D:/Biomedica Vol.26, Jan. – Jun. 2010/Bio-10.Doc P. 34 – 38 (WC)

EVALUATION OF LIPOPEPTIDE (*SURFACTIN*) PRODUCTION BY BACILLUS SUBTILIS

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ABSTRACT
Introduction: *Lipopeptides are the bioactive peptides and some constituents of these compounds are surfactin, fengycin and Turing A, B and C, mycosubtilins and bacillomycins. Among these lipopeptides, surfactin is produced by Bacillus subtilis that has strong anti-microbial properties. Surfactin can be obtained by cultivation of bacteria and possesses various biological activities; anti-microbial, anti-viral, anti-tumour, haemolytic, blood anticoagulant and fibrinolytic activities.* ***Materials and Methods:*** *The present study was evaluated for optimisation of media components (Carbon source, N, P, and K) and environmental factors for the growth and production of lipopeptides by Bacillus subtilis. It was a quasi experimental study. Surfactin production was optimised with different factors including Mannitol, Phosphate, Nitrogen, Carbon, Potassium, and pH by inoculating B. subtilis on standard mineral salt (SMS) medium using fermentation technology.* ***Results:*** *showed that all the optimised factors have contributed their role in the production of lipopeptides by B. subtilis. The increasing concentrations of mannitol and nitrogen produced maximum lipopeptides with O.D 2.110 and 2.375 respectively. Production of surfactin by B. subtilis might be increased by using different factors optimised in medium and these compounds have potential applications both in medical and biotechnological fields.*

Key Word*: Lipopeptides, Surfactin, B. subtilis, production.*

INTRODUCTION

Surfactin is a cyclic acidic lipopeptide produced by *B. subtilis* that is one of the most effective biosurfactants known so far. It contains seven amino residues and is closed by lactone formation. Surfactin is known to be capable of lowering the surface tension from 72 to 27.9mN/m at a concentration of 0.005% (w/v). An important characteristic of this compound is its ability to lyse red blood cells and may act as an antibiotic, antiviral and haemolytic agent (Carillo *et al* 2003).[1] The biosynthesis of surfactin by *B. subtilis* has been extensively studied which is activated by surfactin synthesizing enzyme gramicidin S synthetase that involved in the formation of aminoacyl adenylate and thioester (Kluge *et al.* 1988).[2]

Abu-Ruwaida *et al* (1991)[3] proposed that a large variety of biosurfactants are influenced by the nature of the carbon source, the concentration of nitrogen, phosphorus, magnesium, iron and manganese ions in the medium, and culture conditions including pH, temperature, agitation and dilution rate. Biosurfactant producing bacteria react to changes in their environment by modifying their surface composition and structure.

Surfactin is a useful lipopeptide compound and have potential applications in both medical and biotechnological fields. Most important property of this lipopeptide is their environmental acceptability, because they are readily biodegradable and have lower toxicity than synthetic antibiotics (Lang and Wanger. 1993).[4] Lipopeptide compounds have many pharmacological activities and surfactin has effective antibacterial, antifungal, antiviral, and antimycoplasma, inhibition of the fibrin clot formation and hemolytic properties (Cameotra et al. 2004).[5]

Surfactin has significant antibacterial property because it is capable of penetrating the cell membranes of all types of bacteria (Bergey et al. 1994).[6] This penetration is an essential factor that contributes to surfactin's detergent-like activity as it is able to create a permeable environment for the lipid bilayer and causes disruption that solubilizes the membrane (Heerklotz et al. 2001).[7]

Previously various studies have been conducted for production of lipopeptides using *B. subtilis.* In our study different environmental and salt components are optimised for the production of these medical important compounds.

MATERIAL AND METHODS

Production of Lipopeptides:

Bacillus subtilis strain for surfactin production

One strain of *B. subtilis* – IMBB was provided by Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore, Lahore.

Identification and Confirmation of *B. subtilis*

Bacterial characterization: Colony morphology of *Bacillus subtilis* was circular, smooth, 2 to 4 mm white, off white, serrate and opaque on nutrient agar and shows hemolytic activity on blood agar. Cellular morphology of this was Gram positive rods, present in groups and motile. Spore and capsules staining were positive.

Biochemical Characterization: Biochemical tests included to identify bacterial strain were Catalase, Urease, Indole, Nitrate Reduction and Oxidase.

Inoculation of *B. subtilis* on Mineral Salt Media (MSM) for Surfactin production

Inoculum preparation: A loop full of fresh growth of *Bacillus subtilis*-IMBB was transferred into 100 ml sterilized nutrient broth. The flask was incubated at 37°C for 48 hours. After incubation turbidity of the cultured broth was measured using Unicam spectrophotometer which was 1.201.

Production of Surfactin: Experiment was conducted in three batches for the production of surfactin by fermentation technology. In first batch low and high concentrations of P, K and N were used for the optimising the minerals. In the second batch carbon source was optimized with different concentrations and in the third batch pH was optimised ranging from 6.0 to 8.0. For the production of surfactin 1 ml of growth suspension was inoculated on MSM with different concentrations and composition of minerals. The fermenting flasks were incubated in an orbital shaker at 37°C for 24 hours. After 24 hours 10 ml of media was taken and centrifuge at 10,000 rpm for 20 minutes and optical density (OD) of supernatant was measured at 620 nm for detection and quantification of surfactin. The procedure was performed for upto 120 hours incubation of culture.

Optimisation of Media Components (N, K, P)

MSM with concentration 1.5 and 3.0 g/ 100 ml for nitrogen, 1.5 and 4.0 g/100 ml for phosphate and 1.0 and 2.0 g/dl for potassium was optimised for surfactin production. The growth of *B. subtilis* was monitored as OD at 620 nm by Spectrophotometer.

Optimisation of pH and Carbon source of Medium

The pH of medium is one of the important physical factors affecting bacterial growth and for the production of lipopeptides. To study the affect of pH, inoculm of 100 µl from growth suspension was inoculated on MSM with different pH as 6.0, 6.5, 7.0, 7.5, and 8.0. Carbon is also a source of lipopeptides production and mannitol was optimized with different concentrations from 0.5 to 3.0 g/dl in MSM. A volume of 100 µl of growth suspension was inoculated on MSM. Growth and production of surfactin were compared by OD at 620 nm.

RESULTS

Table 1 shows the higher production of lipopetides by using mannitol as carbon source at maximum time of incubation. Table 1 illustrated that Mannitol showed a positive effect i.e. the higher the mannitol concentration the higher will be the surfactin production. OD of *B. subtilis* is 2.110 at 3.0 g / 100 ml of Mannitol. Table 2 demonstrates the effect of pH and at pH 7.0 the production of lipopeptides is maximum with OD 0.865 after 120 hours of incubation. Effect of nitrogen for surfactin production is depicted in Fig. 1 and Fig. 2. Effects of potassium and phosphate salts in lipopetide production have converse activity and the optical density was higher at low concentration of potassium and phosphate represented in figures from no. 3 to no. 6

The figure 1 and 2 shows that growth was near by ceased after 72 hours due to the unavailability of nitrogen source in the media. The OD was 1.815 after maximum time of incubation (120 hrs). At higher concentration of nitrogen (3.0 g/100 ml) the OD of bacillus *subtilis* was 2.375 after 120 hrs of incubation. In Fig. 3 and 4, lipopeptide production was inversely proportion to the concentration of phosphate salt. At concentrations of 4.0 and at 1.5 g/100 ml the OD was 0.885 and 1.975 respectively. The production of surfactin was high with increasing time period and maximum OD was 3 (Fig. 5).

In Fig. 5 and 6, lipopeptide production is inversely proportion to the concentration of potassium salt. At

Table 1: *Optical Density determined at 620 nm for various Conc. of Mannitol (Carbon source).*

Time (hours)	0.5 g/ 100 ml	1.0 g/ 100 ml	1.5 g/ 100 ml	2.0 g/ 100 ml	2.5 g/ 100 ml	3.0 g/ 100 ml
0	0.133	0.160	0.206	0.271	0.385	0.395
24	0.274	0.350	0.679	0.723	0.888	0.915
48	0.374	0.687	0.763	0.953	1.610	1.580
72	0.484	0.721	1.100	1.619	1.834	1.720
96	0.535	0.926	1.230	1.744	1.882	1.95
120	0.548	0.947	1.310	1.787	1.910	2.110

concentrations of 1.0 and at 2.0 g/100 ml, the OD was 3.37and 1.915 respectively.

DISCUSSION

The genus *Bacillus subtilis* has been widely used in the fermentation industry for the lipopeptide and enzymes production which have a diverse chemical structure and biological activities (Stachelhaus *et al* 1995).[8] The lipopetides produced by *Bacillus subtilis* have haemolytic activity which is directly related to surfactin production (Moran *et al* 2002).[9]

In this study various components in medium were used for optimisation of the medium which was required to check the efficacy of these components in the production of surfactin by *B. subtilis*. Haemolysis produced by *B. subtilis* on blood agar is due to production of surfactin.[9] Mannitol was used as a source of Carbon at concentration 3 g/100ml in the medium which increases the surfactin production with 2.110 OD value. From the obtained results based on biomass production in the presence of mannitol, it was possible to suggest that the utilization of mannitol in this culture was very efficient for the production of surfactin. Results are in accordance as previously it was also observed that when mannitol concentration increased gradually there was efficient increase in the growth of *Bacillus subtillus* producing surfactins.[10]

Other optimizing agent which was utilized for surfactin production was Nitrogen that plays role in cellular metabolism thus affecting its production ability of surface- active compounds. It has been reported that $NaNO_3$ could be the best nitrogen source for the production of surfactin by facultative aerobes.[11] In this study high concentrations of $NaNO_3$ (3 g/100 ml), supplemented with 3 g/ 100 ml mannitol, adjusted to initial pH 7.0 and incubated at 37°C and reflected the biomass production in high quantity on the basis OD was 2.375.

Carbon and nitrogen have significant role in the production of surfactins and lipopeptides. C/N ratio has been reported to affect the surfactin yields; a low C/N ratio being more effective in increased surfactin production (Manresa et al 1991).[12] Results of our study are comparable and suggest a similar trend since an increase in nitrogen concentration implies a low C/N ratio (Davis et al 1999).[13]

Table 2: *Optical density of Bacillus subtilis with different ranges of pH.*

Time (hours)	6.0 pH	6.5 pH	7.0 pH	7.5 pH	8.0 pH
0	0.1955	0.205	0.2125	0.208	0.2185
24	0.263	0.313	0.37	0.283	0.3165
48	0.3825	0.405	0.6295	0.3765	0.344
72	0.415	0.477	0.8375	0.474	0.382
96	0.449	0.485	0.8515	0.488	0.392
120	0.460	0.4925	0.865	0.503	0.413

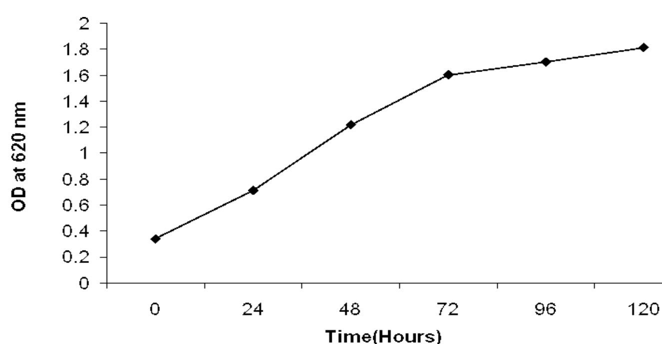

Fig. 1: *Effect of Nitrogen at low level (1.5g/100ml) on the growth of Bacillus subtilis.*

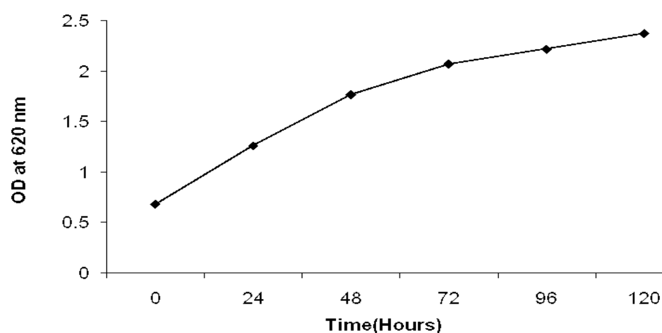

Fig. 2: *Effect of Nitrogen at high level (3g/100ml) on the growth of Bacillus subtilis.*

In contrast, lipopeptides production was reduced by high phosphate (4 g/100 ml) as OD was 0.8885 at 620 while at low phosphate (1.5g/100ml) high OD (1.97) was obtained. One explanation for the reduced surfactin biosynthesis at high phosphate concentration is that phosphate repression might play a role in the regulation of surfactin production, which is consistent with the negative effect of easy utilizable phosphate source on the biosynthesis of antibiotics and other secondary metabolites at the level of transcription (Liras *et al.*,

1990).[14]

It has been concluded that by improving various factors like carbon, nitrogen, phosphate, potassium and pH, the production of medically important lipopeptides could be enhanced which would be a break through in pharmaceutical industry.

ACKNOWLEDGMENT
We acknowledge our gratitude to Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore, for fully supporting this research project.

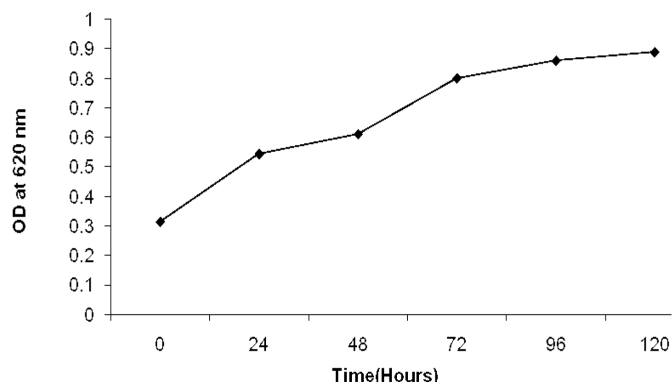

Fig. 3: *Growth of Bacillus subtilis in MSM with high Conc. (4.0 g/100ml) of Phosphate.*

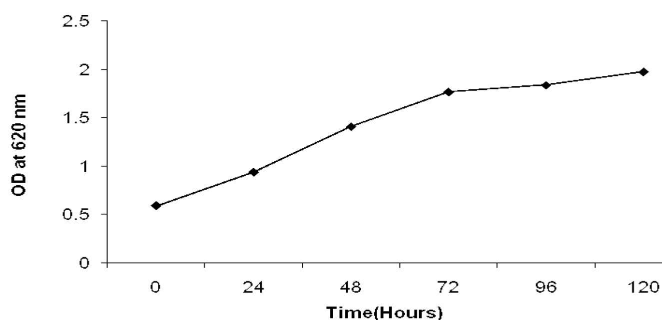

Fig. 4: *Growth of Bacillus subtilis in MSM with low Conc. (1.5 g/100ml) of Phosphate.*

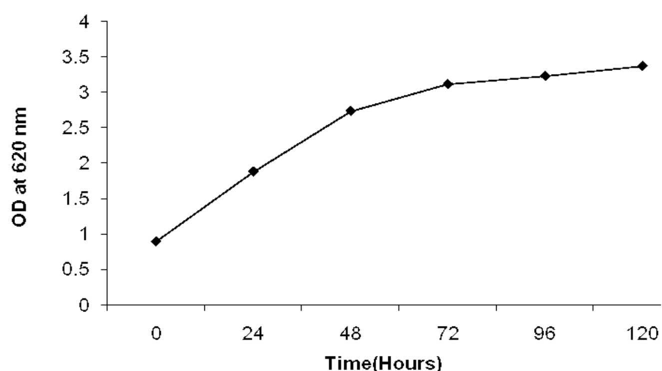

Fig. 5: *Growth of Bacillus subtilis at low concentration (1.0 g/ 100 ml) of Potassium.*

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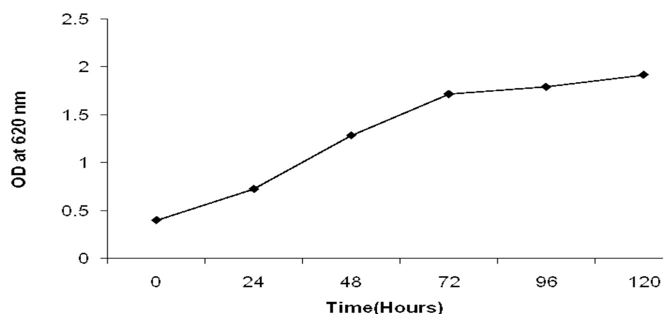

Fig. 6: *Growth of Bacillus subtilis at high concentration (2.0 g/ 100 Ml) of Potassium.*