Kinetic Evidence of a Thermostable β-Amylase from Chemically Improved Mutant Strain of Bacillus subtilis

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Abstract.- In the present article, we report on the kinetic characterization of enhanced β-amylase production from a derepressed mutant strain of Bacillus subtilis under solid-state fermentation (SSF). For this, six bacterial strains were isolated and screened for enzyme production. Of these, IS-4 exhibited relatively better enzyme production (22±4.2 U) and hence selected for further improvement through the treatment with ethyl methane sulphonate (EMS) and nitrous acid (NA). Among the mutants, NA-12 gave the highest enzyme activity (45±1.6 U) and selected for kinetic as well as thermal characterization. M2 (pH 7), as moisture content supported 55% higher amylase activity by the potent mutant in 72 h of incubation. The product yield coefficient ($Y_p\times = 6.4 \text{U/g}$) and the specific rate constant ($q_p = 0.889 \text{U/g/h}$) using starch as a sole carbon source were many fold improved over to the other carbon sources or strains being used. The purified enzyme was most active at 40°C. This enhanced activity remained fairly constant up to a maximum of 44°C. NA-induced mutagenesis markedly improved enthalpy ($\Delta H = 64.5±4.5 \text{kJ/mol}$) and entropy of activation ($\Delta S = -234±18 \text{J/mol/K}$) for β-amylase. The substrate binding ability of enzyme for starch hydrolysis was also potentially increased. SDS-PAGE analysis of purified enzyme revealed a single visible protein band corresponding to about 113 kDa mass showing amylase activity. The results have shown an improvement in the endogenous metabolism of mutant strain for β-amylase hyper production (65.5±5.5 U).

Keywords: Bacillus subtilis / β-amylase / induced mutagenesis / solid-state fermentation / kinetic study / thermal characterization.

INTRODUCTION

The commercial uses of microorganisms as biotechnological foundations for production of potentially useful enzymes have stimulated new interests for the exploration of better activities (Diaz et al., 2003). Amyloytic enzymes hydrolyze α-1,4-glycosidic linkages for the breakdown of starch, glycogen or other polysaccharides into saccharides. They are categorized into three main groups i.e., α-amylase, β-amylase and glucoamylase (Saxena et al., 2007). Among them, β-amylase (EC 3.2.1.2) is an important exo-acting enzyme that cleaves second α-1,4 glycosidic linkages from the non-reducing ends of amylose, amylopectin and glycogen molecules producing maltose. In multiple attacks, the enzyme yields many maltose molecules during a single enzyme-substrate complex (Hossain et al., 2008; Awais et al., 2010). The enzyme β-amylase is of great interest in having extensive applications in starch saccharification, food, brewing, textile, distillery or pharmaceutical industries. It can also be used for the biosynthesis of high conversion and maltose syrups (Li and Yu, 2012). Improvement in enzyme formation, hyper-activity or thermostability has a direct impact in the method development, economics and thus process feasibility. The industrial starch process involves key enzymes. The first step is generally carried out with Bacillus subtilis amylase. It is applied to depolymerize starch into maltodextrins along with corn syrup solids by a process of liquefaction (Sun et al., 2010). The induced mutagenesis involving radiations (like ultraviolet or gamma rays) or chemicals (like alkylating agents or nitrous acid) has been attempted to increase the metabolic performance of bacterial strains for better amylase production (Daba et al., 2013).

Solid state fermentation (SSF) using agricultural by-products such as wheat bran, wheat straw, rice bran, rice straw, rice husk, soybean meal or cassava husk as substrates, have been previously employed (Solimam, 2008; Li and Yu, 2011). These agricultural by products are plentifully available for their utilization in industrial fermentation processes to yield quite useful primary and secondary metabolites. The process of SSF is gaining interest.
due to easy control and better handling, use of a range of raw materials as possible substrate, low energy requirements, low costs and better productivity rates. Cultural conditions, nutritional optimizations and thermophilic or kinetic characterization for a hyper-mutant strain need to be used in order to have an insight into the enzyme potential yield. The present study was planned to explore the kinetic study of β-amylase from an indigenously improved mutant strain of B. subtilis using Arrhenius plots (Aiba et al., 1973).

**MATERIALS AND METHODS**

*Microorganism and culture maintenance*

A total of 6 different strains (Bacillus subtilis) were isolated from the soil samples. Serial dilution method (Clark et al., 1958) was used for culture isolation. One gram of the sample was dispensed in 100 ml of sterilized water. The stock solution was further diluted up to 10³. Approximately 0.5 ml of this diluted soil suspension was transferred to sterile Petri plates having nutrient starch agar medium (pH 7.2) and incubated at 37°C for a period of 24 h. The initial colonies were aseptically picked and inoculated to the agar slopes of same medium. The cultural and morphological characteristics were investigated for strain identification according to Onion et al. (1986). Slant cultures were incubated (37°C, 24 h) for maximum growth. The culture was stored at 4°C in a cold cabinet (510QM, Sanyo, London, UK).

*Strain improvement after induced mutagenesis*

_Ethyl methane sulphonate (EMS) treatment_

EMS was prepared in phosphate buffer (50 mM, pH 7.2) having a range of different concentration _i.e._, 0.02, 0.04, 0.06, 0.08, 0.1 mg/ml. Five millilitre of each EMS grade was transferred to a centrifuge tube having 5 ml of pre-washed bacterial cells. It was shaken until a clear homogenous suspension was formed. EMS solution was replaced with 5 ml of phosphate buffer and treated as control. The cells were centrifuged after regular intervals (10-30 min). These were then washed with 0.02 M acetate buffer, pH 4.5 (Kohno et al., 1989). The treated cells were resuspended in acetate buffer. The suspension was re-inoculated to NB-agar plates.

_Nitrous acid (NA) treatment_

Different NA solutions (0.05-0.25 M) were prepared in acetate buffer (0.02 M, pH 4.5). The washed and centrifuged cells of selected _B. subtilis_ were treated separately. The suspension was then swirled for 10 min. A control was run in parallel. One millilitre of the solution was withdrawn and diluted up to 10-fold using phosphate buffer (50 mM, pH 7.2) to quench the reaction. The treated suspension was inoculated to the agar plates.

The colonies appearing between 24-36 h after incubation were screened independently for better enzyme activity.

_Inoculum preparation_

Inoculum medium (35 ml) containing 8 g/L nutrient broth, 10 g/L starch, 5 g/L lactose and 1.5 g/L NaCl in 50 mM phosphate buffer (pH 7.2) was transferred to a 250 ml Erlenmeyer flask and cotton plugged. It was sterilized at 15 lbs/in² pressure and 121°C temperature for 15 min. The flask was cooled down at ambient temperature of 20°C and inoculated with a loopful of bacterial culture, aseptically. It was incubated in a rotary shaker (200 rpm) at 37°C for 24 h.

_Fermentation procedure_

The production of β-amylase was undertaken using solid-state fermentation (SSF) in 500 ml Erlenmeyer flasks. Wheat bran partially replaced by cottonseed meal at 7.5:2.5 was taken in separate flasks. The substrate was moistened with M2 (being optimized later) as a moistening agent (1:1 ratio). The flask was sterilized in an autoclave (15 lbs/in², 121°C) for 15 min and then cooled at room temperature. Inoculum (1.26×10⁷ CFU/ml) was aseptically seeded to each flask and incubated at 37°C for required time period. The batch culture experiments were run parallel in triplicates.

_Moistening agents_

M1: 3 g/L peptone, 2 g/L beef extract, 10 g/L soluble starch, 5 g/L ammonium sulphate, 10 g/L lactose, 3 g/L CaCl₂, 50 mM phosphate buffer 1000 ml, pH 8 (Saxena et al., 2007).

M2: 2 g/L yeast extract, 2.5 g/L peptone, 8 g/L
soluble starch, 2 g/L ammonium sulphate, 1.2 g/L CaCl$_2$, 0.45 g/L MgSO$_4$·7H$_2$O, 0.2 g/L FeSO$_4$·0.2, pH 7.5 (Hossain et al., 2008).

M3: 2.5 g/L peptone, 2 g/L beef extract, 10 g/L soluble starch, 3 g/L CaCl$_2$, 0.15 g/L MgSO$_4$·7H$_2$O, 0.02 M phosphate buffer 1000 ml, pH 7.2 (Hickman et al., 2009).

M4: 2.5 g/L yeast extract, 2.5 g/L peptone, 10 g/L soluble starch, 1.5 g/L ammonium sulphate, 1.2 g/L CaCl$_2$, 0.45 g/L MgSO$_4$·7H$_2$O, pH 7.2 (Daba et al., 2013).

M5: 3 g/L yeast extract, 12 g/L soluble starch, 1.2 g/L CaCl$_2$, 0.2 g/L FeSO$_4$, 0.12 g/L K$_2$HPO$_4$, 0.05 g/L CuSO$_4$·7H$_2$O, pH 7.5 (Hossain et al., 2008).

M6: 1 g/L peptone, 2.5 g/L beef extract, 10 g/L soluble starch, 3 g/L ammonium phosphate, 2 g/L CaCl$_2$, 0.02 M phosphate buffer 1000 ml, pH 8 (Aiba et al., 1973)

**Enzyme assay**

The enzyme β-amylase was assayed after Hickman et al. (2009). A reaction mixture was prepared by adding 0.5 ml of 1% starch solution with 0.5 ml of diluted enzyme extract in a test tube. A control was run in parallel by replacing enzyme extract with same quantity of distilled water. The incubation was carried out at 60°C for 30 min. The reaction was terminated by adding 0.5 ml of 1N NaOH. The liberated reducing sugars were determined by dinitrosalicylic acid (DNS) reagent. The transmittance was measured at 546 nm by the spectrophotometer (5000 Irmeco GmbH, D-2149 Gee, Germany) against maltose as internal standard.

**Enzyme unit**

One unit of β-amylase is defined as the amount of enzyme which yields 1 mg of maltose (as reducing sugar) under the defined conditions.

**Enzyme purification, kinetic and thermal characterization**

The enzyme solution was concentrated by 10-fold using an ultrafiltration system at 40°C for 2 h. Ammonium sulphate was added to the test solution to attain 60% saturation and swirled overnight (160 rpm) on a magnetic stirring plate with electric stirrer. The suspension was centrifuged at 9,000×g for 20 min (4°C) and decanted off. The partially purified enzyme (25 ml) was then applied to an ion exchange chromatography column system. The proteins were eluted with a NaCl gradient using 30 mM sodium phosphate buffer (pH 6.2). The flow rate was adjusted at 4 ml/min. The effluent was examined by determining $A_{290}$ (Sun et al., 2010).

Batch-culturing kinetics was studied after the procedures laid-down by Pirt (1975). Arrhenius equation was used to ascribe the temperature-dependent irreversible inactivation of β-amylase activity (Aiba et al., 1973; Sun et al., 2010). Temperature ranged from 30-54°C. The specific rate ($q_p$, enzyme units/g cells/h) for enzyme production was used to estimate different parameters following the equations,

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

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

The plot of $\ln(q_p / T)$ vs 1/T exhibited a straight line.
The slope was \( -\Delta H/R \) and intercept was \( \Delta S/R + \ln(K_B/h) \), where \( h \) (Planck’s constant) = 6.63×10\(^{-34}\) Js and \( K_B \) (Boltzman constant) \[ R/N = 1.38×10^{-23} \text{ J/K} \] where \( N \) (Avogadro’s No.) = 6.02×10\(^{23}\) per mol.

**Statistical analysis**

The treatment effects were equated after Snedecor and Cochran (1980). Duncan’s multiple ranges (Sps-21, version 12, USA) were applied using I-way analyses of variance (I-ANOVA). The significance of results has been presented as probability (p≤0.05) values.

**RESULTS AND DISCUSSION**

The present study deals with the kinetic characterization of improved β-amylase production from a potent mutant strain of *Bacillus subtilis* using solid-state fermentation (SSF). A total of six wild-cultures of bacteria were isolated from the soil samples of various industrial zones of Lahore. The isolates were picked-up by observing clear zones formed due to starch hydrolysis in the nutrient agar plates. Nevertheless, the zones be correlated quantitatively with the β-amylase yielded during the batch-process because of the hydrolytic potential of some other enzymes particularly α-amylase and glucoamylase (Todaka and Kanekatsu, 2007; Daba et al., 2013). Consequently, the screening of bacterial strains having β-amylase activity using starch-agar plates remained only a partially selective process. Therefore, these isolates were screened for better enzyme activity using SSF technique in 250 ml Erlenmeyer flasks (Table I). The isolate *B. subtilis* IS-4 exhibited comparatively higher enzyme activity (22±4.2 U with 0.242 mg/ml protein). The selected culture was improved after treatment through ethyl methane sulphonate (EMS), soon after followed by nitrous acid (NA) exposure to further enhance its hydrolytic potential for β-amylase activity (Table I). Among the various mutants examined, the derepressed NA-12 gave the highest activity (45±1.6 U) and thus was selected for its kinetic and thermodynamic characterization in batch-culture. Total protein content was noted to be 0.934 mg/ml by the selected mutant. All of the rest of mutant variants gave almost insignificant enzyme productivity under the same set of fermentation conditions. This work is substantiated with the findings of Ajayi and Fagade (2003) that also isolated some extremely aerobic bacteria (including JF1, JF2 and D) from the Chinese koji rice and thereafter identified as two different *Bacillus* spp. which produced a thermostable enzyme in the culture broth.

**Table I.-** Screening of *B. subtilis* strains (wild and mutant variants) for β-amylase production.

<table>
<thead>
<tr>
<th><em>B. subtilis</em> strains</th>
<th>Protein contents (mg/ml)</th>
<th>β-Amylase (U)</th>
<th>Specific growth rate, ( \mu ) (per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild isolates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS-1</td>
<td>0.242</td>
<td>6±3.2</td>
<td>0.125</td>
</tr>
<tr>
<td>IS-2</td>
<td>0.165</td>
<td>18±2.3</td>
<td>0.375</td>
</tr>
<tr>
<td>IS-3</td>
<td>0.089</td>
<td>12±2.8</td>
<td>0.252</td>
</tr>
<tr>
<td>IS-4</td>
<td>0.242</td>
<td>22±4.2</td>
<td>0.453</td>
</tr>
<tr>
<td>IS-5</td>
<td>0.204</td>
<td>17±4.6</td>
<td>0.354</td>
</tr>
<tr>
<td>IS-6</td>
<td>0.182</td>
<td>11±2.5</td>
<td>0.229</td>
</tr>
<tr>
<td><strong>EMS-induced mutants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMS-7</td>
<td>0.192</td>
<td>22±3</td>
<td>0.453</td>
</tr>
<tr>
<td>EMS-8</td>
<td>0.178</td>
<td>19±1.3</td>
<td>0.381</td>
</tr>
<tr>
<td>EMS-9</td>
<td>0.294</td>
<td>31±4.2</td>
<td>0.646</td>
</tr>
<tr>
<td>EMS-10</td>
<td>0.456</td>
<td>25±1.6</td>
<td>0.512</td>
</tr>
<tr>
<td><strong>NA-induced mutants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA-11</td>
<td>0.528</td>
<td>34±1.5</td>
<td>0.708</td>
</tr>
<tr>
<td>NA-12</td>
<td>0.986</td>
<td>45±1.6</td>
<td>0.934</td>
</tr>
<tr>
<td>NA-13</td>
<td>0.645</td>
<td>31±3.5</td>
<td>0.646</td>
</tr>
<tr>
<td>NA-14</td>
<td>0.262</td>
<td>26±1.8</td>
<td>0.535</td>
</tr>
</tbody>
</table>

Incubation period 48 h, temperature 37°C

Each value is an average of three parallel replicates. ± Indicate standard deviation from mean value.

*EMS Ethyl methane sulphonate*  
**NA Nitrous acid (HNO\(_2\), unstable & readily oxidizable)**

The selection of suitable moisture content for β-amylase activity by *B. subtilis* IS-4 and NA-12 was carried out (Fig. 1). The medium M2 containing 8 g/L soluble starch, 2.25 g/L peptone, g/L yeast extract, 2 g/L ammonium sulphate, 1.2 g/L CaCl\(_2\), 0.55 g/L MgSO\(_4\), 7H\(_2\)O, 0.25 g/L FeSO\(_4\) 0.2 at pH 7.5 gave maximal β-amylase productivity (54 U) by the mutant strain. Yeast extract and peptone were used as organic nitrogen sources and ammonium sulphate acted as an inorganic nitrogen source. The other moistening agents offered comparatively...
lower enzyme yield. It was possibly due to the fact that these agents lacked some of the macronutrients which were essential for the proper growth and subsequent enzyme production. During the early first growth period, microorganism utilized nitrogen source, while maximum enzyme remained associated with the cell lysates as reported previously (Clark et al., 1958; Li and Yu, 2012). In the second period, the carbohydrate source (lactose) was utilized and the enzyme peaked during early phase of growth. The strain IS-4 gave almost insufficient β-amylase productivity by all the moistening agents used. Kohno et al. (1989) isolated B. flavothermus that supported β-amylase activity to a maximum of 12.8 U with 40 g/L lactose and 20 g/L yeast extract (pH 6) as moisture contents. The enzymes are highly sensitive to pH variations (Fazekas et al., 2013). In the present investigation, effect of pH range (6-8.5) of the moistening agent on enzyme production was also studied alternatively by both the strains (Fig. 2). β-Amylase production was the best (59 U with the mutant) at a neutral pH of 7. Further increase in pH leads to the decreased enzyme activity. The rate and secretion of enzyme was expressively inhibited at a slightly alkaline pH shift (8-8.5).

The time for incubation of B. subtilis (both wild-culture IS-4 and mutant NA-12) for β-amylase biosynthesis was studied (Fig. 3). The enzyme activity was amplified with the rise in incubation period i.e., from 8-96 h and reached to a maximal level, 72 h after incubation by the mutant (while 80 h by the wild-culture). Thus NA-12 exhibited over 2.5 fold improved enzyme productivity compared to IS-4. In the present study, the production was proceeding after lag phase (about 8-12 h) reaching maximum at the onset of stationary phase. It was followed by a steady decline during the death phase (probably due to the proteolysis effect). The work is substantiated with the reports of Klosowski et al. (2010). A further increase in incubation period other than the optimal resulted in a sharp decline in enzyme activity. It was possibly due to the accumulation of some by-products (such as toxins or cellular debris) and also exhaustion of nutrients along with mineral ions from the medium. The
undesirable microbial by-products further inhibited the growth of bacterial cells and consequently the enzyme yield as reported earlier (Li and Yu, 2012).

The comparison of kinetic parameters emphasized that $q_p$ (specific rate of enzyme production) value is highly substantial ($p \leq 0.05$) in the presence of soluble starch but remained almost insignificant with glucose or xylose (at sugar level 1.5%, w/v irrespective of the source of carbohydrate moiety) by the mutant (NA-12). Similarly, the values for $Y_{p/x}$ (the enzyme produced per cell being formed) were considerably decreased by adding glucose or xylose as the sole carbon sources (Fig. 4). It is due to carbon catabolite repression that resulted in a lower level of the enzyme being produced, as reported by Pirt (1975). Additionally, when the starch was supplemented with some complex agricultural by-products particularly wheat bran, it acted as an inducer for microbial growth. Initially, the organism hydrolysed complex carbohydrates notably wheat bran for its choice food and also growth purposes with the concomitant excretion of β-amylase into the production medium (Ajayi and Fagade, 2003). The strain NA-12 may also require a little more starch for the proper initial growth with major enzyme activity (0.842 mg/ml protein). The present results are substantiated with Pirt (1975) and Mikami et al. (1999); however, the values for $Y_{p/x}$ (U/g) and $q_p$ (U/g/h) remained between 15-20 fold better than the previous workers.
ENHANCED B-AMYLASE PRODUCTION BY BACILLUS SUBTILIS

Fig. 5. Thermophilic behavior of B. subtilis wild-culture IS-4 and mutant NA-12 for β-amylose activity. Incubation temperature was maintained at 37°C for a period 48 h at substrate to diluent’s ratio of 1:1. Each value is an average of three parallel replicates. Y-error bars indicate standard deviation from mean value (B. subtilis IS-4 ■, B. subtilis NA-12 □).

Thermophilic characterization of wild-culture (B. subtilis IS-4) and mutant strain (NA-12) for β-amylose production was also carried out. The temperature was ranged from 30-54°C (Fig. 5). The purified enzyme (65.5±5.5 U) from the mutant was most active at 40°C. The enzyme activity remained fairly constant up to a maximum of 44°C (regardless of the higher temperature). More importantly, the temperature deviation up to a certain limit has no adverse effect on the enhanced enzyme activity. Thermal inactivation of enzyme was characterized by the activation enthalpy (ΔH₀ 86±6* kJ/mol), which was much lower than that of the wild culture (Table II). The value of ΔH₀ was considerably higher than other bacterial cultures employed by some previous workers (Aiba et al., 1973). The activation entropy by the mutant cells (−234±18thk J/mol/K) is marginally lower and could be compared conveniently with some other amylose production processes. The negative symbol reflects that the inactivation phenomenon implicit a little disorderness during the microbial growth following enzyme formation. Essentially this value was lesser than those estimated for amylose activity reported by other systems used (Sato and Park, 2006). This suggested better protection exerted by the mutant strain compared to that of wild cells against the thermal inactivation. Hensely et al. (1980) investigated the cell growth kinetics involved in β-amylose production by Bacillus spp. The growth kinetics and production rates were studied revealing the dominance of mutant cells over the free bacterial cells.

Table II. Comparison of thermodynamic parameters* of B. subtilis wild-culture IS-4 and mutant NA-12 for β-amylose activity.

<table>
<thead>
<tr>
<th>Thermodynamic parameters</th>
<th>Enzyme production</th>
<th>Thermal inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation enthalpy, ΔH₀* (kJ/mol)</td>
<td>Parental (IS-4) 34.5±3.3bc 67±3.3bc</td>
<td>Mutant (NA-12) 64.5±4.5ab 86±6ab</td>
</tr>
<tr>
<td>Activation entropy, ΔS** (J/mol/K)</td>
<td>Parental (IS-4) 31.28±7ab 56±4a</td>
<td>Mutant (NA-12) (−)234±18thk (−)195±3cd</td>
</tr>
</tbody>
</table>

Each value is an average of three parallel replicates. ± Indicate standard deviation from mean value. Values followed by different letters in each row are significantly different from each other at p<0.05.

*Thermodynamic parameters were determined using the following equation,
\[ \ln(q_0/T) = \ln(k_0 h) + \Delta S^* R - \Delta H_0^* R / T \] ……….,
where q₀, h, T, k₀, h, ΔS*, ΔH₀*, 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 k₀, h and R are 1.38×10⁻²³ J/K, 6.63×10⁻³⁵Js and 8.314 J/K/mol, respectively. ΔH* was calculated as slope and Ln (k₀/h) + ΔS*/R as intercept on Y-axis.

The enzyme was purified from the culture broth. The elution profiles of both Q-sepharose and sephacryl-S200HR chromatography depicted one peak with amylose activity. The fraction was collected, dialyzed, and further concentrated by lyophilization. The enzyme was then purified to homogeneity with over 6-fold increase in specific activity (yield ~16%) compared to the clear supernatant (Table III). The SDS-PAGE analysis of purified enzyme revealed a single protein band corresponding to approximately ~113 kDa that showed β-amylose activity (Fig. 6).
purified enzyme revealed a single protein band of ~113 kDa which confirmed amylase activity. However, metabolic engineering of NA-12 is in progress to further increase the enzyme stability prior to scale up studies in a bioreactor.

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**Conflict of interest declaration**

The authors have declared no conflict of interest.

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