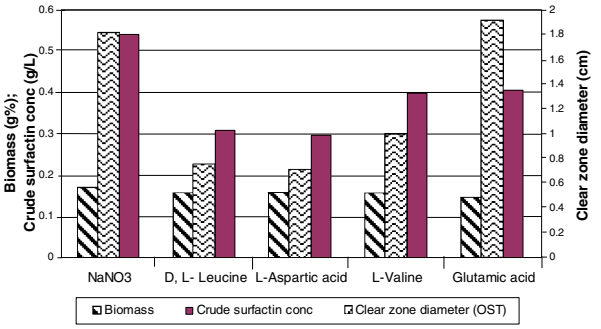

Fig. 10 Effect of different amino acids on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM after 72 h incubation. Surfactin activity was measured using the clear zone diameter as determined by the OST

Time Course of Surfactin Production by B. subtilis Isolate BS5 in MMSM

The results in Fig. 16 showed that surfactin production started early in the exponential phase and the production kinetics paralleled the biomass kinetics up to 2 days of incubation. On the basis of these facts, it could be concluded that surfactin production is growth-associated, the same finding was observed when using the basal medium (MSM). It was found that the maximum level of cell biomass was obtained after 48 h of incubation; however, maximum surfactin concentration was obtained 12 h later, i.e., after 60 h of incubation. After those periods, a sharp reduction in either biomass or surfactin production levels was observed.

Detection of Plasmid(s) in B. subtilis Isolate BS5

The plasmid extraction process was conducted on culture grown in both Luria–Bertani (LB) broth and in MSM medium. Plasmids were not detected (Fig. 17). Thus, it can be concluded that neither high nor low molecular weight plasmids do exist in the tested isolate.

Discussion

The chemical composition of the culture medium and environmental factors influence cell growth and biosurfactant production [17]. A better understanding of the medium

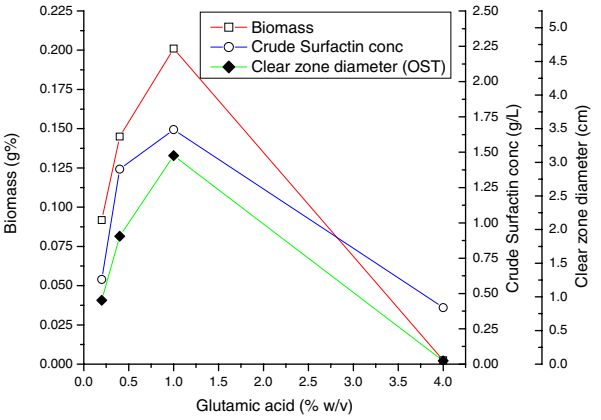

Fig. 11 Effect of different concentrations of glutamic acid on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM after 72 h incubation. Surfactin activity was measured using the clear zone diameter as determined by the OST

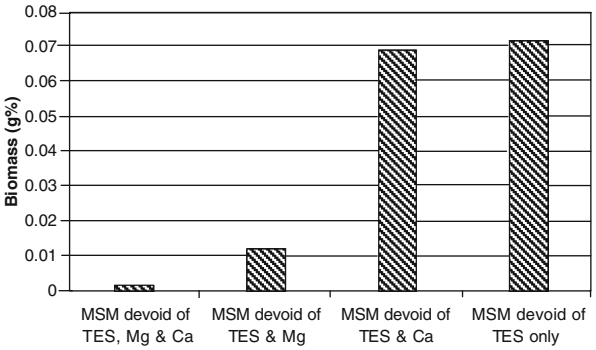

Fig. 12 Growth of *B. subtilis* isolate BS5 in MSM with and without different multivalent cations after 48 h incubation

components and environmental factors and their optimal control can, therefore, be used to improve the biosurfactant production. A basal synthetic medium (MSM supplemented with 2% glucose) was applied for studying different factors affecting biosurfactant production by *B. subtilis* isolate BS5.

Time Course Experiments in MSM

The results (Fig. 1) showed that surfactin production started early in the exponential phase and the production kinetics paralleled the biomass kinetics through the logarithmic phase. Accordingly, surfactin production by this isolate is growth-associated. Growth-associated production of biosurfactant has been reported for *B. subtilis* [6]. In subsequent experiments, results were taken after 72 h of incubation as production was increasing at a slower rate thereafter.

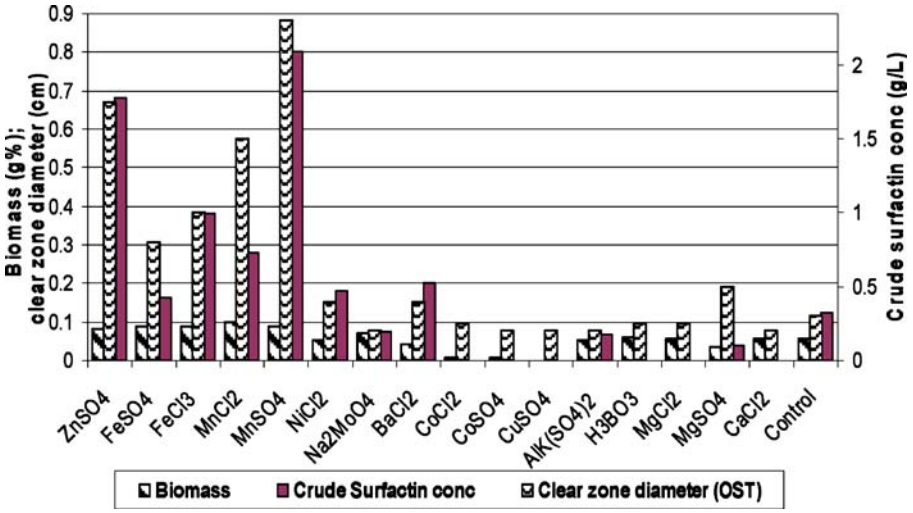

Fig. 13 Effect of addition of different multivalent cations on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in modified MSM after 72 h incubation. Surfactin activity was measured using the clear zone diameter as determined by the OST

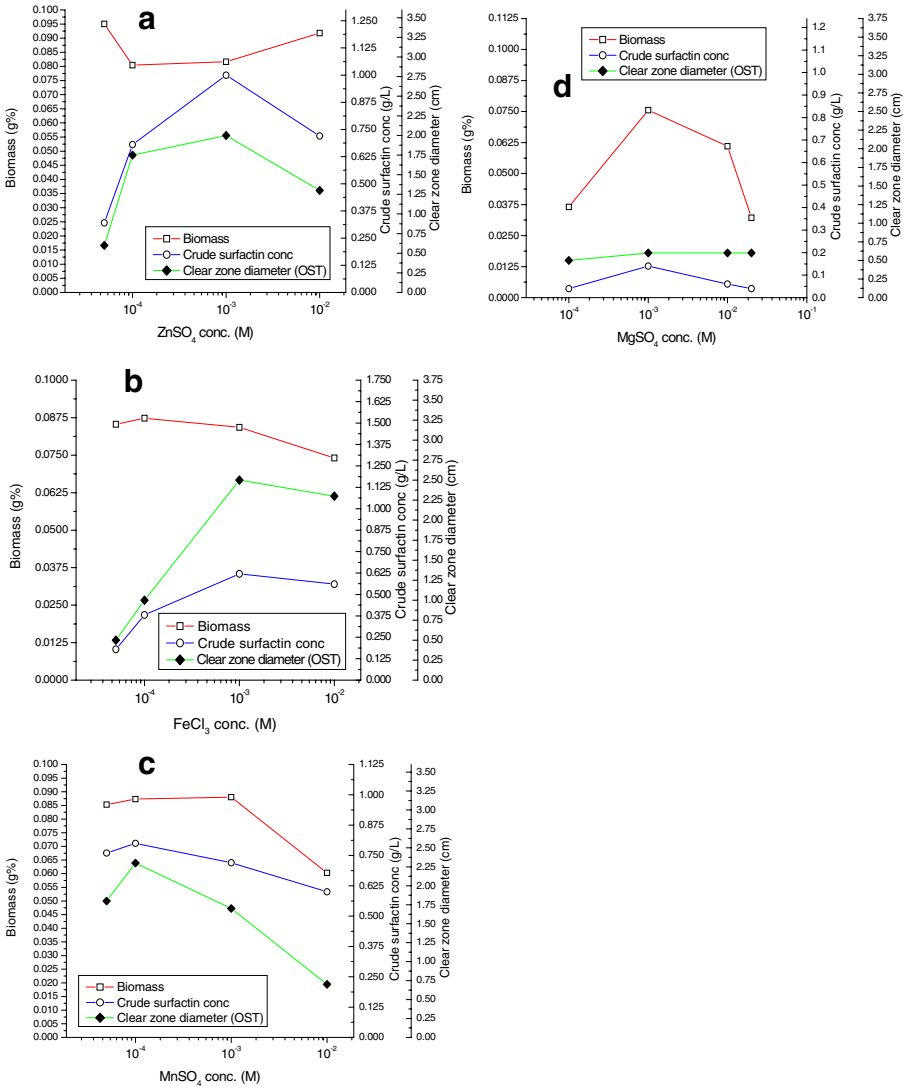

Fig. 14 Effect of different concentrations of **a** zinc sulfate, **b** ferric chloride, **c** manganese sulfate, **d** magnesium sulfate on growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in modified MSM after 72 h incubation. Surfactin activity was measured using the clear zone diameter as determined by the OST

Effect of Different Environmental Fermentation Conditions on Surfactin Production

The environmental factors: pH, temperature, and aeration significantly influence the bacterial cell growth and biosurfactant production [17]. Inoculum size and inoculum condition also have paramount effects on bacterial growth and productivity [29].

The study of the effect of aeration on surfactin production (Fig. 2) revealed that a linear relationship between aeration and production existed and a sharp decline in surfactin

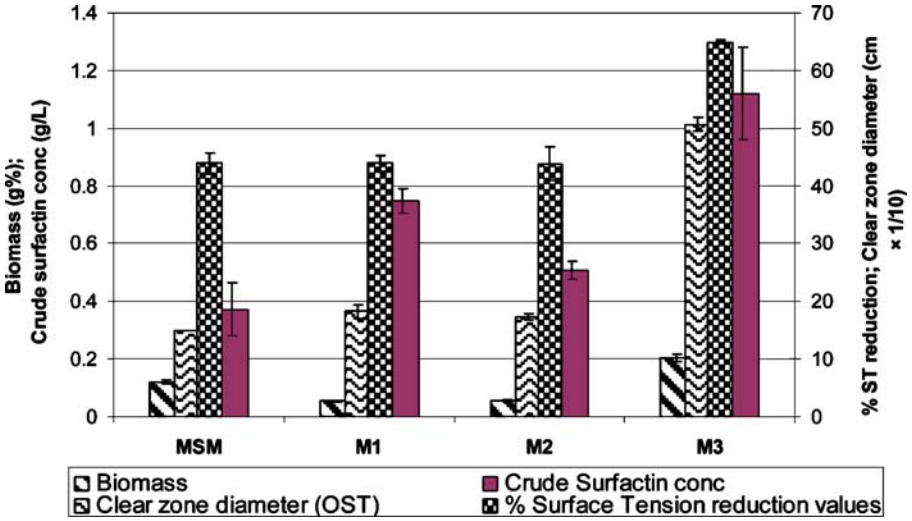

Fig. 15 Growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MSM and in different modified culture media (M1, M2, and M3) after 72 h incubation. Surfactin activity was measured in CFS using the clear zone diameter as determined by the OST and the percentage of the reduction of ST

production occurred upon decrease of aeration. This is in accordance with the oxygen requirements for such an aerobic microorganism.

The effect of different inoculum sizes on growth and surfactin production by the tested isolate was investigated. There was a gradual decrease in growth accompanied by a gradual slow increase in surfactin production upon increasing the inoculum size up to 6% *v*/*v* (Fig. 3). As there was no dramatic increase in surfactin production upon increasing the inoculum size, 2% *v*/*v* inoculum size, which gave reasonably high productivity, was applied in subsequent experiments.

The pH of the medium is one of the environmental factors that may affect surfactin production and bacterial growth. From the results (Fig. 4), it appeared that the test *Bacillus* isolate grew at pH 6 to 9.0. Highest cell growth and surfactin production was recorded at pH 6.8.

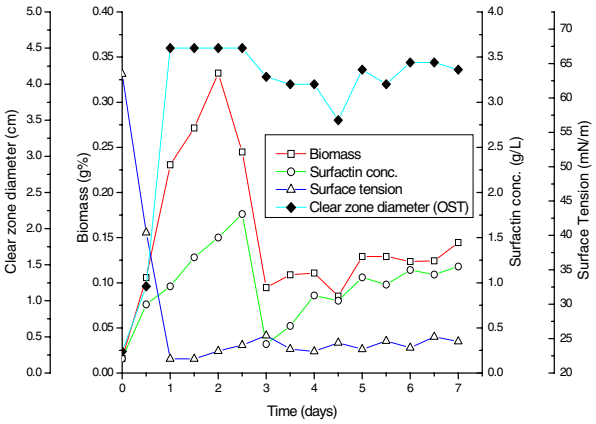

Fig. 16 Time course of growth and surfactin production (in terms of concentration and activity) by *B. subtilis* isolate BS5 in MMSM. Surfactin activity was determined by measuring the clear zone diameter as determined by the OST and measuring the surface tension of the CFS stalagmometrically

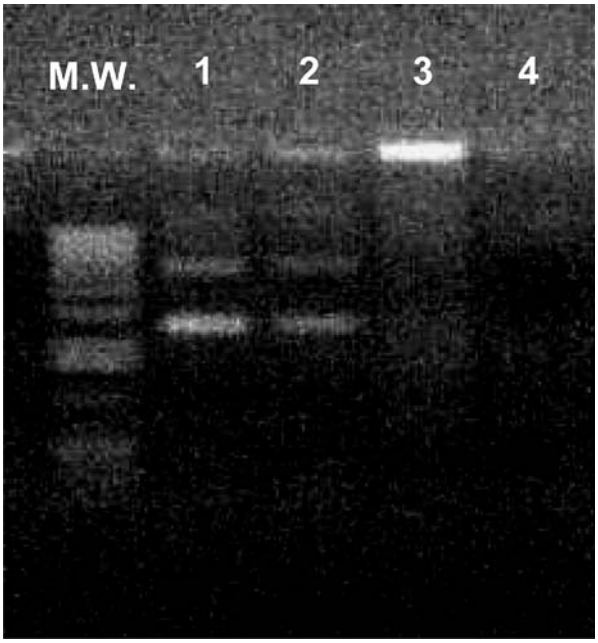

Fig. 17 Agarose gel (0.7%) electrophoresis of the plasmid preparations of *B. subtilis* isolate BS5 and *E.coli* DH5α/pUC18. *Lanes 1* and *2* plasmid preparation from *E. coli* DH5α/pUC18 grown in LB medium (as a positive control); *lanes 3* and *4* plasmid preparation from *Bacillus* isolate BS5 grown in LB medium and MSM, respectively; *M.W.* 1 Kb DNA ladder

There is always an optimum temperature at which the growth rate of a microorganism is fastest [30]. In this study, the results (Fig. 5) showed that *B. subtilis* isolate BS5 could grow at all tested temperatures with relatively high cell growth and biosurfactant production being obtained at an incubation temperature of 30°C. This temperature was similar to the incubation temperature commonly used for surfactin production by *B. subtilis* [7, 6, 31].

Accordingly, by the end of the study of the effect of different environmental fermentation conditions, it can be concluded that the optimum fermentation conditions for high surfactin production by *B. subtilis* isolate BS5 in shake flasks are: incubation period, 72 h; volumetric oxygen percentage, 90%; inoculum size, 2% *v*/*v*; initial pH, 6.5–6.8; and incubation temperature, 30 °C.

Effect of Different Medium Components on Surfactin Production

The basic nutritional requirements of microorganisms are an energy or carbon source, an available nitrogen source, inorganic elements, and for some cell types, specific growth factors [32].

During the course of studying the effect of nutritional factors on surfactin production, a protocol of three phases was followed: first, screening of the different nutritional sources belonging to each nutritional category (carbon sources, nitrogen sources, and minerals) that give maximum surfactin production, then selecting the promising sources for production; second, studying the effect of the selected sources on biosurfactant production at different concentrations. Finally, collective combination of the different nutritional sources at their optimum concentrations in a newly formulated medium and studying their collective effect on surfactin production.

The carbon source used in bacterial culture has a very pronounced effect on biosurfactant production [19, 20]. The carbon sources generally used in biosurfactant

production can be divided into three categories: carbohydrates, hydrocarbons, and vegetable oils. In this study, the carbon sources tested were carbohydrate sources (D-glucose, D-fructose, sucrose, maltose, lactose, galactose, D(+)-mannose, D-sorbitol, glycerol, glucose syrup, molasses, and malt extract), vegetable oils (soybean oil and olive oil), and finally, hydrocarbons (hexadecane and paraffin oil).

In this study, the results revealed that molasses is the best carbon source for surfactin production and growth (Fig. 6). However, sucrose, maltose, D-sorbitol, and malt extract gave levels of surfactin and cell growth comparable to those of glucose. The sugars, lactose and galactose, sharply inhibited bacterial growth and resulted in lower surfactin productivities compared to glucose. Growth and surfactin production increased remarkably compared to glucose and steadily by increasing molasses concentration up to 16 g% (Fig. 7c), however, malt extract at 4 g% showed biosurfactant productivity comparable to that of glucose; this finding is very promising because both molasses and malt extract are cheaper and promising alternatives to glucose.

It is worthy to note the inhibitory effect of vegetable oils (soy bean oil and olive oil) and hydrocarbon (hexadecane and paraffin oil) on surfactin production (Fig. 6), although it was postulated that hydrocarbons enhance the production of biosurfactants by bacteria [33]. However, many authors documented that, unlike the microorganisms producing glycolipid, polymeric, or fatty acid type biosurfactants, *Bacillus* sp. requires only carbohydrates to produce lipopeptide-type biosurfactant [34, 35, 36, 37, 38]. The fact that some biosurfactants are produced even when the cells are not grown on hydrophobic substrates suggests that their function is not solely restricted to the stimulation of substrate availability but they perform other defensive and biological functions [39].

Therefore, at the end of this study, the highest surfactin production by this *Bacillus* isolate BS5 can be achieved by the use of molasses at 160 ml/l for *B. subtilis* isolate BS5. Alternative carbon sources can also be effectively used for high surfactin production; these include malt extract (40 g/l) and glucose (40 g/l).

The nitrogen source plays an important role in the production of surface-active compounds by microorganisms [21]. In this study, the nitrogen sources tested were organic (ammonium oxalate, urea, yeast extract, peptone, tryptone, and corn steep liquor) and inorganic sources (sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium bromide, ammonium carbonate, and ammonium sulfate).

In this study, it was found that sodium nitrate was the best nitrogen source for surfactin production by *Bacillus* isolate (Fig. 8). However, other tested nitrogen sources decreased surfactin production with different degrees and such decrease was more pronounced in case of protein sources.

No advantage was gained concerning surfactin production by the use of amino acids, although glutamic acid gave surfactin productivity comparable to that of sodium nitrate in terms of activity only (Fig. 10). Similar results were reported where different amino acids did not show any notable difference in surfactin production when they were separately added to the culture medium [40]. Although the approach was different, similar results were also reported by Cooper et al. [6]. They reported that no improvement in surfactin production was obtained when a mixture of all of the amino acids entering in the structure of the surfactin lipopeptide was added to the medium. When the effect of glutamic acid on surfactin production was further studied at different concentrations, maximum surfactin production and maximum bacterial growth were obtained at 1 g% (Fig. 11). However, the level of surfactin production at this concentration (1 g% glutamic acid) was still comparable (in terms of activity) to that produced by sodium nitrate at its optimum concentration (0.5 g%). The use of glutamic acid as the sole nitrogen source was previously reported by some authors [41].

Therefore, at the end of this study, the highest surfactin production by this *Bacillus* isolate BS5 can be achieved by the use of sodium nitrate at 5 g/l. Alternative nitrogen sources can also be effectively used for high surfactin production; these include glutamic acid (10 g/l), ammonium oxalate (10 g/l), and ammonium nitrate (5 g/l).

The study of the effect of some inorganic salts or minerals revealed that growth and surfactin production were greatly enhanced in the presence of the following multivalent cations: zinc, iron (II), iron (III), and manganese (II) at 0.1 mM concentrations (Fig. 13) with iron (III) showing better results than iron (II) in terms of surfactin production. The observed stimulatory effect of iron (II) and iron (III) on growth and surfactin production by a *Bacillus* species was previously reported by Wei and Chu [42]. They recommended raising iron concentrations from the micromolar to the millimolar level to greatly enhance the surfactin production from *B. subtilis* ATCC 21332. In 2002, the same authors reported that a great enhancement of surfactin productivity was obtained by adding manganese (II). It is established that this metal is a "key" metal for the production of secondary metabolites by *Bacillus* species without having an effect on cell growth [6].

Concerning the inhibitory effect of metal ions, Cu^2 appeared to be the most potent inhibitor of both growth and surfactin production by the test isolate (Fig. 13); this finding was in agreement with that reported by Wei and Chu [43].

Consequently, a number of trace elements could be used for optimum surfactin production by the test isolate. These trace elements were $ZnSO_4$ (1.0 mM), $FeCl_3{\cdot}6H_2O$ (1.0 mM), and $MnSO_4{\cdot}H_2O$ (0.1 mM).

Based on the results accumulated from the previous study on nutritional elements and media components affecting surfactin production, modified media were formulated. These media combined the optimum elements selected from each nutritional category, i.e., carbon and nitrogen sources as well as trace elements. Among the different formulated media, MMSM, which contained optimum carbon source (molasses 160 ml/l), optimum nitrogen source ($NaNO_3$ 5 g/l), and optimum trace elements achieved obvious increase in surfactin productivity of about threefold compared to MSM.

Time Course of Surfactin Production by *Bacillus* Isolate BS5 in MMSM

It was necessary at the end of the optimization phase to redetermine the optimum incubation period required for maximum growth and surfactin production in MMSM. This is because the optimum incubation period for maximum surfactin production may probably differ in the modified medium from those in the basal medium (MSM). The results (Fig. 16) showed nearly similar profiles to those obtained in MSM (Fig. 1) where production was growth-associated, however, a shorter incubation period was required in MMSM.

Detection of Plasmid(s) and its Relation to Surfactin Production

Neither high nor low molecular weight plasmids were observed in the test isolate by the technique used (Fig. 17). Thus, it can be concluded that the surfactin-coding gene is located in the chromosomal DNA of the tested isolate and not plasmid-associated. Similarly, studies conducted by Fleck et al. [44] on biosurfactant production by *B. subtilis* B1 and *Pseudomonas aeruginosa* P1 showed that none of the strains presented plasmids, either of low or high molecular weights. They concluded that the coding genes for biosurfactant production are located in the chromosomal DNA. This finding may be advantageous from the industrial point of view because one of the major drawbacks that prevent a producing bacterium from being a candidate industrial strain is genetic instability. Genetic instability

and, consequently, fluctuation of production level is commonly encountered among strains that have the production genes carried on plasmids.

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