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Kinetic Characterization of Extracellular α-Amylase from a Derepressed Mutant of *Bacillus licheniformis*

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Abstract

Three strains of *Bacillus licheniformis* were isolated and screened for α-amylase production by solid-state fermentation. Of these, IS-2 gave relatively higher enzyme production (32 ± 2.3 U/[g·min]) and was selected for improvement after treatment with *N*-methyl *N*-nitro *N*-nitroso guanidine (NG) or nitrous acid (NA) to enhance its hydrolytic potential. Among the mutant variants, NA-14 gave higher enzyme production (98 ± 1.6 U/[g.min]), and hence, was selected for kinetic and thermal characterization. M1 as a moistening agent (pH 7.0, optimized) supported 2.65-fold improved amylolytic activity by the derepressed mutant 72 h after inoculation. The values of product yield coefficient ($Y_{p/x}$ = 1833.3 U/g) and specific rate constant (q_p = 25.46 U/[g·h]) with starch were severalfold improved over those from other carbon sources and the other cultures. The purified enzyme from NA-14 was most active at 40°C; however, the activity remained almost constant up to 44°C. The NA-induced random mutagenesis substantially improved the enthalpy (ΔH_D = 94.5 ± 11 kJ/mol) and entropy of activation (ΔS = –284 ± 22 J/[mol·K]) for α-amylase activity and substrate binding for starch hydrolysis. The results of this study (117.8 ± 5.5 U/[g·min]) revealed a concomitant improvement in the endogenous metabolism of the mutant culture for α-amylase production.

Index Entries: *Bacillus licheniformis*; α-amylase; mutant; solid-state fermentation; wheat bran; thermal characterization.

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Introduction

The potential uses of microorganisms as biotechnological sources for industrial enzymes have stimulated renewed interest in the exploration of improved activities *(1)*. α-Amylase, an extracellular enzyme, degrades the α1-4 linkage of starch, and its spectrum of applications has widened in many sectors such as clinical, medicinal, and analytic chemistry *(2)*. It is a highly thermostable starch-degrading enzyme and has been extensively studied inboth academic and industrial laboratories. The enzyme also finds applications in the food, baking, brewing, detergent, textile, and paper industries *(3)*. Therefore, any improvement in the enzyme's production, thermostability, or activity will have a direct impact on process performance, economics, and feasibility. The industrial starch process requires several enzymes. The first step is carried out with *Bacillus licheniformis* α-amylase, which is used to depolymerize starch to maltodextrins and corn syrup solids by controlled hydrolysis, a process known as liquefaction. Random mutagenesis using radiation (ultraviolet [UV] or gamma rays) or chemicals (alkylating agents or nitrous acid [NA]) has been employed to improve the metabolic behavior of *Bacillus* strains in relation to α-amylase production *(4,5)*. *N*-methyl *N*-nitro *N*-nitroso guanidine (NG) is a powerful mutagen, and it causes permanent changes in the genome of microbes. UV radiation causing a death rate of 99% was found to be the most effective in the development of mutants with improved α-amylase production.

The basic part of cell structure and proteins, carbon contents affect not only the mode of amylase biosynthesis but also the rate with which the carbohydrates are metabolized *(6)*. Solid-state fermentation using moist agricultural polymeric substrates such as wheat, rice, soy, or cassava has been employed *(7)*. These agricultural byproducts are abundantly available in Pakistan and are exploited in microbial fermentations to produce useful primary or secondary metabolites. Solid-state fermentation is gaining interest owing to its easy control and handling, use of a wide range of raw materials as substrate, low energy requirements, and higher productivity rates. The cultural conditions, nutritional requirements, and thermophilic characterization for a selected strain need to be exploited in order to obtain insight into the enzyme kinetics and yield potential. Therefore, in the present study, we investigated the kinetic characterization of extracellular α-amylase from a locally developed derepressed mutant of *B. licheniformis* following Arrhenius plots *(8)* by solid-state fermentation.

Materials and Methods

Moistening Agents

1. M1: 2.0 g/L of yeast extract, 2.5 g/L of peptone, 8.0 g/L of soluble starch, 2.0 g/L of ammonium sulfate, 1.2 g/L of $CaCl_2$, 0.45 g/L of $MgSO_4 \cdot 7H_2O$, 0.20 g/L of $FeSO_4$, pH 7.5 *(4)*.

2. M2: 3.0 g/L of peptone, 2.0 g/L of beef extract, 10.0 g/L of soluble starch, 5.0 g/L of ammonium sulfate, 10.0 g/L of lactose, 3.0 g/L of $CaCl_2$, 1000 mL of phosphate buffer (0.02 *M*), pH 8.0 *(2)*.
3. M3: 2.5 g/L of peptone, 2.0 g/L of beef extract, 10.0 g/L of soluble starch, 3.0 g/L of $CaCl_2$, 0.15 g/L of $MgSO_4 \cdot 7H_2O$, 1000 mL of phosphate buffer (0.02 *M*), pH 7.2 *(5)*.
4. M4: 2.5 g/L of yeast extract, 2.5 g/L of peptone, 10.0 g/L of soluble starch, 1.5 g/L of ammonium sulfate, 1.2 g/L of $CaCl_2$, 0.45 g/L of $MgSO_4 \cdot 7H_2O$, pH 7.2 *(9)*.
5. M5: 3.0 g/L of yeast extract, 12.0 g/L of soluble starch, 1.2 g/L of $CaCl_2$, 0.20 g/L of $FeSO_4$, 0.12 g/L of K_2HPO_4, 0.05 g/L of $CuSO_4 \cdot 7H_2O$, pH 7.5 *(4)*.
6. M6: 1.0 g/L of peptone, 2.5 g/L of beef extract, 10.0 g/L of soluble starch, 3.0 g/L of ammonium phosphate, 2.0 g/L of $CaCl_2$, 1000 mL of phosphate buffer (0.02 *M*), pH 8.0 *(10)*.

Microorganism

Three different strains of *B. licheniformis* were isolated from soil by the serial dilution method *(9)*. One gram of soil sample was suspended in 100 mL of sterilized distilled water. The soil suspension was diluted up to 10^4– 10^5 times, and 0.5 mL of the diluted suspension was transferred to Petri plates containing nutrient broth starch agar medium (pH 7.2). The Petri plates were incubated at 40°C for 24–36 h. The newborn colonies were aseptically picked up and transferred to the nutrient broth agar slopes. Cultural and morphologic characteristics were studied. The strains were identified *(11)*. The slants were incubated at 37°C for 24 h until maximum growth and thereafter stored at 4°C in a cooled incubator (Griffin, London, UK).

Culture Improvement After Random Mutagenesis

NG Treatment

Different concentrations of NG (i.e., 0.02, 0.04, 0.06, 0.08, 0.10 mg/mL) were prepared in acetate buffer (0.02 *M*, pH 4.5). Five milliliters of each NG solution was added to individual centrifuge tubes containing 5.0 mL of washed bacterial cells and shaken until a homogeneous suspension was achieved. In the control, 5.0 mL of NG solution was replaced with acetate buffer. After a specific time interval (20–60 min), the cells were centrifuged and washed in 0.02 *M* phosphate buffer, pH 7.2 *(12)*. The NG-treated cells were resuspended in 5.0 mL of phosphate buffer. Approximately 0.1 mL of the suspension was inoculated to nutrient broth agar (pH 7.2) plates. Colonies appearing 24–36 h after incubation at 40°C were screened independently for enzyme activity.

NA Treatment

Five milliliters of each NA solution (0.05–0.20 *M*) prepared in acetate buffer (0.02 *M*, pH 4.5) was added to the washed and centrifuged cells of *B.*

licheniformis. The suspension was thoroughly shaken for about 10 min. Afterward, 1.0 mL of solution was withdrawn and diluted fivefold in phosphate buffer (0.02 *M*, pH 7.2) to stop the reaction. Approximately 0.1 mL of the suspension was inoculated to the nutrient broth agar plates. The control was also run parallel. Colonies appearing 24–36 h after incubation at 40°C were screened independently for enzyme activity.

Preparation of Inoculum

Fifty milliliters of inoculum medium containing 9.0 g/L of nutrient broth, 10 g/L of soluble starch, 5.0 g/L of lactose, 5.0 g/L of NaCl, and 2.0 g/L of $CaCl_2$ in 1000 mL of 0.02 *M* phosphate buffer (pH 7.5) was transferred to a 250-mL cotton-plugged Erlenmeyer flask and sterilized at 15 psi of pressure (121°C) for 15 min. After cooling to an ambient temperature, a loopful of bacteria was aseptically transferred to the flask and agitated in a rotary shaking incubator (200 rpm) at 40°C for 24 h.

Fermentation Procedure

All microbial fermentations were carried out using solid-state fermentation in 250-mL Erlenmeyer flasks. Ten grams of wheat bran partially replaced with cottonseed meal (7.5:2.5) was transferred to individual flasks and the flasks were plugged with cotton. The substrate was moistened with moistening agent (M1 optimized) in a ratio of 1:1. The flasks were sterilized in an autoclave for 15 min (15 psi, 121°C) and cooled at room temperature. One milliliter of inoculum (1.26×10^7 cells) was aseptically transferred to each flask, and the flasks were placed in an incubator at 40°C for 72–96 h. All the experiments were run parallel in triplicates.

Enzyme Extraction

After incubation, 100 mL of phosphate buffer with 0.02% (v/v) Tween-80 was transferred to each flask containing the fermented mash. The flasks were rotated in an incubator shaker at 200 rpm for 1 h. The contents of the flasks were then centrifuged at 9000 rpm (8331*g*) for 15 min. The substrate-free supernatant was used for estimation of α-amylase.

Determination of Biomass

Biomass was determined turbidimetrically at 650 nm using a spectrophotometer and compared with the standard for dry weight vs absorbance at the same wavelength. A blank was run parallel replacing biomass solution with distilled water. Biomass was converted into grams per gram according to the method of Hariuchi et al. *(10)*.

Protein Contents

Bradford reagent (5.0 mL) was added to a test tube containing 0.1 mL of the appropriately diluted enzyme extract. A blank was run parallel. The

tubes were vortexed and absorbance was noted at 595 nm on a spectrophotometer. The concentration of protein in each sample was obtained using bovine serum albumin according to Bradford *(13)*:

$$\text{Protein (mg/mL)} = \text{Slope} \times 5.0 \times \text{Dilution factor}$$

Enzyme Assay

α-Amylase activity was estimated according to Rick and Stegbauer *(14)*. One unit of activity is equivalent to the amount of enzyme that in 1 min liberates the reducing group from 1.0% Linter's soluble starch correspond-ing to 1.0 mg of maltose hydrate. One milliliter of enzyme extract (pH 7.5) was incubated at 60°C using 1.0% (w/v) soluble starch solution. The reduc-ing sugars were measured by adding 3,5-dinitro salicylic acid reagent, boiling for 5 min, cooling, and measuring the optical density at 540 nm in a UV/Vis double-beam scanning spectrophotometer (Model CE-7200, CECIL, London, UK) vs maltose as the standard. A blank was also run parallel replacing the enzyme extract with 1.0 mL of distilled water.

Purification of Enzyme

The culture filtrate was concentrated about 10-fold using an ultrafiltration unit at 40°C for 2 h (Millipore, Bedford, MA). Thereafter, ammonium sulfate was added to the concentrate to reach 60% saturation (w/v) and stirred overnight (120 rpm) on a magnetic stir plate. The precipitated suspension was centrifuged at 16,000*g* for 30 min at 4°C and decanted. The pellet was dissolved in 30 m*M* sodium phosphate buffer (pH 6.0), dialyzed against distilled water. Proteins were eluted from the column with an NaCl gradient (0–0.5 m*M*) in 30 m*M* sodium phosphate buffer (pH 6.0) at a flow rate of 4 mL/min. Protein in the effluent was monitored by determin-ing the absorbance at 290 nm *(15)*.

Kinetic and Thermal Characterization

The kinetic parameters were obtained according to Pirt *(16)*. The values for specific growth rate, μ (per hour), were calculated from the plots of ln(*X*) vs time of fermentation. The product yield coefficient ($Y_{p/X}$) and specific rate constant for enzyme (q_p) were determined by using the following relationships:

$$Y_{p/X}\,(\text{U/g}) = dP/dX$$

$$q_p(\text{U/[g·h]}) = \mu \times Y_{p/X}$$

The empirical approach of Arrhenius equations was used to describe the relationship of temperature-dependent irreversible inactivation of α-amylase production *(8)*. The temperature ranged from 32 to 48°C. Specific

rate for thermophilic enzyme production (q_p, enzyme units/[g of cells·h]) was used to calculate different variables following these equations:

$$q_p = \mathrm{T}\cdot k_B/he^{\Delta S^*/R}\mathrm{e}^{-\Delta H^*/RT}$$

$$\ln(q_p/T) = \ln(K_B/h) + \Delta S^*/R - \Delta H^*/RT$$

The plot of $\ln(q_p/T)$ vs $1/T$ gave a straight line whose slope was $-\Delta H/R$ and intercept was $\Delta S/R + \ln(K_B/h)$, in which h (Planck's constant) = $6{\cdot}63 \times 10^{-34}$ Joules seconds (Js) and K_B (Boltzmann constant [R/N]) = $1{\cdot}38 \times 10^{-23}$ J/K, in which N (Avogadro's number) = $6{\cdot}02 \times 10^{23}$/mol.

Statistical Analysis

Treatment effects were compared using the method of Snedecor and Cochran *(17)*. Duncan's multiple range tests (Spss-10.5, version 4.0) were applied under one-way analyses of variance. Significance was presented in the form of probability ($p < 0.05$) values.

Results and Discussion

Nutritional Conditions

Three wild cultures of *B. licheniformis* were isolated from soils of different areas of Lahore (Pakistan) by observing clear zones of starch hydrolysis in nutrient broth agar plates. However, the zones cannot be correlated quantitatively with the amount of α-amylase produced because of the hydrolytic potential of other enzymes such as glucoamylase *(4)*. Therefore, screening of strains producing α-amylase using starch plates may be only partially selective. Hence, these cultures were screened for enzyme production using solid-state fermentation in 250-mL Erlenmeyer flasks (Table 1). *B. licheniformis* isolate IS-2 gave relatively higher enzyme activity (32 ± 2.3 U/[g·min], 0.160 mg/mL of protein). This culture was mutated after treatment with NG or NA to further enhance the hydrolytic potential in terms of α-amylase activity (Table 1). Among the mutants, the derepressed NA-14 was found to give the highest enzyme activity (98 ± 1.6 U/[g·min]) and selected for kinetic and thermodynamic characterization. The total protein content was recorded to be 0.862 mg/mL with the mutant. The rest of the variants produced insignificant amounts of enzyme under the same set of conditions. Our work is substantiated by the findings of Ginka et al. *(10)*, who isolated aerobic bacteria (JF1, JF2, and D) that were from Chinese koji and identified as two different *Bacillus* spp. that produced a thermostable α-amylase.

Selection of an appropriate moistening agent for α-amylase production by *B. licheniformis* IS-2 and NA-14 was undertaken (Fig. 1). The use of M1, containing 2.0 g/L of yeast extract, 2.5 g/L of peptone, 8.0 g/L of soluble starch, 2.0 g/L of ammonium sulfate, 1.2 g/L of $CaCl_2$, 0.45 g/L of $MgSO_4{\cdot}7H_2O$, and 0.20 g/L of $FeSO_4$ (pH 7.5), resulted in the maximum production of α-amylase (99.5 U/[g·min]) by the putative mutant. Yeast

Table 1
Screening of B. licheniformis Strains (Wild and Mutant Variants) for α-Amylase Production[a]

B. licheniformis strain	Protein contents (mg/mL)	α-Amylase (U/[g·min])	Specific growth rate, μ (per h)
Wild isolates			
IS-1	0.272	18 ± 3.2	0.175
IS-2	0.160	32 ± 2.3	0.067
IS-3	0.096	19 ± 2.8	0.196
NG-induced mutants			
NG-4	0.224	31 ± 4.2	0.046
NG-5	0.200	37 ± 4.6	0.171
NG-6	0.489	31 ± 2.0	0.142
NG-7	0.092	12 ± 3.0	0.150
NG-8	0.086	19 ± 1.3	0.090
NG-9	0.234	30 ± 4.2	0.025
NG-10	0.568	34 ± 1.6	0.108
NG-11	0.321	16 ± 1.5	0.233
NG-12	0.220	47 ± 1.0	0.079
NA-induced mutants[b]			
NA-13	0.489	32 ± 3.0	0.067
NA-14	0.862	98 ± 1.6	0.042
NA-15	0.078	11 ± 2.2	0.129
NA-16	0.342	28 ± 2.4	0.083

[a]The incubation period was 48 h and the temperature was 37°C. Each value is an average of three parallel replicates. Plus/minus indicates the SD from the mean value.
[b]Nitrous acid (HNO_2, unstable and readily oxidizable).

extract and peptone acted as organic nitrogen sources while ammonium sulfate acted as an inorganic nitrogen source. Other moistening agents gave comparatively lower enzyme production. This might be owing to the fact that these agents lacked any of the macronutrients that may be essential for growth and subsequent enzyme secretion. In the first growth phase, the microorganism utilized the nitrogen source and the maximum enzyme was associated with cell lyses *(7,13)*. In the second phase, the carbohydrate source (i.e., lactose) was utilized and enzyme peaked in the early stage of growth. IS-2 gave insufficient production of amylase by all the moistening agents. Hariuchi et al. *(10)* isolated a culture of *Bacillus flavothermus* that gave α-amylase activity (28.6 U/[g·min]) with 40 g/L of lactose and 20 g/ L of yeast extract (pH 6.0) as moistening agent. The enzymes are highly sensitive to the pH optima *(18)*. In the present study, the effect of different pH values (6.0–8.5) of moistening agent on α-amylase production was also studied using IS-2 and NA-14 (Fig. 2). α-Amylase production was found to be the best at pH 7.0. A further increase in pH resulted in decreased enzyme activity. Moreover, the rate and secretion of enzyme were greatly inhibited at an alkaline pH (8.0–8.5).

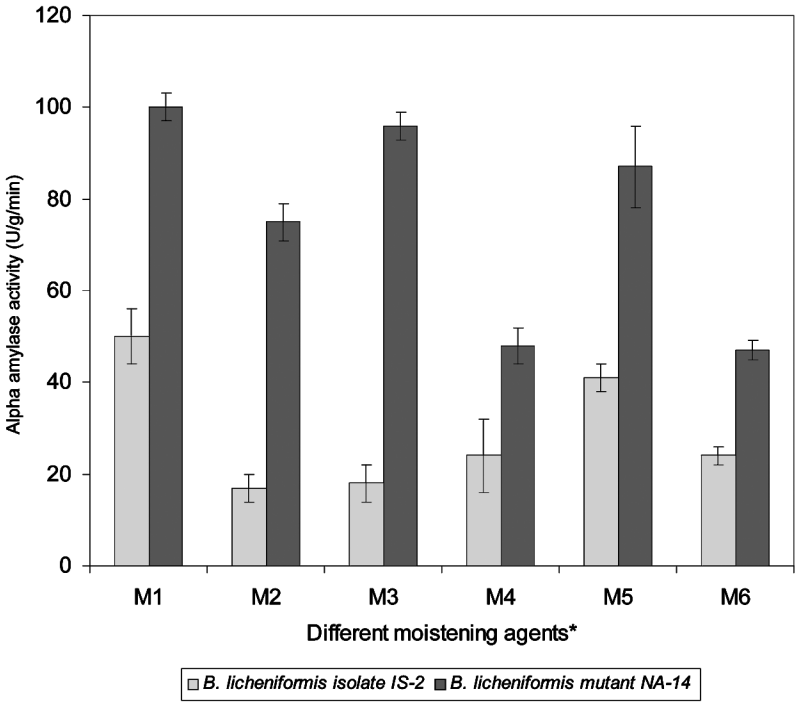


Fig. 1. Selection of moistening agent for α-amylase production by *B. licheniformis* wild culture IS-2 and mutant NA-14. The incubation period was 48 h and the temperature was 37°C. Each value is an average of three parallel replicates. Y-error bars indicate the SD from the mean value. *See Materials and Methods for the composition of each of the moistening agent media.

Production and Characterization of Enzyme

The time course comparison of *B. licheniformis* wild culture IS-2 and mutant NA-14 for α-amylase production was studied (Fig. 3). α-Amylase biosynthesis was increased with an increase in incubation period from 0 to 96 h and reached a maximum 72 h after inoculation by the mutant (80 h by the parental). Thus, NA-14 gave about 2.2-fold higher enzyme production than IS-2. α-Amylase production started after a lag phase of about 8–12 h and reached maximum at the late stationary phase, followed by a steady decline at the onset of the death phase (owing to the proteolysis effect). These findings are substantiated by those of Ramesh and Lonsane *(19)*. A further increase in the incubation period beyond optimal resulted in decreased enzyme activity. This might be owing to the accumulation of other byproducts (toxins and cellular debris) and exhaustion of nutrients from the medium. Undesirable microbial byproducts inhibit the growth of cells and, hence, enzyme production *(20)*.

Comparison of kinetic parameters highlighted that the value of q_p (specific rate of enzyme production) was highly significant ($p < 0.05$) in the presence of starch but almost insignificant in the case of glucose or xylose

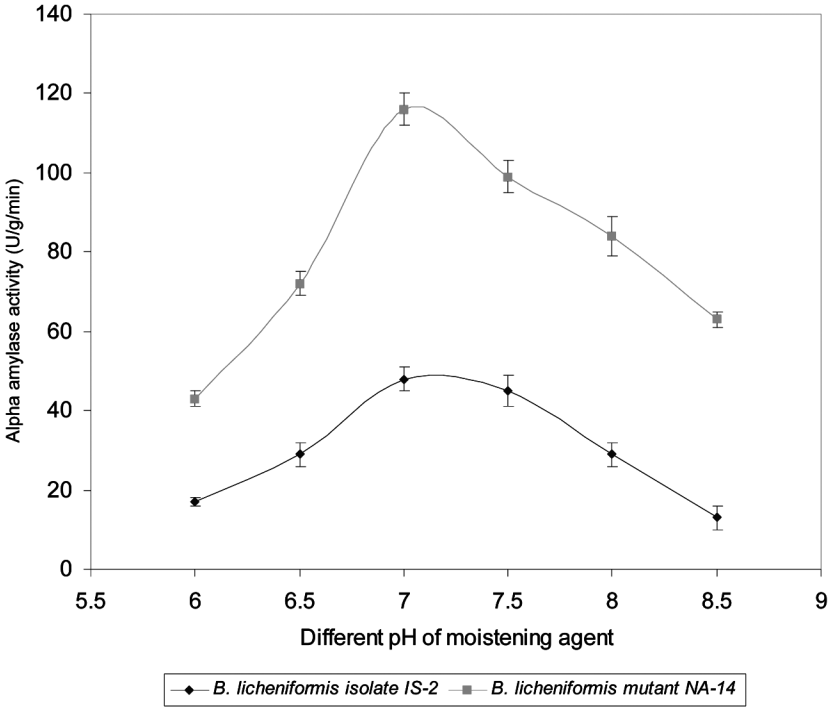


Fig. 2. Comparison of different pH values of moistening agent for α-amylase production by *B. licheniformis* wild culture IS-2 and mutant NA-14. The temperature was 37°C, and the substrate to diluent ratio was 1:1. Each value is an average of three parallel replicates. Y-error bars indicate the SD from the mean value.

(sugar level of 1.5% [w/v] regardless of the carbohydrate source) by the mutant NA-14. Similarly, the values of $Y_{p/x}$ (enzyme produced per cell mass formed) were significantly decreased by the addition of glucose or xylose into the medium (Fig. 4). This might be owing to carbon catabolite repression, which results in decreased enzyme production *(19)*. In addition, when starch was supplemented with complex agricultural byproducts such as wheat bran, it acted as an inducer for growth of the microorganism. In the initial growth phase, the organism can hydrolyze complex carbohydrates such as wheat bran for its food and growth purposes with concomitant secretion of α-amylase into the medium *(21)*. Thus, strain NA-14 may require slightly more starch for its initial growth with concomitant significant enzyme production (0.862 mg/mL of protein). Pirt *(16)* and Converti and Dominguez *(22)* have reported similar findings; however, the present values of $Y_{p/x}$(U/g) and q_p(U/[g·h]) are ≈8- to 10-fold higher than those of previous studies by other researchers.

Thermophilic characterizatio1n of *B. licheniformis* wild culture IS-2 and mutant NA-14 for α-amylase activity was carried out at a temperature range of 32–48°C (Fig. 5). The purified enzyme from NA-14 was most active (117.8 ± 5.5 U/[g·min]) at 40°C; however, the activity remained almost

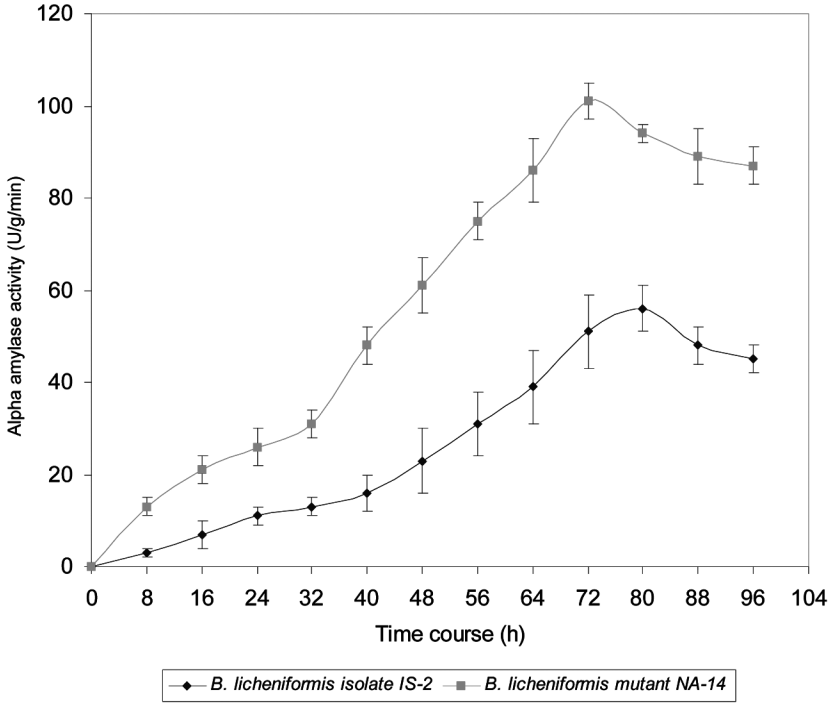
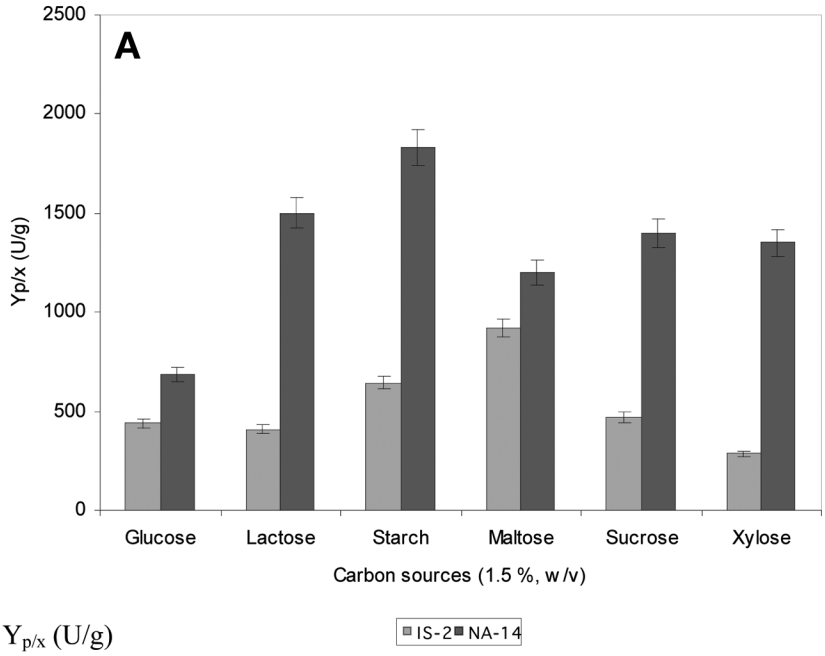
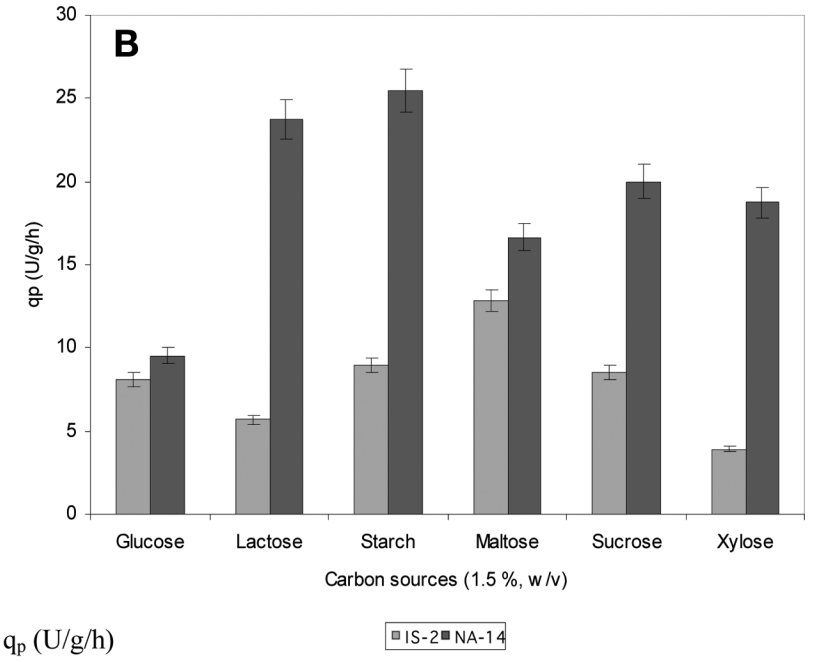


Fig. 3. Time course comparison profile of *B. licheniformis* wild culture IS-2 and mutant NA-14 for α-amylase production. The temperature was 37°C, and the substrate to diluent ratio was 1:1. Each value is an average of three parallel replicates. Y-error bars indicate the SD from the mean value.

constant up to 44°C. Therefore, notably the temperature variation up to a certain extent has no bearing on enzyme activity. Thermal inactivation of α-amylase was characterized by an activation enthalpy (ΔH_D) of 94.5 ± 11 kJ/mol, which was remarkably lower for the mutant (Table 2). The value of ΔH_D (94.5–128 kJ/mol) was significantly lower than the values of bacterial cultures used by previous investigators *(12,23)*. The activation entropy of α-amylase production by mutant cells (–284 ± 22 J/[mol·K]) was very low and compared favorably with that of xylitol formation reactions. The negative symbol reflects that the inactivation phenomenon implies a slight disorderness during growth and subsequent enzyme formation. Practically this value is lower than those estimated for amylase production by other systems *(22)*. This suggests more protection exerted by the mutant cell system compared with wild cells against thermal inactivation. Converti and Borghi *(23)* investigated cell growth kinetics and α-amylase production by a *Bacillus* species. The kinetics of the growth rate was studied, and the superiority of mutant cells over free cells was demonstrated.



$Y_{p/x}$ (U/g)



q_p (U/g/h)

Fig. 4. Comparison of kinetic parameters of carbon source utilization for α-amylase production by *B. licheniformis* wild culture IS-2 and mutant NA-14: **(A)** $Y_{p/x}$ (U/g); **(B)** q_p (U/g·h). The incubation period was 48 h, the temperature was 37°C, and the substrate to diluent ratio was 1:1. Each value is an average of three parallel replicates. Y-error bars indicate the SD from the mean value.

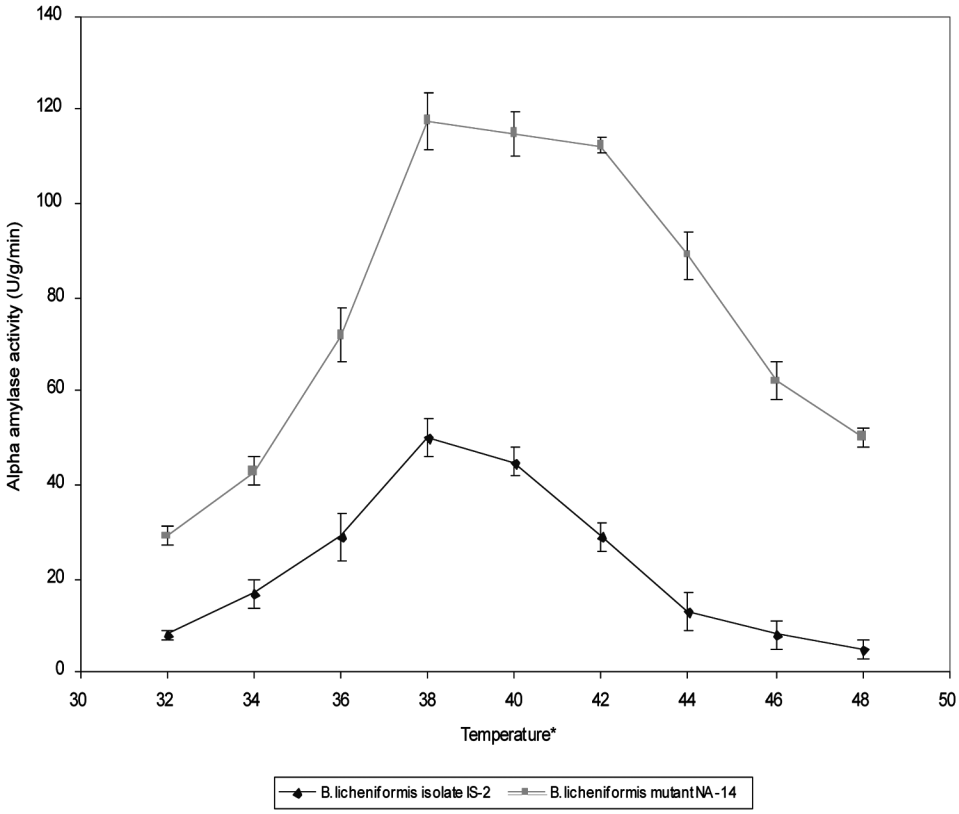


Fig. 5. Thermophilic characterization of *B. licheniformis* wild culture IS-2 and mutant NA-14 for α-amylase activity. The incubation period was 48 h, and the substrate to diluent ratio was 1:1, °C*. Each value is an average of three parallel replicates. Y-error bars indicate the SD from the mean value.

Conclusion

A putative mutant strain of *B. licheniformis* NA-14 was developed after treatment with NA for enhanced α-amylase production. Cultural condi-tions and nutritional requirements were optimized. Enzyme from the mutant was purified and found to be most active at 40°C. More notably, the activity remained almost constant up to 44°C and declined thereafter. NA-induced random mutagenesis improved the enthalpy (ΔH_D = 94.5 ± 11 kJ/mol) and entropy of activation (ΔS = !284 ± 22 J/[mol·K]) for α-amylase activity and substrate binding for starch hydrolysis. However, in order to increase the stability of amylase, microbial and biochemical engineering of NA-14 is currently in progress.

Table 2
Comparison of Thermodynamic Parameters of *B. licheniformis* Wild Culture IS-2 and Mutant NA-14 for α-Amylase Activity[a]

Parameter[b]	Enzyme production	Thermal inactivation
Activation enthalpy, ΔH_D^* (kJ/mol)		
Parental (IS-2)	128 ± 5.5	97 ± 6
Mutant (NA-14)	94.5 ± 11	66 ± 8.5
Activation entropy, ΔS^* (J/[mol·K])		
Parental (IS-2)	112 ± 10	62 ± 12
Mutant (NA-14)	(–)284 ± 22	(–)295 ± 15

[a]Each value is an average of three parallel replicates. Plus/minus indicates the SD from the mean value. Values followed by different letters in each row are significantly different from each other at $p \leq 0.05$.

[b]Thermodynamic parameters were determined using the following equation:

$$\mathrm{Ln}(q_p/\mathrm{T}) = \mathrm{Ln}(k_B/h) + \Delta S^*/R - \mathrm{D}H_D^*/R{\cdot}1/T \ldots,$$

in which q_p, T, k_B, h, ΔS^*, ΔH_D^*, and R are specific activity, absolute temperature, Boltzmann constant, Planck's constant, entropy of activation, enthalpy of activation, and gas constant, respectively. The values of k_B, h, and R are 1.38×10^{-23} J/K, 6.63×10^{-34} Js, and 8.314 J/(K·mol), respectively. ΔH^* was calculated as slope and $\mathrm{Ln}(k_B/h) + \Delta S^*/R$ as intercept on the Y-axis.

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