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Induction and catabolite repression of alpha-amylase synthesis in *Bacilluslicheniformis* SKB4

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ABSTRACT

Microbial amylases have an exciting potentiality and are being used extensively in different industries. In this study, regulation of amylase biosynthesis was examined in Bacillus licheniformis SKB4 (wild type) and its mutant strain (8b). The mutant strain was developed by using UV exposure. Expression of the α-amylase gene of Bacillus licheniformis was activated by inducer and subject to catabolite repression. Addition of exogenous glucose or sucrose repressed bio-synthesis of α-amylase which was concentration (0.05-1.0% w/v) dependent. However, mutant strain could enable to overrule the glucose mediated repressive effect. Supplementation of second messenger like cyclic adenosine 3',5'-monophosphate (cAMP, 5 mM) along with glucose could a little bit improve amylase synthesis in wild strain. Antibiotics like rifampicin and tetracycline (ribonucleic acid and protein synthesis inhibitor; 100μg/ml) had stopped the release of enzyme in both wild and mutant strain. Amylase production was also inhibited in presence of respiratory inhibitor 2,4-dinitrophenol (uncoupler) at (5mM) concentration. Thus, the pattern of regulation of α-amylase production in the present strain was in multiple forms; it showed the classical glucose effect without stimulation of second messenger system.

Key Words: Amylase, regulation of biosynthesis, *Bacillus licheniformis* SKB4.

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INTRODUCTION

Microbes are able to grow in various environments and derive energy from various organic substrates present in their surrounding environment by secretion of specific enzyme. Starch degrading microbes secret amylolytic enzyme for hydrolysis of starch to obtain energy. Several researchers (1-3) had studied the regulation of extracellular α-amylase synthesis in different bacteria and they indicated that several factors were involved in this process. They also mentioned that an appropriate structural gene was responsible for synthesis of such enzyme and there was a specific mechanism for enzyme induction rather than direct adaptation to intake nutrient.

The bacterial cells growing in the medium containing easily obtainable carbon sources had shown a global alteration of gene expression, the process termed carbon catabolite repression (CR) (4). Catabolite repression (CR) of gene expression is universal phenomenon found in virtually all living organisms (5,6). Many cellular and developmental processes including sporulation, enzyme transport systems, and extracellular enzyme synthesis are subject to catabolite

repression in *Bacillus* species (7). The role of cyclic AMP in some prokaryotes is well accepted. Cyclic AMP mediated gene regulation of catabolite-repressible genes is common in gram-negative bacteria (8).

It is known that the majority of extracellular enzyme synthesized by genus *Bacillus* appears to be at least partially inducible and many workers (9-13) had reported the inducible nature of α-amylase formation. The evidence for substrate induction (eg. starch or α 1-4 linked oligosaccharides) had been found in studies with *B. stearothermophilus* (14), *B. licheniformis* (15,16), and *Bacillus* Sp. (11). Basically, bacterial amylase biosynthesis is controlled by carbon catabolite repression especially in presence of glucose (17). Huek and Hillen (18) had shown that catabolite repression of glucose on the synthesis of α-amylase could be reversed in the addition of cyclic AMP and it is able to eliminate the repressive effect in the gram-positive bacterium *Bacillus subtilis*. The present investigation deals with the exploration of some aspect of regulatory mechanism of α-amylase synthesis in *Bacillus licheniformis* SKB4 in relation to catabolite repression.

MATERIALS AND METHODS

Bacterial strain and growth media

Bacillus licheniformis SKB4, a soil isolate, (19) was used for the present study. Submerged fermentation was carried out for amylase production in 250 ml flask having 50 ml enriched medium (pH 6.5) which contains (w/v, g l^{-1}): starch, 5.0; peptone, 10.0; beef extract, 5.0; KH_2PO_4, 3.0; $MgSO_4$ 0.5; $CaCl_2$; 0.02. The medium was inoculated by addition of 1% (v/v) freshly prepared inoculum and incubated on a rotary shaker (120 rpm) at 42°C for 28 h. The culture supernatant was used to determine the amylase activity.

Preparation of mutant strain

The mutant strain of *Bacillus licheniformis* SKB4 was prepared by UV irradiation. Fresh culture of bacteria was serially diluted in sterilized distilled water up to 10^{-9} concentration; 0.5 ml of diluted culture spread uniformly over solid starch-agar plate. The petriplates had kept under UV radiation (4-watt UV lamp, General Electric USA; emitting the energy of 1.6×10^2 J/ m^2/s) up to 10 min. The irradiated plates were immediately kept in ice bath and then incubated at 42°C for 24 h. The survival percentage of microorganisms on the UV treated plates was determined by comparing the number of colonies appeared on the control culture plates with UV treated plates.

Screening of high yielding amylase producing mutants

The deformed colonies along with higher clearing zone around them on starch agar plate were selected and preserved separately in starch agar slant. These colonies were then repeatedly cultivated (10 cycle) in starch-agar plate to confirm the stability of mutation followed by culture in the liquid medium. Among them, one consistent higher α-amylase producing colony had been chosen. Antibiotic assay (cup method) had also been performed to detect the sensitivity of antibiotics in both wild and mutant strain.

Assay of amylase

Amylase activity was determined by studying its saccharolytic properties according to Bernfield (20). Briefly, the reaction mixture consisted 0.5 ml of 1% (w/v) starch, 0.4 ml of phosphate buffer (10 mM, pH 6.5) and 0.1 ml of enzyme solution and incubated for 5 min in 90°C. The reaction was stopped by addition of 1 ml of 3, 5 dinitrosalicylate (DNS) reagent. The quantity of reducing sugar was measured colorimetrically at 530 nm using glucose as standard sugar. The unit of amylase was defined as the amount of enzyme which produced 1 µmol of reducing sugar as glucose in 1 min under specified condition.

Effect of additional carbon sources on synthesis of amylase

Effect of different concentration of glucose (0.05-1.0% w/v) and sucrose (0.05-1.0% w/v) on amylase production by *Bacillus licheniformis* SKB4 were studied. These sugars were added as additional carbon source in starch (0.5% w/v) containing basal media. Fermentation was carried out at pH 6.5 and at 42°C for 28 h.

Effect of Cyclic Adenosine Monophosphate (cAMP) on amylase synthesis

The effect of cAMP was carried out on catabolically repressed bacterial cells to notice the change in amylase synthesis. This was done by culturing the bacteria in the media of following combinations: (1) basal media; (2) basal media + 1 % glucose; (3) basal media + 1 % glucose + cAMP (5 mM); (4) basal media + cAMP (5 mM).

Study of different inhibitors on amylase synthesis

Inhibitor like antibiotics (rifampicin, tetracycline), respiratory blocker (uncupler) like 2, 4 DNP (5mM) were used in culture broth to reveal their effects both in wild and mutant strain respectively. These inhibitors were added during active log phase (10 h) of cell growth.

RESULTS AND DISCUSSION

Selection of mutant strain

The survival percentage of the *Bacillus licheniformis* SKB4 was counted after irradiation of the organism under UV rays (1.6×10^2 J/ m^2/s) for 10 min (Fig 1). It was observed that most of the bacteria were killed after 5 min of treatment. The enzyme producing capacities of the 8 deform mutated colonies of *Bacillus licheniformis* SKB4 were studied in liquid culture medium (Table 1). It has been found that four mutated strains viz. 8b, 4c, 4b and 8c produced higher amount of amylase than others. Among the four mutated strains, 8b produced maximum amylase in liquid medium and selected for further study. Varalakshmi et al. (21) also reported that α-amylase production had been increased in UV induced mutant strain of *Bacillus* sp. 1.

The antibiotic sensitivity test of both strains (wild and mutant) revealed that the mutant strain (8b) became resistant against tetracycline and refampicin (100µg/ml) (Plate 1and 2). This result indicated that UV radiation not only changes the chromosomal DNA materials but also the extrachrosomal plasmid DNA.

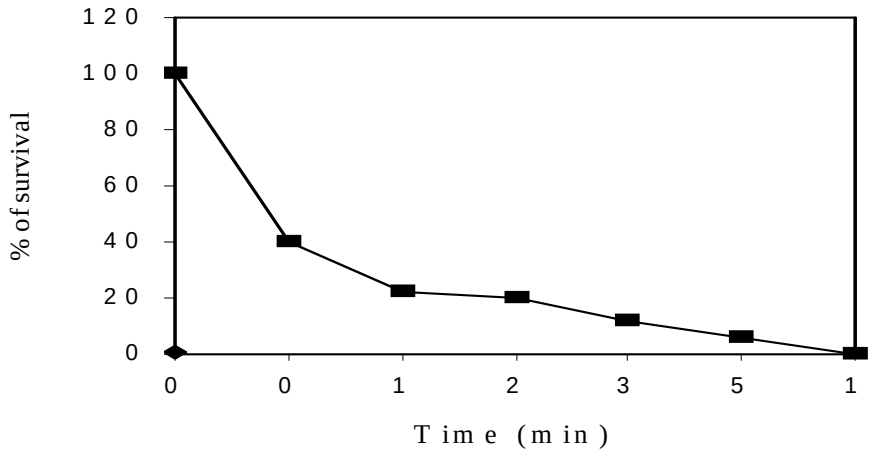

Figure 1: Effect of UV radiation and % of survival of *Bacillus licheniformis* SKB4

Table 1: Amount of amylase produced by the mutated colonies of *B. licheniformis* SKB4 after 10th repeated cycle

Strains Mutant	Relative Activity (%)
6a	53
8a	69
8b*	180*
4c	150
6c	80
6b	42
4b	111
8c	123
Wild	100

(*) Maximum amount of enzyme produced

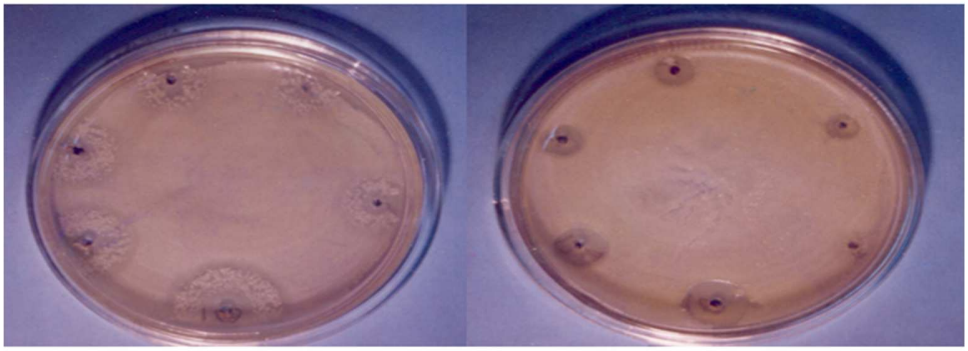

Plate 1: Effect of antibiotics on wild type strain of *Bacillus licheniformis* SKB4

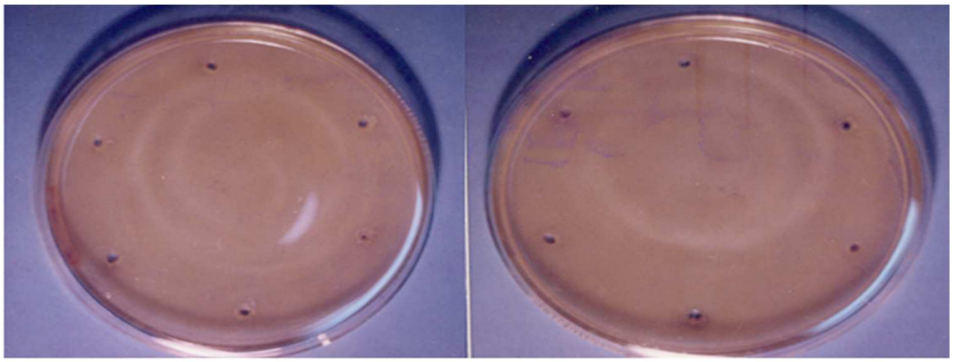

Plate 2: Effect of antibiotics on mutant strain (8b) of *Bacillus licheniformis* SKB4

Effect of the addition of antibiotics and respiratory inhibitors on amylase formation

It was found that the organism stopped the synthesis of α-amylase after addition of antibiotics like rifampicin, tetracycline (100 µg/ml) in the medium during 10 h growth period in both wild and mutant strain (Fig. 2 and 3). As the growth proceeds, the wild strain did not overcome the inhibitory effect of antibiotics (Fig. 2), while mutant strain gradually trying to do so (Fig. 3). This study indicated that the release of enzyme in culture medium was the product of de novo protein synthesis and the immediate inhibition of amylase secretion after addition of antibiotics, inhibitory to ribonucleic acid and protein synthesis (rifampicin, tetracycline) had proved de novo process of formation of α-amylase by *Bacillus licheniformis* SKB4. The instant cessation of amylase secretion without any lag period indicated that the amylase of *Bacillus licheniformis* SKB4 did not accumulate within the cell after its synthesis. This result was very similar with the findings of other reports (22,23).

The enzyme synthesis was also stopped immediately in both strains after addition of respiratory inhibitor 2, 4-dinitrophenol at 5 mM concentration in the culture medium of both wild and mutant strain (Fig. 2 and 3). Basically, 2,4-dinitrophenol is a synthetic uncoupler with a lipid-solubility property. This compound has been able to dissolve in the inner mitochondrial membrane and function as H^+ carriers. Thus, DNP shuttles H^+ across inner membrane that leads to dissipate electrochemical gradient of H^+ between intermembrane space and matrix of mitochondria. The ultimate result is inhibition of oxidative phosphorylation and ATP synthesis. This finding indicated that energy production by aerobic system is essential for active secretion of amylase. Previously, Saito and Yamamoto (15) had reported that addition of 2, 4-dinitrophenol or KCN (5mM) to the cultures had stopped the enzyme synthesis immediately.

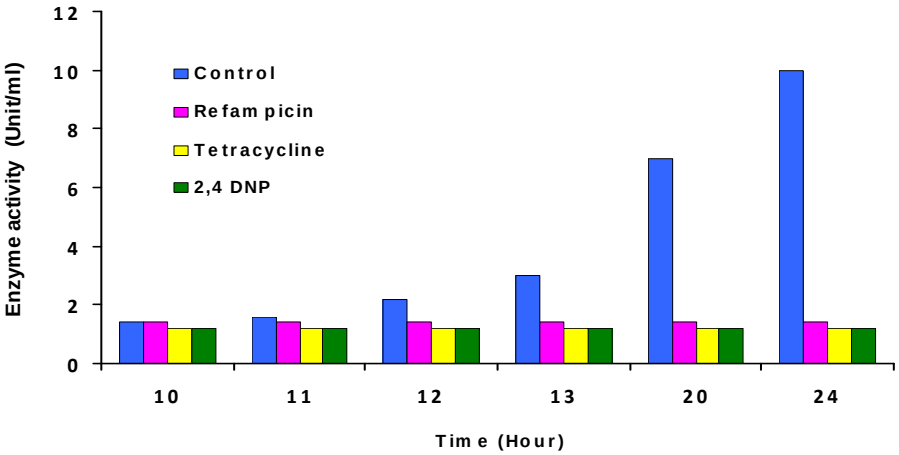

Figure 2: Effect of antibiotics and respiratory inhibitors on amylase synthesis by wild strain of *Bacillus licheniformis*SKB4.

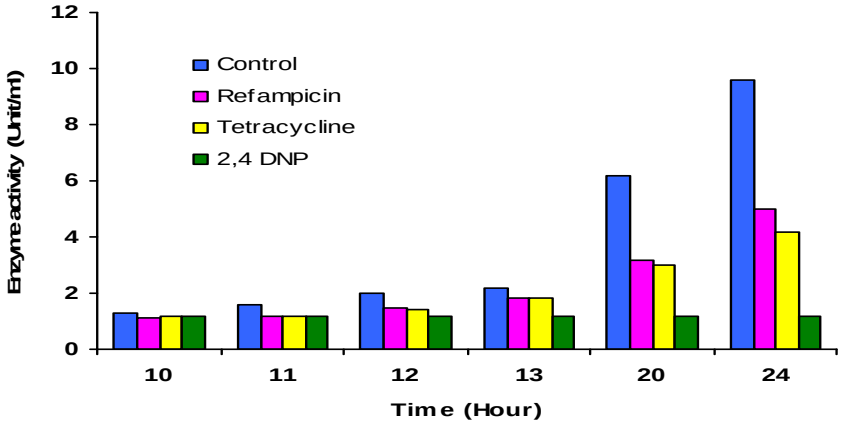

Figure 3: Effect of antibiotics and respiratory inhibitors on amylase synthesis by mutant strain (8b) of *Bacillus licheniformis*SKB4

Table 2: Effect of glucose sucrose on growth and amylase synthesis by *Bacilluslicheniformis* SKB4

Concentration (% w/v)	Cell mass growth (620 nm)	Enzyme activity (%)
Control (Without glucose)	2.8 ± 0.214	100 ± 4.56
	Glucose	
0.05	3.4 ± 0.176	94 ± 1.763
0.1	3.5 ± 0.260	72 ± 2.666
0.3	3.8 ± 0.321	37 ± 1.855
0.5	4.0 ± 0.230	6.0 ± 2.403
1	4.0 ± 0.346	6.0 ± 1.527
	Sucrose	
0.05	2.6 ± 0.260	62 ± 4.163
0.1	2.5 ± 0.348	62 ± 3.527
0.3	2.7 ± 0.233	12 ± 2.905
0.5	3.0 ± 0.176	6.0 ± 2.027
1	3.2 ± 0.404	6.0 ± 2.403

Effect of glucose and sucrose on synthesis of amylase

Production of the α-amylase was greater during growth on starch in contrast with the growth on glucose and sucrose; while the growth of the organism increased along with the increasing concentration of these simple sugars as they were readily assimilated as carbon sources. Addition of different concentration (0.05%-1.0% w/v) of glucose and sucrose had diminished to 6% of amylase production by *Bacillus licheniformis* SKB4. The enzyme synthesis was partially better (94%) in presence of glucose at lower concentration (0.05%) (Table 2). It is due to early utilization of readily usable sugar. Saito (24) previously reported that a strain of *Bacillus licheniformis* completely stopped the α-amylase synthesis when glucose was added to the growing cultures. Haseltine (17) reported that the presence of excess amount of glucose (0.1% w/v) in starch containing culture medium did not allow α-amylase synthesis by the cells, while exhaustion of glucose resulted in immediate production of enzyme. He also observed that decreasing amounts of glucose concentrations in the starch containing culture medium promoted early appearance of α-amylase in the culture supernatant.

The bacterial cells growing in the medium containing easily obtainable carbon source such as glucose or sucrose did not need to waste valuable energy in the biosynthesis of amylase. Therefore, the gene for amylase is turned off (25). Starch mediated induction of α-amylase synthesis was also argued as a sensory system for exogenous starch detection (17). Previously variety of soluble sugars were used to determine their stimulatory effect on amylase production by *Bacillus licheniformis* SKB4 and the results had indicated that starch (0.5% w/v) was the best inducer for amylase synthesis followed by others (26). The induction of α-amylase synthesis by starch and repression by glucose suggested that the α-amylase secretion by *Bacillus licheniformis* SKB4 was subjected to multiple forms of regulation. Soluble starch was preferably used as the carbon source in the standard medium, but starch itself cannot be an inducer due to its large size. It is predicted that malto-oligosaccharides either be present in soluble starch or forms after hydrolysis of starch by secreting amylase, acts as inducer. Saito and Yamamoto (15) observed that hydrolysable products of amylose (maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose) by α-amylase of *Bacillus licheniformis* 584 had been found to be superior to maltose as inducers of α-amylase synthesis in the same strain. Alpha amylase synthesis was also stimulated by malto-oligosaccharides in *B. stearothermophilus* (22).

The mutant strain of *Bacillus licheniformis* SKB4 produced greater amount of amylase in presence of glucose (upto 0.3%); but sucrose had no major effect on enzyme production in the same mutant strain (Table 3). This finding indicated that mutant strain could partially be able to overcome the glucose repression effect. Higher amylase yielding mutant strain of *Bacillus licheniformis* was successfully developed by Saito (24). It was found that mutant strain was able to produce the amylase during growth on a medium containing either starch or glucose as the sole carbon source, indicating resistance to catabolite repression.

Catabolite repression of α-amylase synthesis

Catabolite repression is the repression of enzyme synthesis that occurs when bacterial cultures are grown in the presence of a readily metabolized carbon source, such as glucose (27) and expression of the *Bacillus licheniformis* α-amylase gene, amyL, was also subject to catabolite repression (7). Amylase synthesis was completely inhibited after addition of glucose (0.5%) in starch containing medium during active log phase (10 h). This observation suggested that the secretion of alpha amylase by this strain was under the control of catabolite repression. While, addition of cyclic AMP (cAMP) in the growing culture containing glucose slightly stimulated the enzyme production (Fig. 4.2). These findings indicated that cAMP could try to overcome glucose repression effect by about 50%. But cAMP itself was not able to stimulate the enzyme synthesis when added to standard growing culture medium (Fig. 4). Catabolite repression of *Bacillus licheniformis* SKB4 was also similar to findings of other workers (13,15,17,28-30). Presence of glucose in starch containing medium along with cAMP enhanced amylase production in mutant strain. Thus, glucose mediated catabolite repression was not fully active in mutant strain. Earlier, several reports indicated that mutant strains of *Bacillus subtilis* (31), *Bacillus cereus* (32) and *Bacillus licheniformis* (33) were also resistant to glucose mediated catabolite repression of α-amylase synthesis in presence of glucose.

Table 3:- Effect of glucose sucrose on growth and amylase synthesis by mutant strain (8b) of *B. licheniformis*KB4

Concentration. (% w/v)	**Cell mass growth (620 nm)**	**Enzyme activity (%)**
Control (Without glucose)	3.8± 0.4.26	100 ± 3.34
Glucose		
0.05	4.1 ± 0.276	110 ± 4.664
0.1	4.4 ± 0.460	118 ± 6.212
0.3	4.8 ± 0.652	134 ± 5.342
0.5	5.4 ± 0.442	122 ± 2.778
1	5.6 ± 0.512	114 ± 7.254
Sucrose		
0.05	3.0 ± 0.260	66 ± 3.152
0.1	3.2 ± 0.348	72 ± 3.542
0.3	3.5 ± 0.233	82 ± 4.612
0.5	3.7 ± 0.176	48 ± 2.127
1	3.8 ± 0.404	44 ± 5.324

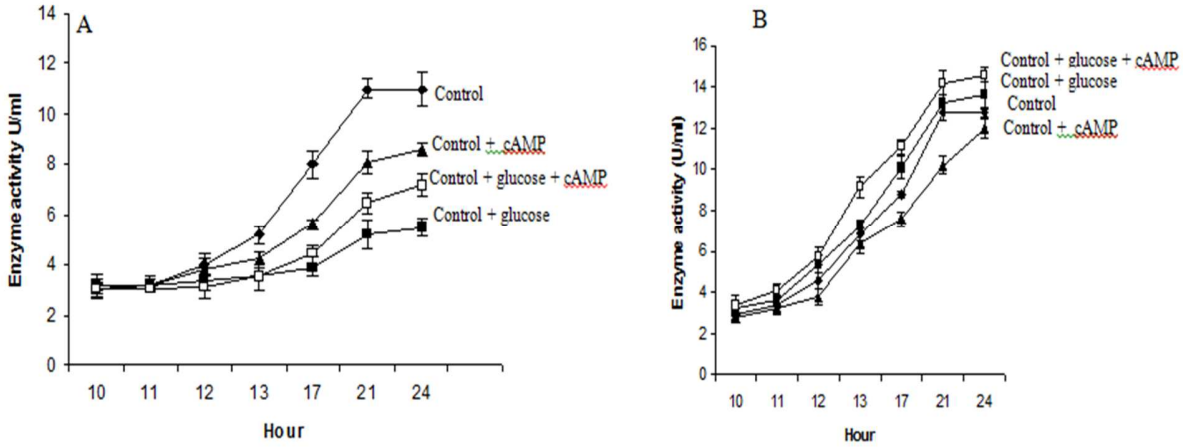

Figure4:Study of catabolic repression on amylase synthesis by *Bacilluslicheniformis*SKB 4, Control (♦), cAMP (▲), Glucose + cAMP (□), Glucose (■). The catabolite effectors were added at active state of growth (10th h of cultivation). A-Wild strain; B-Mutant strain (8b)

The glucose mediated catabolite repression effect was initially described as the glucose effect (34,35), observed in both gram-positive and gram-negative organisms. Shih and Labbe (36) suggested that glucose mediated catabolite repression of enzyme synthesis is very common in *Bacillus* sp. There was no significant stimulation of α-amylase synthesis after addition of cyclic AMP in culture medium of *Bacillus licheniformis* SKB4 as exogenous cAMP could not overcome the repression effect of glucose. This finding indicated that catabolite repression mediated by glucose in our strain was not related to the cAMP like second messenger. The role of cAMP in some prokaryotes is soundly accepted. Early studies on *Escherichia coli* had shown the rapid loss of intracellular cyclic AMP after addition of glucose to bacterial cultures (37) and glucose induced repressive effects were overrode by adding exogenous cAMP (38). Cyclic AMP binds to a catabolite activator protein (CAP) and this complex (CAP-cAMP) is a mediator of positive control on catabolic operons (39,40). Although, cyclic AMP has not been established as the sole mediator of catabolite repression because it plays an important role in regulating the transcription of many genes. Generally, *Bacillus* species do not contain cyclic AMP under physiological growth conditions and thus, catabolite control is not similar to *E. coli* (7). Alternatively, cyclic AMP had been established as an effector in the corresponding catabolite response to the gram-positive bacterium *Bacillus subtilis* (18). So, it can be stated that the expression of the α-amylase in *Bacillus licheniformis* SKB4 is regulated in a different manner as compared with the gene specifying cellular protein formation.

CONCLUSION

From the above findings it is concluded that amylase from *Bacillus licheniformis* SKB4 was an

inducible enzyme. The enzyme was synthesized through de novo process under control of catabolic repression. The mode of induction and repression is not linked with cAMP as second messenger.

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Conflict of interest
The authors declare that there are no conflicts of interest.

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