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ENHANCED AMYLOLYTIC ACTIVITY OF INTRACELLULAR α-AMYLASE PRODUCED BY BACILLUS TEQUILENSIS

[1]Prasanth Manohar, [1]Shanthini T, [2]Kodiveri Muthukalianan Gothandam, [3]Velu Rajesh Kannan, [1]Nachimuthu Ramesh*

Address(es):
[1]Department of Biomedical Sciences, School of Biosciences and Technology, VIT University, Vellore, Tamil Nadu, India.
[2]Department of Biotechnology, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India.
[3]Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

*Corresponding author: drpnramesh@gmail.com

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ABSTRACT

The amylolytic enzyme plays a very important role in industrial applications. This study aimed to screen amylase producing *Bacillus* sp. and to promote its amylolytic activity by mutagenesis. Samples were collected from coastal mud samples and starch hydrolyzing isolates were screened. A single isolate having the highest enzyme activity was identified as *Bacillus tequilensis* by 16S rRNA analysis. A starch medium was optimized and fermentation period studies revealed that the mutant strain (after 60 sec of UV exposure) had higher activity (868 U/mL/min) than the parental strain (418 U/mL/min) after 36 hours of incubation at 37°C, pH 7.0. It was also found that amylase from intracellular mutant strain had maximum activity; on the other side parental strain had maximum activity with an extracellular enzyme. Optimized temperature, pH and salt concentration revealed that the intracellular amylase from mutant strain had the maximum activity of 978 U/mL/min, 985 U/mL/min, 960 U/mL/min respectively. Varying the source of carbon in the medium had a significant impact on enzyme activity. Metalloenzymes like amylases were reported to have strong activity towards metal ions, so amylase activity was analysed by adding different metal ions in the medium and found that calcium ions strongly promoted amylase activity and Fe^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} inhibited the activity. SDS-PAGE results showed that the molecular weight of isolated amylase to be approximately 55.0 kDa. Our study showed the capability of mutant *B. tequilensis* strain to produce double the amount of intracellular amylase than the parental strain.

Keywords: Amylolytic, mutagenesis, metalloenzymes, metal ions, zymogram

INTRODUCTION

Amylases are extracellular enzymes which hydrolyze the alpha 1, 4 glycosidic linkages of starch into sugars. Based on the cleavage site, amylase was classified into three types namely α- amylase, β- amylase and γ- amylase. Among them, α-amylase is one of the significant industrial enzymes which are widely utilized for the purpose of brewing, baking, textile and detergent **(Gupta *et al*., 2003)**. The biologically active enzymes are immensely present in natural resources such as plants, animals and microorganisms **(Mageswari *et al*., 2012)** of which microbial source plays a vital role since it can be produced in huge quantity meeting the demands of the market **(Pandey *et al*., 2000)**. *Bacillus tequilensis* is a Gram-positive, motile rods, produces central endospore in unswollen sporangia and its pathogenicity was undetermined **(Gatson *et al*., 2006)**. Several studies on ultraviolet (UV) light have been reported to be mutagenic in a variety of organisms. The impact of UV radiations on alpha amylase producing ability of *Bacillus* species were studied earlier and attempts have been made for the overproduction of microbial enzyme by induced mutagenesis **(Demirkan, 2011)**. The increase in amylase activity after exposing to UV light may be due to the changes in the promoter zone of a gene encoding for this enzyme and also the transcription of mRNA corresponding to the enzyme might have deregulated **(De Nicolas-Santiago *et al*., 2006)**. The impact of ultraviolet radiations on *Bacillus tequilensis* producing α-amylase was studied for the first time and the comparison of mutant and wild (parental) strain for increased α-amylase activity was performed.

MATERIALS AND METHODS

Sample collection

Samples were collected from the coastal environment in Pudukkottai district, (10° 1' 12" North, 79° 13' 35" East) Tamil Nadu, India. Under sterile conditions, mud samples were collected and the samples were transferred to the laboratory and stored at -20°C until further processing. The samples were collected from three different spots; [I] from the top layer (PDU-1), [II] from 10cm depths (PDU-2) and [III] near the trees (PDU-3).

Isolation, screening and identification of α-amylase producer

Collected samples were used for the isolation of amylase producing bacteria. Samples were processed by taking one gram of mud sample suspended in 9.0 mL of sterile water and agitated for a min. 0.1 mL of the suspended sample was spread plated onto nutrient starch agar (2.0% starch, 0.75% peptone, 0.5% beef extract, 0.5% NaCl, 1.8% agar) and incubated at 37°C for 24 hours. To identify the amylolytic bacteria, plates were overlaid with iodine reagent (0.01 M I_2-KI). Based on the starch hydrolysis properties, isolates were identified by the clear zone formed around the isolates. All the isolates that produced a clear zone of hydrolysis (Amy+ and Amy-) were further selected and screened for amylase production. Selected positive isolates (Amy+) were sub-cultured twice for purity and pre-cultures were maintained in nutrient agar slants (-20°C). Single selected isolate B- PDU2/2 was further identified by 16S rRNA sequencing. DNA was isolated using Hi-media bacterial DNA mini-prep kit as per manufacturer's protocol and amplified using universal 16S rRNA primers; 27F: 5'-AGAGTTTGATC MTGGCTCAG- 3' and 1492R: 5' -CGGTTACCTTGTTACGACTT- 3' **(Prasanth *et al*., 2016)**. Species level identification was done at www.ncbi.nlm.nih.gov/BLAST/ and BLAST nucleotide sequence similarity with 98% or above was deemed as sufficient for species identification.

Inoculum and crude enzyme preparation

Inoculum preparation was done by inoculating overnight grown culture from starch agar plates into 50 mL of starch broth and incubated at 37°C for 24 hours.

Exponential phase was achieved that contained $3.0X10^8$ CFU/mL and suitable volume from this suspension was used for the tests. To prepare crude enzymes, inoculated overnight grown cultures in starch broth were centrifuged at 10,000 rpm for 15 min and the supernatant was used as a crude enzyme for the enzyme assay for determination of activity.

Enzyme assay

The enzyme activity was assayed by using the DNS method. Briefly, a reaction mixture contained 0.5 mL crude enzyme solution and 0.5 mL soluble starch (1 g/100 mL- prepared using 0.01M phosphate buffer) that was incubated for 5 min and reducing sugar was measured. The reaction mixture without crude enzyme served as a control **(Miller, 1959)**. One unit of amylase activity is defined as the amount of enzyme that produces one micromole of reducing sugar in one minute at a constant temperature using soluble starch as substrate. Lowry's protein estimation method was used for total protein content determination **(Lowry *et al*., 1951).**

Mutagenic studies

The grown overnight bacterial culture was centrifuged at 10,000 rpm for 15 min and the bacterial cells were resuspended in 50 millilitres of peptone water and further diluted to 10^6 times and dilutions were plated, that served as a control. Ten millilitres from the diluted culture was transferred to another sterile petri plate. Mutagenesis by UV rays was studied by exposing the petri plates at different time intervals (30s, 60s, 90s and 120s) under the UV lamp. After exposing to pre-determined time intervals, 0.5 mL of bacterial culture was plated onto a starch agar plate and incubated at 37°C for 24-48 hrs and further used for enzyme assay. The intracellular and extracellular activity of amylase was studied for both parental and mutant strains. To extract the intracellular amylase, cells were sonicated and centrifuged to collect the intracellular crude enzyme.

Optimization of physiochemical and nutritional parameters

Parameters that can influence the amylase production were optimized independently and individually. So, optimization conditions were used subsequently for all the experiments both for the parental and mutant strain. For temperature optimization, the basal medium was inoculated and incubated at 35, 40, 45, 50, 55°C under standard assay conditions and for every 12 hours samples were withdrawn to study the effect of the incubation period. For pH optimization, the culture medium was prepared with variable pH 5.0 to 9.0 and assayed for standard assay conditions. The growth medium was supplemented with varying concentrations of starch (1% to 4%) and also with different carbon sources including maltose, sucrose, lactose, glucose and fructose for enzyme production. To analyse the effect of salt concentration on enzyme production varying concentrations of sodium chloride (1% to 6%) was used in the growth medium.

Effect of metal ions on enzyme activity

Metal ions have a strong influence on enzyme production and activity. To investigate the role of metal ions towards amylase activity various metal ions, $FeSO_4$, KCl, $CuSO_4$, $ZnSO_4$, $CaCl_2$, $MgSO_4$ were used at 25 mM concentration. Different metal ions were added to the medium and enzyme activity was assayed in standard assay conditions **(Demirkan, 2011)**.

SDS-PAGE and zymogram analysis

The molecular weight of the enzyme was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The prepared enzyme from both parental and mutant strain was loaded along with protein marker in 12% gel and purity of the protein was confirmed **(Laemmli, 1970)**. Approximate molecular weight was determined after staining with CBB and de-staining. Zymogram was done with native-PAGE by incubating in 1% starch for 1 hour and staining with iodine solution.

Kinetic properties of α-amylase and statistical analysis

To assess the kinetic properties, hydrolysis by DNS method with different concentrations of starch was used. K_m and V_{max} were calculated using the Michaelis-Menten equation. All the experiments were performed in triplets and the resulting values were presented as the mean of three independent observations. GraphPad Prism 5.0 software was used for calculating mean-standard deviation for each experimental result.

RESULTS AND DISCUSSION

Isolation, screening and identification of amylase producing bacterial cultures

Bacterial isolates producing clear amylolytic zones were initially identified by staining the plates with iodine solution. 16 isolates were found to produce clear zones in the starch-iodine plate out of which six isolates produced >1.7 cm zones that are considered as significant. Selected isolates were reassessed for the zone of clearance by a well-diffusion method. Isolates that showed the constant zone of clearance within 5-6 hours of incubation at 37°C was selected for further analysis. Isolate B-PDU2/2 was selected and found to be Gram-positive, motile and facultative aerobe. It was found to grow at pH 6.0, 7.0, 8.0 and salt concentrations up to 7%. Analysis of 16S rRNA sequence revealed its 98% homology with *Bacillus tequilensis* (KT760402). 16S rRNA is a powerful molecular marker for species-specific identification at the microscopic level was provided earlier **(Tiwari *et al*., 2014)**.

Medium selection and mutagenic experiments

Growth curve experiments revealed that B-PDU2/2 had a steady stationary phase after 30 hours in starch broth while other nutrient broths had 24 hours. Extracellular amylase activity was found to have maximum activity/productivity at 36 hours in starch broth (Fig.2). Mutagenic studies with UV exposure at different time intervals revealed that after 60 secs of UV exposure maximum amylase activity was achieved in the intracellular enzyme (Fig.1). Interestingly, the parental strain had a maximum activity of 418 U/mL/min with extracellular enzyme whereas intracellular enzyme from mutant strain had a maximum activity of 868 U/mL/min. Hence, the starch broth with the extracellular enzyme from parental strain and intracellular enzyme from a mutant strain (60 secs of UV exposure) was further taken for optimization. Similarly, higher activity was observed in the mutant strain than in its parental strain in *B. subtilis* **(Zhao and Qirong, 1994; Allan *et al*., 1997)**. In some cases, UV mutagenesis also inhibited the activity of parental strain that showed lower activity than parental strain in contrast chemical mutagenesis had higher activity **(Haq *et al*., 1997)**.

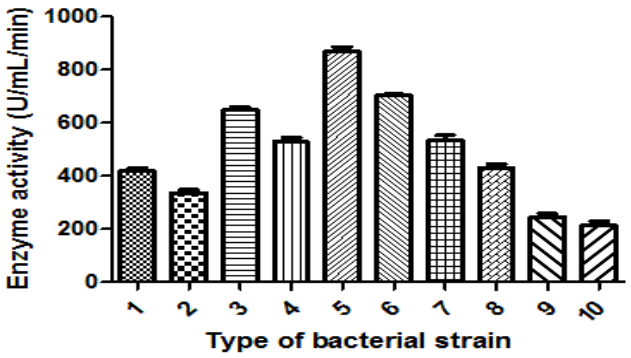

Figure 1 Comparison of amylase produced by parental and wild type strains of *Bacillus tequilensis* in starch medium at 45°C, pH 7. 1-parental extracellular, 2-parental intracellular, 3-after 30s of UV exposure intracellular, 4-after 30s of UV exposure extracellular, 5-after 60s of UV exposure intracellular, 6-after 60s of UV exposure extracellular, 7-after 90s of UV exposure intracellular, 8-after 90s of UV exposure extracellular, 9-after 120s of UV exposure intracellular, 10-after 120s of UV exposure extracellular.

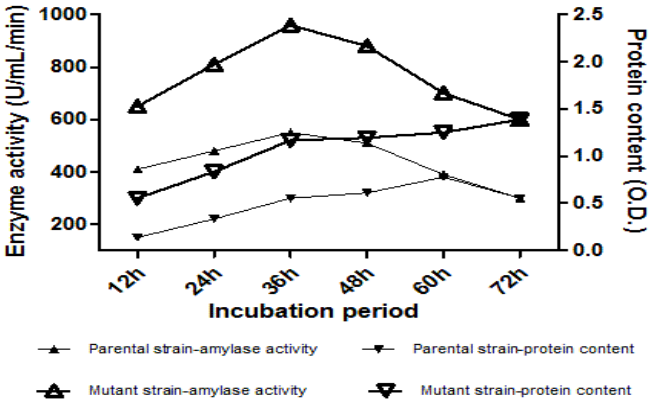

Figure 2 Total protein content of *Bacillus tequilensis* compared with production of amylase in mutant (60 sec UV exposure, intracellular) and parental strain (extracellular). Fermentation period analysis was made in starch medium at 45°C, pH 7.0.

Effect of different temperature

Evaluation of amylase activity at different temperatures (35-65°C) showed that both parental extracellular and mutant intracellular enzyme had a maximum activity of 522 and 978 U/mL/min at 45°C respectively. It also showed that

increase in temperature gradually decreases the activity of amylase (Fig.3,4). The temperature was also found to have a profound effect on *Bacillus* sp. in amylase production **(Raul *et al*., 2014)**. An incubation temperature of 37°C was optimum for most *Bacillus* sp. to produce maximum activity of amylase though some thermostable amylase can sustain temperatures up to 45 – 90°C **(Sodhi *et al*., 2005; Asgher *et al*., 2007)**.

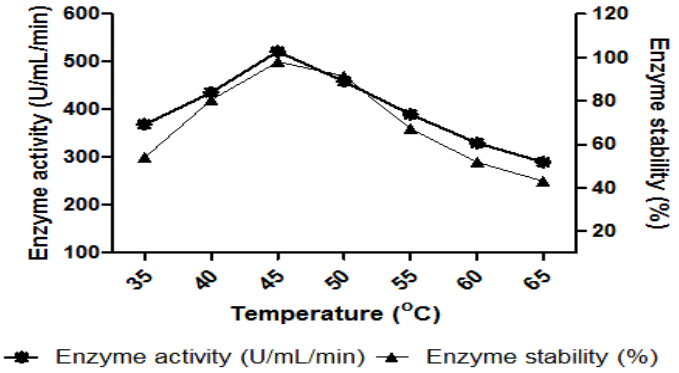

Figure 3 Effect of different temperature on enzyme activity and enzyme stability of parental strain (extracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates.

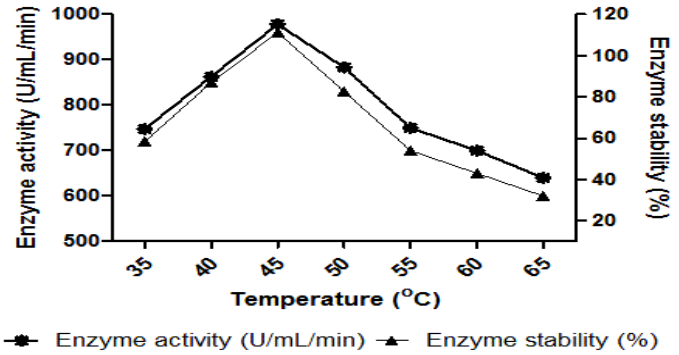

Figure 4 Effect of different temperature on enzyme activity and enzyme stability of mutant strain (60 sec of UV exposure, intracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates. Mutant strain was found to have more enzyme activity as well as production rate comparing with parental strain.

Effect of different pH

Optimization with different pH (5.0 – 9.0) revealed that both extracellular and intracellular amylase were not stable at stronger acidic as well as alkaline conditions. Also, pH of the medium had a strong influence on enzyme activity (Fig.5,6). At neutral pH 7.0 extracellular amylase of parental strain had an activity of 538 U/mL/min and intracellular amylase from the mutant strain (985 U/mL/min) had maximum activities. *Bacillus* sp. used for industrial amylase production was found to have optimum pH of 6.0 to 7.0 **(Haq *et al*., 2010)**. Variation in pH also indicates the initiation and end of enzyme synthesis **(Friedrich *et al*., 1989)**. The incubation period of 36 hours was found to have maximum enzyme activity (fig.2) and increase in incubation time decreases the enzyme activity that may be due to an interaction of synthesized enzyme with other components in the medium **(Ramesh and Lonsane, 1987)**.

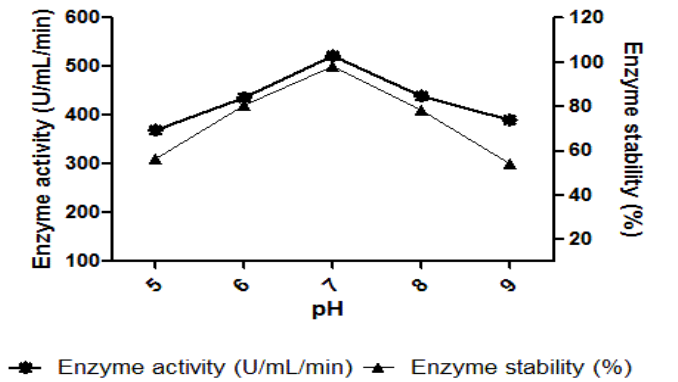

Figure 5 Effect of different pH on enzyme activity and enzyme stability of parental strain (extracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates.

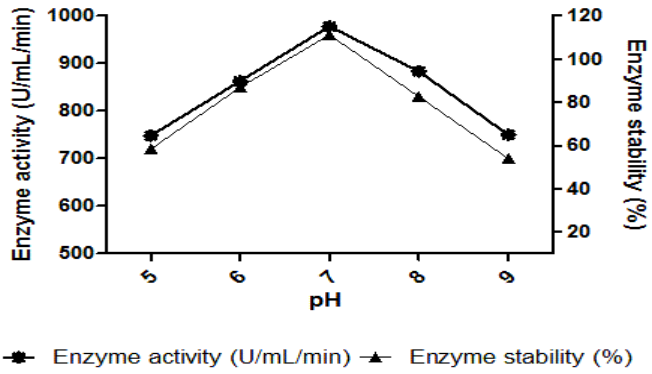

Figure 6 Effect of different pH on enzyme activity and enzyme stability of mutant strain (60 sec of UV exposure, intracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates. Mutant strain was found to have more enzyme activity as well as production rate compared to parental strain.

Effect of salt concentration on enzyme production

Enzyme activity was found to be decreasing as salt concentration (NaCl) increases from 1%. Though bacterial growth was observed at higher salt concentrations up to 7% enzyme activity was inhibited with an increase in salt concentration (Fig.7). Accordingly, the parental strain had maximum activity at 1% (510 U/mL/min) as same as the mutant strain (960 U/mL/min).

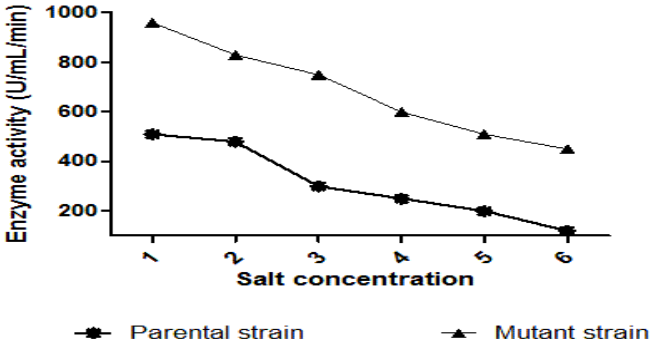

Figure 7 Effect of different salt concentration (NaCl) on enzyme activity of parental strain (extracellular) and mutant strain (60 sec of UV exposure, intracellular). Each individual point is the representation of mean with standard deviation for each experimental result done in triplicates.

Effect of different carbon sources

The starch broth was found to give maximum enzyme activity/production. Any increase or decrease in starch concentration (1% to 4%) had a lesser effect on enzyme activity on both parental and mutant strains (510 U/mL/min and 960 U/ml/min) whereas 2% starch had maximum activity after 36 hours of incubation (Fig.8). Supplemented with other carbon sources had affected the enzyme activity in that case glucose and fructose gave lesser enzyme activity than maltose, sucrose and lactose (maltose>lactose>sucrose>glucose>fructose). Some reports found maltose to be a good inducer of amylase activity **(Goto *et al*., 1988; Narang and Satyanarayana, 2001)**. In some cases, glucose was found to repress amylase activity and also glucose was reported to be an inducer **(Normurodova *et al*., 2007)**. Varying starch concentration was also reported influencing the enzyme activity **(Tiwari *et al*., 2014)**.

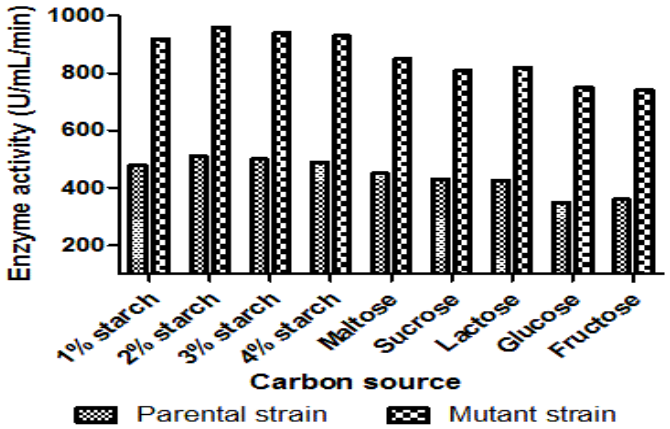

Figure 8 Effect of different carbon sources on enzyme activity of parental strain (extracellular) and mutant strain (60 sec of UV exposure, intracellular). Each individual bars are the representation of mean with standard deviation for each experimental result done in triplicates.

Effect of different metal ions

For the study of metal ions, 25mM each of $FeSO_4$, KCl, $ZnSO_4$, $CaCl_2$, $CuSO_4$, $MgSO_4$ were used in the medium at optimum temperature, pH and incubation period. Out of which calcium ions was found to be best for amylase production. Other ions Fe^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} had slightly decreased the enzyme activity compared to Ca^{2+} ions (Fig.9). It was found that calcium ions increase the activity of amylase compared to control (without ions). The catalytic activity of amylolytic enzymes can be affected by mono- and divalent metal ions but this metalloenzyme has up to six Ca^{2+} atoms at its active site so that can be activated by calcium ions **(Asgher *et al*., 2007; Normurodova *et al*., 2007)**.

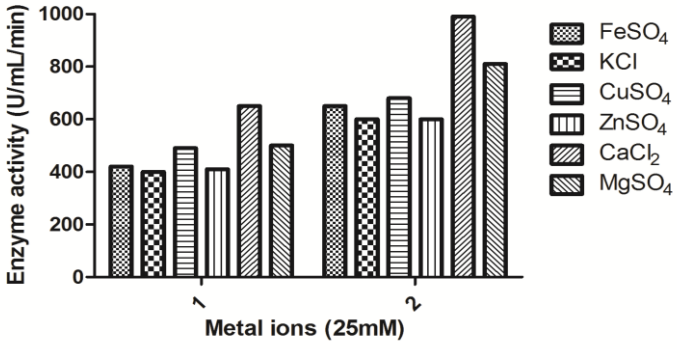

Figure 9 Effect of different metal ions on enzyme activity of parental strain (extracellular) and mutant strain (60 sec of UV exposure, intracellular). 1- Parental strain and 2- Mutant strain. Each individual bars are the representation of mean with standard deviation for each experimental result done in triplicates.

Stability of amylase and kinetic properties

Stability of enzyme was particularly important for its application in industries. Amylase produced from *B. tequilensis* was found to be active and stable at 45°C and pH 7.0 that correlates with its higher production rate (Fig.3,4,5,6). Protein content and amylase activity were found to be strongly influenced by the temperature and pH, although the temperature was controlled by incubation conditions and pH in the medium. Amylase from *Bacillus* spp. was also reported being stable at acidic as well as alkaline conditions **(Demirkan *et al*., 2005; Demirkan, 2011)**. Michaelis-Menten kinetic parameters were calculated to be 1.27 mg/mL and 1.97 mg/mL for K_m and 121 U/mL and 187 U/mL for V_{max} respectively.

SDS-PAGE analysis and zymography

The molecular weight was determined to be approximately 55.0 kDa (Fig.10) and a single band in SDS-PAGE confirmed the purity of amylase produced from *B. tequilensis*. Alpha-amylase from *Bacillus* spp. was found to have a molecular weight ranging from 50-60 kDa. A similar study using *B. tequilensis* reported molecular weight to be 67 kDa for synthesized amylase **(Tiwari *et al*., 2014)**. Zymogram results also indicated the presence of an amylolytic enzyme that hydrolysis the starch. The clear zone indicates the presence of an enzyme (protein) in zymography (Fig.11).

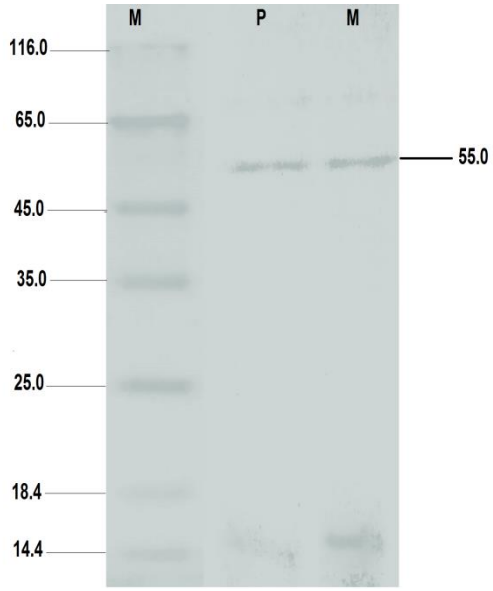

Figure 10 SDS-PAGE results for amylase from parental and mutant strains of *Bacillus tequilensis*. M- Protein marker, P-extracellular amylase from parental strain, M- intracellular amylase from mutant strain. Molecular weights were represented in kDa and produced amylase was ~55.0 kDa.

Figure 11 Zymogram result for amylase from *B. tequilensis* in native PAGE that hydrolysed soluble starch. A clear white region around the black background indicates the amylolytic activity. Lane-1; extracellular amylase from parental strain, Lane-2; intracellular amylase from mutant strain.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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