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EFFECT OF SURFACTANTS ON THE BIOSYNTHESIS OF ALPHA AMYLASE BY *BACILLUS SUBTILIS* GCBM-25

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Abstract

The present study was concerned with the selection of suitable surfactant for the production of alpha amylase by *Bacillus subtilis* GCBM-25 in 250 ml shake flask containing 50 ml of fermentation medium. Different surfactants (laundry soap, detergent powder, sulphonic acid, acyle benzene sulphonic acid, liquid soap, Tween 80, sodium silicate, bath soap, sodium tri-polyphosphate, sodium lauryl ether sulphate or sodium lauryl sulphate) @ 2.0 % (w/v) were tested for enzyme production. Of all the surfactants, laundry soap gave better production of alpha amylase (605 U/ml/min) 44 h after inoculation (4.0 % inoculum size). The production of enzyme was found to be optimum (857 U/ml/min) when Millon soap @ 3.2 % (w/v) was added to the medium. The thermostability of the enzyme was decreased from 70 to 50°C as the surfactant was added to the fermentation medium.

Introduction

Alpha amylase degrades α, 1-4 linkage of starch in an endo-fashion and produces glucose and maltose (maltotriose, maltotetrose, maltopentose, maltohexose). This enzyme is extensively used in food, textile, pharmaceutical and syrup industries (Nigam & Singh 1995). The liquid culture in submerged fermentation is preferable since it allows supplementation of specific growth components such as carbon, nitrogen or inorganic sources that affect microbial growth and product formation (Prescott & Dunn, 1987). Ivanava *et al*., (1993) have reported that alpha amylase obtained from *Bacillus* spp., was stable at pH 6.5-8.0 and at 90°C whereas Castro *et al*., (1999) detected the maximum alpha amylase activity at 45°C (pH 7.0) in the late exponential phase. The production and thermostability of alpha amylase is greatly effected by the addition of surfactants to the fermentation medium (Ledent *et al*., 1999). The surfactants reduce the surface tension of the liquid medium and also provide essential nutrients for the growth of organism and increase the secretion of alpha amylase from the bacterial cells (Uelger & Cirakoglu, 2001). The present study was concerned with the selection of suitable surfactant for the production of alpha amylase by *Bacillus subtilis* GCBM-25.

Material and Methods

Organism: The mutant strain of *Bacillus subtilis* GCBM-25 was obtained from Biotechnology Research Centre, Department of Botany, Government College University Lahore. The strain was maintained on nutrient starch agar slopes.

Inoculum preparation: Vegetative inoculum was used in the present studies. Fifty ml of inoculum medium containing (% w/v): nutrient broth 1.0, soluble starch 1.0, lactose 0.5, NaCl 0.5, $CaCl_2$ 0.2 in 100 ml of phosphate buffer was transferred to each of 250 ml cotton plugged Erlenmeyer flask and sterilized at 15 lb/in^2 pressure (121°C) for 15 min.

After cooling at room temperature, a loopful of bacteria was aseptically transferred to each flask. The flasks were then rotated in the rotary shaking incubator (200 rpm) at 40°C for 24 h.

Fermentation technique: Fifty ml of the fermentation medium containing (g/l): starch 10, lactose 0.5, nutrient broth 10.0, $(NH_4)_2SO_4$ 5.0, $MgSO_4.7H_2O$ 2.0, $MnSO_4.7H_2O$ 2.0, NaCl 3.0, $CaCl_2$ 2.0 in 1000 ml of phosphate buffer (pH 7.5) were transferred to 250 ml cotton plugged Erlenmeyer flask. The surfactants were also added to the medium. After sterilization, the flasks were cooled at room temperature. One millilitre (1.2×10^7 cells) of inoculum (24 h old) was transferred to each flask. The flasks were placed in the rotary incubator shaker (200 rpm) at 40°C for 48 h. The fermented broth was then centrifuged at 7,000 rpm for 15 min., and the supernatant was used for the estimation of alpha amylase. All the experiments were run parallel in triplicates.

Enzyme assay: Alpha amylase was estimated according to the method of Rick & Stegbauer (1974). The enzyme solution was incubated at 60°C using 1.0 % (w/v) soluble starch. The reducing sugars were measured by adding 3,5-dinitro salicylic acid reagent, boiled for 5 min., cooled and the optical density measured at 546 nm using the spectrophotometer (Model: CECIL CE7200) against maltose as standard. One unit of activity is equivalent to the amount of enzyme, which in 10 min., librates reducing group from 1.0 % Lintner's soluble starch corresponding to 1.0 mg maltose hydrate.

Statistical analysis: Treatment effects were compared after Snedecor & Cochran (1980). Significance was presented in the form of probability ($p<0.05$) values.

Table 1. Effect of surfactants on the biosyntheses of ∝–amylase by *Bacillus subtilis* GCBM-25 in shake flask.

No.	Surfactants (2%)	U/ml/min
1.	Control	500 ± 5^b
2.	Laundry Soap (Sufi soap)	605 ± 3^a
3.	Detergent Powder (Express power)	523 ± 6^b
4.	Sulphonic acid	445 ± 2^c
5.	Acyle benzene sulphonic acid	441 ± 3^c
6.	Liquid soap	432 ± 6^c
7.	Tween 80	432 ± 4^c
8.	Sodium silicate	391 ± 2^d
9.	Both soap (Dettol soap)	366 ± 1^d
10.	Sodium tripolyphosphate	321 ± 6^d
11.	Sodium lauryl ether sulphate	321 ± 2^d
12.	Sodium lauryl sulphate	190 ± 4^e

Each value is an average of three replicates. ± indicate standard deviation from mean value. The values denoted by letters differ significantly at $p< 0.05$.

Results and Discussion

In the present study, the effect of different surfactants was tested for the production of alpha amylase (Table 1). Among all the surfactants tested, laundry soap (anionic surfactant) gave enhanced production of alpha amylase. The laundry soap may contain fatty alcohols as well as silicates that decrease the surface tension and increase the air

supply of the medium (Pederson & Nielson, 2000). The accumulation of the enzyme was greatly inhibited with the addition of sodium lauryl sulphate. It might have been due to the fact that sodium ions along with sulphate ions were toxic for the bacterial growth and enzyme production (Milner *et al*., 1996). However, Ramgren *et al*., (1988) have reported that Tween 80 was excellent surfactant for the production of alpha amylase by the bacterium.

The laundry soaps of different brands were tested for the production of alpha amylase (Table 2). Of all the soaps tested, Millon soap gave higher production of alpha amylase. It might have been due to the fact that this soap contained adequate concentrations of fatty acids and silicates, which facilitates the secretion of enzyme from the bacterial cells (Rao & Satyanarayana, 2002). The production of the enzyme was greatly inhibited with the addition of Chaman, Dulhan or Rani soap to the fermentation medium (40%) because these soaps normally contain sodium silicate at a higher level, which is toxic for the growth of bacteria. The enzyme obtained from this bacterium was found to be highly active at pH 7.5 (Haq *et al*., 1997). Further, increase in the pH inhibited the production and stability of the enzyme (Khajeh *et al*., 2001). The different concentrations (0.4-4.0 %) of Millon Soap were tested for the biosynthesis of alpha amylase (Fig. 1). The production of the enzyme was found encouraging in the presence of laundry soap at the level of 3.2%. Further increase in the concentration of laundry soap resulted in decrease in the production of enzyme. It might have been due to the toxic inhibitory effects of the laundry soap on the bacteria at high concentration.

Table 2. Effect of different laundry soaps on the biosynthesis of alpha-amylase by *Bacillus subtilis* GCBM-25 in shake flask.

No.	Surfactants (2%)	U/ml/min
1.	Control	500 ± 5^{cd}
2.	Millon soap	675 ± 4^{a}
3.	Sufi soap	605 ± 3^{b}
4.	Tata soap	576 ± 9.3^{bc}
5.	Ghai soap	510 ± 2.8^{c}
6.	Roshan soap	497 ± 5.2^{d}
7.	Khajoor Marka soap	484 ± 2.1^{d}
8.	Chaman soap	296 ± 6.7^{e}
9.	Dulhan soap	296 ± 1.7^{e}
10.	Rani soap	284 ± 2.4^{e}

Each value is an average of three replicates. ± indicate standard deviation from mean value. The values denoted by letters differ significantly at $p< 0.05$.

The time course of alpha amylase was carried out with the addition of surfactant and in the controlled flasks (Fig. 2). The production of enzyme increased with increase in the time period and found optimum 48 h after inoculation in the control flasks. However, the production of the enzyme with the addition of surfactant to the fermentation medium was found to be optimum at 44 h after inoculation due to the bacteria which reached in the stationary phase earlier than the control flask (Lealam & Gashe, 1994). Further, increase in the incubation period resulted into decrease in the production of alpha amylase due to the production of proteases at the end of stationary phase. The accumulation of proteases caused proteolysis of the bacteria as well as alpha amylase. The effect of time of addition of surfactant was studied for the optimum production of alpha amylase (Fig. 3). The production of enzyme was found to be maximum when the surfactant was added at the time of inoculation.

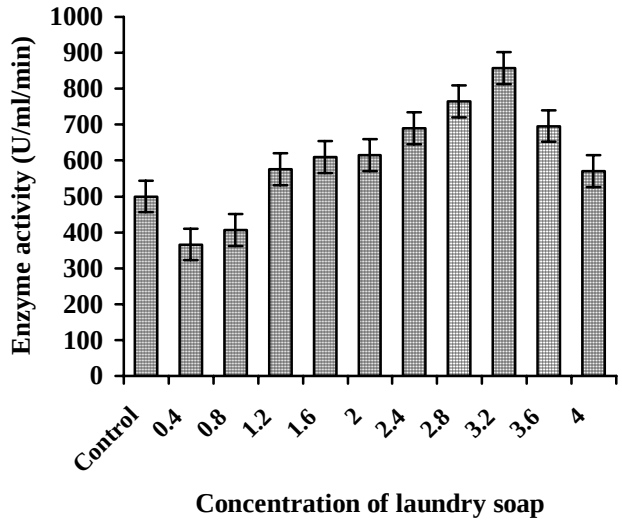

Fig. 1. Effect of different concentrations of Millon soap on the production of alpha-amylase by *Bacillus subtilis* GCBM-25 in shake flask

Each value is an average of three replicates. Y error bars indicated the standard error from mean value.

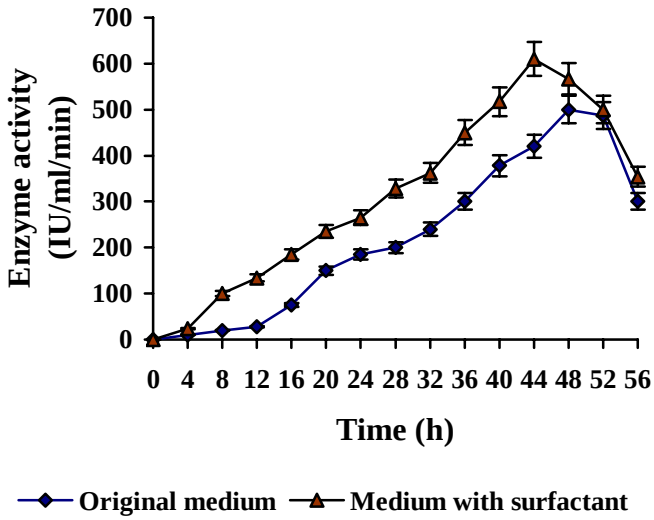

Fig. 2. Comparison between original medium and the medium with surfactant for time course study of alpha amylase production by *Bacillus subtilis* GCBM-25.

Each value is an average of three replicates. Y error bars indicated the standard error from mean value.

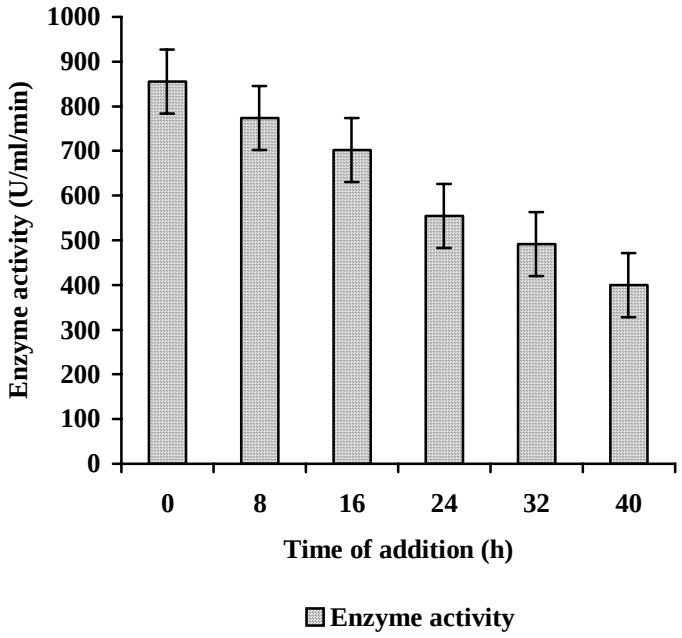

Fig 3. Effect of the time of the addition of the Millon soap on the biosynthesis of ∝–amylase by *Bacillus subtilis* GCBM-25 in shake flask.

Each value is an average of three replicates. Y error bars indicated the standard error from mean value. The values in figure vary significantly at $p< 0.05$.

The different inoculum levels (0.1-0.4 %) were tested for the production of alpha amylase (Fig 4). The production of enzyme was increased with increase in the size of inoculum and found to be optimal at 4% inoculum. As the inoculum level was further increased, the production of the enzyme was gradually inhibited. It is likely that at a higher inoculum level, the bacteria grow rapidly and the nutrients essential for the growth of bacteria were consumed at the initial stages that resulted in the accumulation of other by products in the fermentation medium. The insignificance of the results at low level of the inoculum may have been due to the slow growth of the organism and increase in the time period for the bacteria to reach in the stationary phase. Thus, the production of the enzyme was low at low level of inoculum as already found in another study (Lin *et al*., 1998).

The crude enzyme was tested to optimize the thermostability (Fig. 5). The enzyme substrate complex incubated at different temperatures of 30-75^{o}C showed that the enzyme was found to be most active at 50^{o}C. The activity of the enzyme was greatly inhibited at high temperature. In controlled flask, the enzyme was found to be highly active at 60-70^{o}C. So, the addition of surfactant to the fermentation medium resulted in the decreased thermostability of the enzyme. It may have been due to decrease in the binding property of the enzyme's protein with activator Ca^{2+}. Haq *et al*., (2002) have reported that the thermostability of the alpha amylase was Ca^{2+} dependent. Brumm & Teague (1990) have also reported that the alpha amylase was denatured at high temperature with the addition of detergents to the fermentation medium.

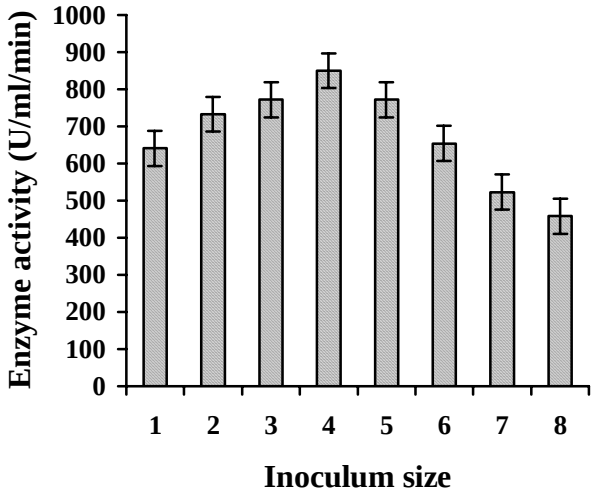

Fig. 4. Effect of different sizes of inoculum on the biosynthesis of ∝–amylase by *Bacillus subtilis* GCBM-25 in shake flask.

Each value is an average of three replicates. Y error bars indicated the standard error from mean value. The values in figure vary significantly at $p< 0.05$.

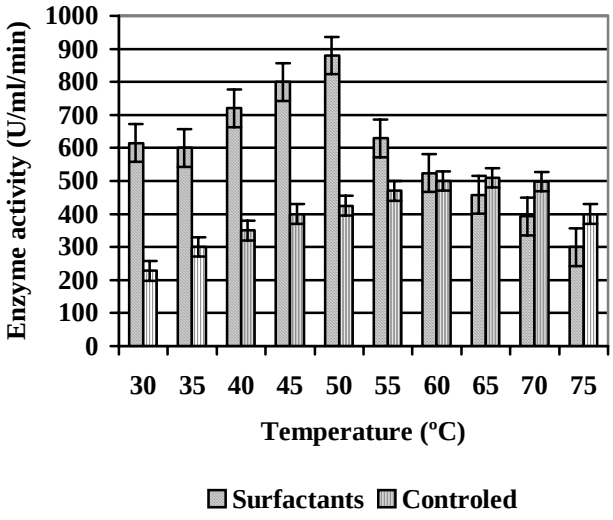

Fig. 5. Effect of surfactants on the thermostability of ∝–amylase by *Bacillus subtilis* GCBM-25 in shake flask.

Each value is an average of three replicates. Y error bars indicated the standard error from mean value. The values in figure vary significantly at $p< 0.05$.

From the present study, it is concluded that surfactants may enhance the production of alpha amylase as they reduce the surface tension of the fermentation medium. However, enzyme thermostability was decreased with the addition of surfactants to the medium.

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