Pakistan J. Zool., vol. 46(5), pp. 1415-1423, 2014.

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 ($Y_{p/x}$ = 6.4 U/g) and the specific rate constant (q_p = 0.889 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 (ΔH_D = 64.5±4.5[a] kJ/mol) and entropy of activation (ΔS = −234±18[ghk] 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). Amylolytic 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 sacchrification, 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

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0030-9923/2014/0005-1415 $ 8.00/0

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 10^6. 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 upto 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/in^2 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/in^2, 121°C) for 15 min and then cooled at room temperature. Inoculum (1.26×10^7 CFU/ml) was aseptically seeded to each flask and incubated at 37^{o}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 $CaCl_2$, 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 $MgSO_4.7H_2O$, 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 $MgSO_4.7H_2O$, 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 $MgSO_4.7H_2O$, 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_2HPO_4, 0.05 g/L $CuSO_4.7H_2O$, 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 extraction

After required incubation, 100 ml of phosphate buffer along with 0.02% (v/v) Tween 80 was added to the flask and agitated in an incubator shaker at 240 rpm for 1 h. Afterwards, the contents were centrifuged at 8,000 rpm (7,330×g) for 15 min. The substrate free clear supernatant was selected for enzyme assay.

Biomass determination

Biomass was measured turbidimetrically at 660 nm using a UV/Vis spectrophotometer. It was later compared with standard for dry cell mass vs. optical density. A control was also run in parallel replacing the biomass with distilled water. The final values were calculated as g/g following Kohno *et al.* (1989).

Protein contents

Five milliliter of Bradford reagent was transferred to a test tube having 0.1 ml of diluted enzyme solution. A control was run in parallel also. The tubes were vortex and A_{595} was measured by the spectrophotometer. The protein concentration was determined using bovine serum albumin (BSA) after Solimam (2008).

$$\text{Protein (mg/ml)} = \text{Slope} \times 5 \times \text{Dilution factor}$$

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 60ºC 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,

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

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} J/K where N (Avogadro's No.) = 6.02×10^{23} per mol.

Statistical analysis

The reatment effects were equated after Snedecor and Cochran (1980). Duncan's multiple ranges (Spss-21, version 12, USA) were applied using I-way analyses of variance (I-ANOVA). The significance of results has been presented as probability ($p\leq0.05$) 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.

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

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 2.5 g/L peptone, g/L yeast extract, 2 g/L ammonium sulphate, 1.2 g/L $CaCl_2$, 0.55 g/L $MgSO_4.7H_2O$, 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

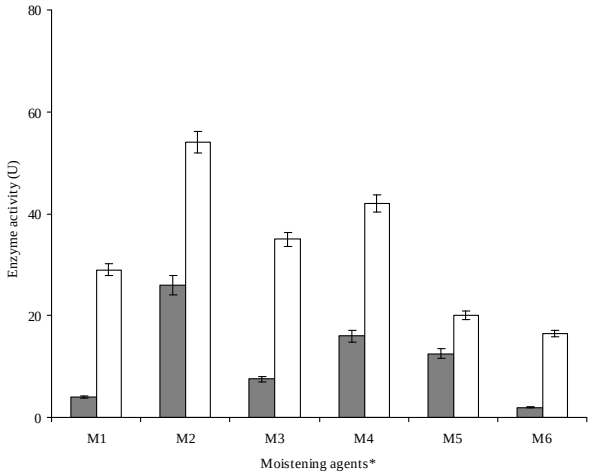

Fig. 1. Selection of moistening agent for β-amylase production by *B. subtilis* wild-culture IS-4 and mutant NA-12. Incubation temperature was maintained at 37°C for a period 48 h. Each value is an average of three parallel replicates. Y-error bars indicate standard deviation from mean value (*B. subtilis* IS-4 -■-, *B. subtilis* NA-12 -□-). *Composition of each of the moistening agent medium is given in the methodologies section.

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 lyses 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).

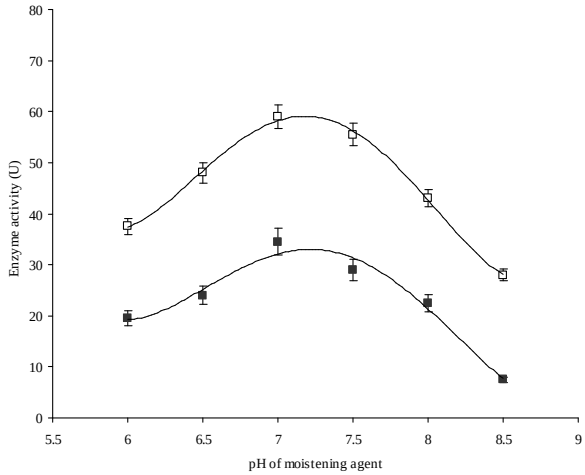

Fig. 2. Comparison of different pH of moistening agent for β-amylase production by *B. subtilis* wild-culture IS-4 and mutant NA-12. Incubation temperature was maintained at 37°C for a period 48 h at substrate to diluent's ratio of 1:1. Each value is an average of three parallel replicates. Y-error bars indicate standard deviation from mean value (*B. subtilis* IS-4 -■-, *B. subtilis* NA-12 -□-).

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).

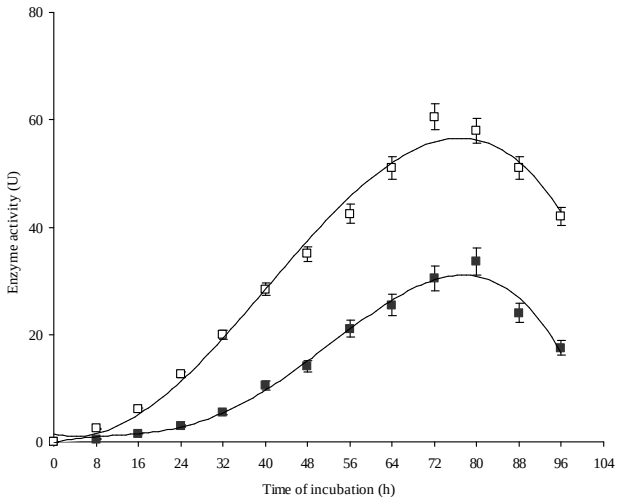

Fig. 3. Time of incubation of *B. subtilis* wild-culture IS-4 and mutant NA-12 for β-amylase production. Incubation temperature was maintained at 37°C at substrate to diluent's ratio of 1:1. Each value is an average of three parallel replicates. Y-error bars indicate standard deviation from mean value (*B. subtilis* IS-4 -■-, *B. subtilis* NA-12 -□-).

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.

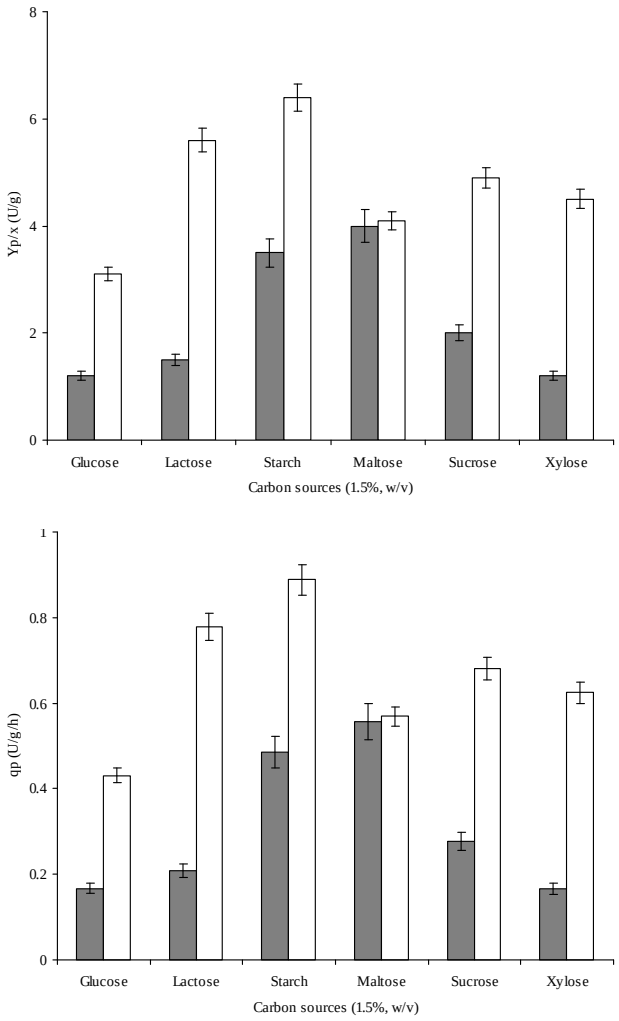

Fig. 4. Comparison of kinetic parameters of carbon source utilization for β-amylase production by *B. subtilis* wild-culture IS-4 and mutant NA-12, a) $Y_{p/x}$ (U/g), b) q_p (U/g/h). Incubation temperature was maintained at 37°C for a period 48 h at substrate to diluent's ratio of 1:1. Each value is an average of three parallel replicates. ± Indicate standard deviation from mean value (*B. subtilis* IS-4 -■-, *B. subtilis* NA-12 -□-).

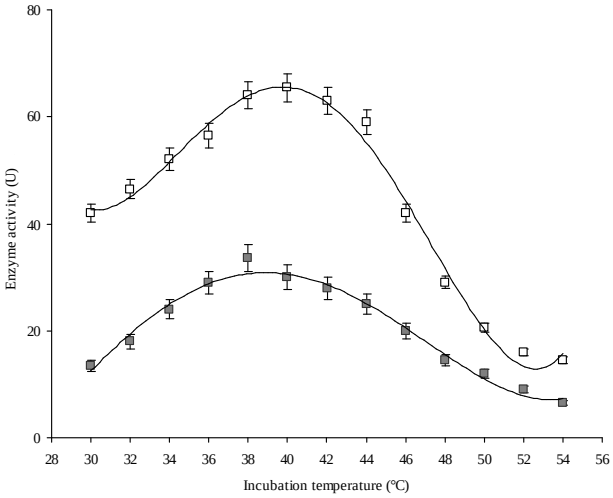

Fig. 5. Thermophilic behavior of *B. subtilis* wild-culture IS-4 and mutant NA-12 for β-amylase activity. Incubation temperature was maintained at 37°C for a period 48 h at substrate to diluent's ratio of 1:1. Each value is an average of three parallel replicates. Y-error bars indicate standard deviation 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-amylase 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 (ΔH_D 86±6^{a} kJ/mol), which was much lower than that of the wild culture (Table II). The value of ΔH_D was considerably higher than other bacterial cultures employed by some previous workers (Aiba *et al*., 1973). The activation entropy by the mutant cells (−234±18^{ghk} J/mol/K) is marginally lower and could be compared conveniently with some other amylase 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 amylase 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-amylase 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-amylase activity.

Thermodynamic parameters	Enzyme production	Thermal inactivation
Activation enthalpy, ΔH_D* (kJ/mol)		
Parental (IS-4)	34.5±3.5^{bc}	67±3.3^{b}
Mutant (NA-12)	64.5±4.5^{a}	86±6^{a}
Activation entropy, ΔS** (J/mol/K)		
Parental (IS-4)	31.28±7^{ab}	56±4^{a}
Mutant (NA-12)	(−)234±18^{ghk}	(−)195±3^{cd}

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 $p \leq 0.05$.

*Thermodynamic parameters were determined using the following equation,

$Ln(q_p/T)=Ln\ (k_B/h)+\Delta S^*/R-\Delta H_D^*/R.1/T$ ……..,

Where 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 $Ln\ (k_B/h)+\ \Delta S^{**}/R$ 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 amylase 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-amylase activity (Fig. 6).

Table III.- Overall summary of the purification steps of β-amylase activity.

Purification steps	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Supernatant	671	247	91.25	1	100
Q-Sepharose	202	108	58	3	26.5
P-95RD column	116	60	44.5	4	15.5

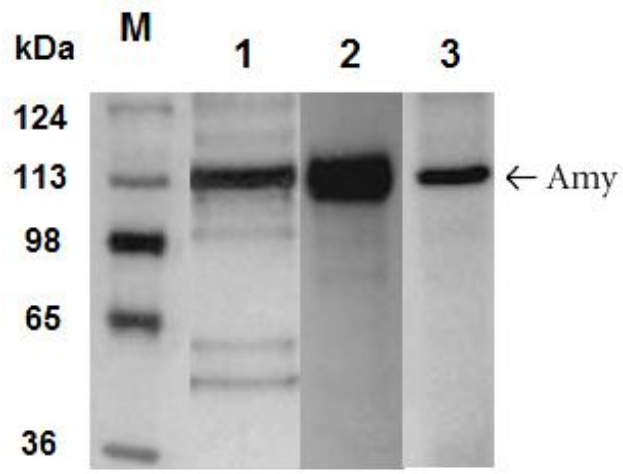

Fig. 6. Purified β-amylase on 8% SDS-PAGE; M, Marker proteins; separate lane 1 & 2, protein ion exchange on Q-Sepharose column; separate lane 3, enzyme after gel filtration on Sephacryl P-95RD column.

CONCLUSIONS

In the present study, a mutant strain of *Bacillus subtilis* (NA-12) was developed through treatment with NA. The cultural conditions and nutritional requirements were adjusted for the enhanced β-amylase production. The enzyme from the mutant was then purified and found to be the most active at 40°C. Notably, the activity was almost constant up to 44°C and thereafter declined gradually. The NA-induced mutagenesis improved both the enthalpy (ΔH_D= 64.5±4.5^{a} kJ/mol) and entropy of activation (ΔS= −234±18^{ghk} J/mol/K) for enzyme activity and subsequent substrate binding for starch hydrolysis. The SDS-PAGE analysis of 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.

ACKNOWLEDGEMENTS

Vice Chancellor and Director IIB are gratefully acknowledged for their contributions in promoting research culture in the University.

Conflict of interest declaration

The authors have declared no conflict of interest.

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(Received 30 April 2014, revised 17 May 2014)