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Vol. 8(21), pp. 2168-2173, 21 May, 2014
DOI: 10.5897/AJMR2013.6586
Article Number: 3E4C50945029
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Production and characterization of alkaliphilic alpha-amylase from *Bacillus subtilis* A10 isolated from soils of Kahramanmaras, Turkey

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Received 24 December, 2013; Accepted 26 February, 2014

Bacillus subtilis **A10 isolated from orchard soil, Kahramanmaras, Turkey. The enzyme synthesis was observed between pH 7.0-11.0, with an optimum 37°C. The amylase was purified by fractional ammonium sulfate precipitation and sephadex G-100 column. Analysis of the enzyme with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single band which was estimated as 46.9 kDa. The enzyme presented an optimum activity at pH 8.5 and 45°C. Its thermal stability between 20-50°C was about 89.5% for 30 min. The pH stability was observed between pH 7.0 and 10.0 with an average of 84.9% of retaining activity for 15min. The activity of the enzyme was inhibited by SDS and EDTA by 48.4 and 75.9%, respectively. On the other hand, Na_2SO_3 and β-Mercaptoethanol did not effect the enzyme activity. This alkaliphilic amylase is suitable for waste-paper, starch and bioethanol industries.**

Key words: *Bacillus subtilis,* alpha amylase, alkaliphilic, enzyme.

INTRODUCTION

Amylase (EC 3.2.1.1) is one of the hydrolytic enzyme that catalyzes the hydrolysis of α-glucosidal bonds in starch, glycogen and related polysaccharides (Reddy et al., 2003). They are widely used in various industries such as food, textile, paper, detergent, and bewerage etc. Amylases constitute approximately 25% of the todays enzyme market (Elayaraja et al., 2011; Haq et al., 2012). Alpha amylases degrade α-1,4-glucosidic linkages by endo acting and produce oligosaccharides such as maltose, glucose and alpha limit dextrin. Common sources of the enzyme for industrial need is microorganisms because of their short fermentation period and bulk production of enzyme (Gupta et al., 2003). Microbial amylases are also applicative in pharmaceutical and biotechnological industries (De Souza et al., 2010; Naidu and Saranraj, 2013). Among the microorganisms, the genus *Bacillus* is one of the highly potential industrial agents since they are extracellular enzyme producers and generally regarded as safe etc. (Schallmey et al., 2004).

Amylases working at pH values 8.0 or higher have potentials for harsh conditions as in textile and detergent industries (Saxena et al., 2007). Most of the amylases from bacterial and fungal (Comlekcioglu et al., 2010) strains have an optimum pH between 5.0-7.5, therefore they are not appropriate to use in many industrial applications (Das et al., 2004). So, alkaline amylases to meet

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industrial demand have temptated the search for microbial strains propagating the enzymes with desired properties. In this study, we report purification and some properties of an alkaliphilic alpha amylase produced by a newly isolated *Bacillus* sp.

MATERIALS AND METHODS

Organisms and cultivation conditions

Bacillus sp. A10 isolated from the soil samples collected from orchard located at hillside of the Ahir mountain, Kahramanmaraş, Turkey. The soil samples were pasteurised in water bath at 80ºC for 10 min for selection of Gram-positive spore forming bacteria, *Bacillus* sp. (Lennete et al., 1985; Hamilton et al., 1999). The isolated strains were monitored for amylase production on agar plate composed of Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g, $MgSO_4$ 0.24 g, $CaCl_2$ 0.01 g, peptone 3 g, 1% (w/v) soluble starch (Merck), and Agar 15 g (Shibuya et al., 1986). The starting pH of the medium was 9.5 arranged with 10% Na_2CO_3 after sterilization. Amylase producing strains were selected after allowing the plates to iodine solution's vapour (Hols et al., 1994). Amylase positive strains were stored at 4°C on agar slope until enzyme production processes.

Identification of the microorganisms

Microorganisms were identified on the basis of 16S rDNA sequence analysis as well as its morphological and biochemical properties such as colony morphology, gram staining, spore bearing, motility, catalase production and acid production from glucose, xylose and manitol (Ratanakhanokchai et al., 1999). The polymerase chain reaction (PCR) product was sequenced by a commercial company (Refgen, Ankara, Turkey) using automatic sequencer. The analyses of the nucleotide sequence were performed by Clone Manager 5 and homology search was carried out by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of the *B. subtilis* A10 was registered on GenBank, NCBI (Accession Number: KJ433550).

Enzyme production

The selected strain, *Bacillus* sp. A10, was cultivated in minimal medium (M9) containing 1% soluble starch. The initial pH of the medium was adjusted to 9.0 with 10% Na_2CO_3 after sterilization. The medium was inoculated with a 10% of fresh overnight cell culture. The medium was then left incubation for 3 days at 37°C with shaking at 250 rpm. After removal of bacterial cells by centrifugation (Hettich Mikro 22R) (4020 *g*) for 30 min at 4°C. The supernatant was used for further investigations.

Purification of enzyme

The cell free supernatant was applied to fractionated ammonium sulfate precipitation for enzyme purification. The ammonium sulfate crystals were added to the supernatant to make the desired saturation (40-90%) at 4°C. Four hours later, the precipitate was removed by centrifugation at 4020 *g* and 4°C, for 30 min. The precipitates produced in different ammonium sulfate saturation were resuspended with 100 mM phosphate buffer at pH 7.6 (McTigue et al., 1995). They were then dialysed against the same buffer for 48 hours with several changing. The fractions were checked for amylase activity on agar-starch (1%) petri dish by dropping 10 μL. Then the fractions showing amylase activity were pooled and concentrated in Amicon Ultra-15 Filter Units tubes by centrifugation at 4020 *g*. The concentrated suspension was applied to Sephadex G-100 gel Filtration Column (1cm Diameter × 40 cm Height). First of all, the column was equilibrated with 100 mM phosphate buffer at pH 7.6. The enzyme suspension (1.5 mL) was eluted at a flow rate 14 mL/h using the same buffer at room temperature. The fractions (0.5 mL each) were checked again on a agar-starch petri dish to determine the fractions including enzyme. The fractions giving the largest hydrolytic zones were then gathered together.

Homogeneity, molecular weight determination and zymography

The homogeneity and molecular weight of the enzyme was tested by SDS-PAGE (10%) (Laemmli, 1970). As a molecular weight marker, protein mixture SDS6H2 (SIGMA) containing porcine myosin (200 kDa), *E. coli* β-Galactosidase (116 kDa), rabbit muscle Phosphorylase b (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), bovine erythrocytes carbonic anhydrase (29 kDa) was used and the bands were detected by staining with Coomassie brilliant blue R-250.

Zymography analysis of the enzyme was carried out by SDS-PAGE containing soluble starch (1%). After electrophoresis process, the gel was subjected to the renaturation solutions (Saul et al., 1990) prior to incubation. The incubation of gel was carried out in a plastic storage box after lining the gel on a glass plate at 45°C for 2 h. Activity bands were obtained by soaking the gel in iodine solution (KI: 5 g/L, I_2:0.5 g/L) (Hashim et al., 2004).

Enzyme assay

α-Amylase activity of the supernatant and the purified enzyme were assayed by the detection of reducing sugars (Miller, 1951). The reaction mixture contained 400 μL of 1% soluble starch and 100 μL of enzyme solution. The enzymatic reaction was stopped with 500 μL of 3,5-dinitrosalicylic acid after 30 min incubation at 45°C, and absorbance was measured at 550 nm in a Perkin Elmer Lambda EZ 150 Spectrophotometer. One unit of amylase activity was defined as the amount of enzyme liberating 1μmol reduced sugars per min under assay conditions.

Effect of pH and temperature on activity and stability

The optimum pH and optimum temperature for amylase activity were assayed at different pH values ranging from 3 to 11.5 and temperatures from 4-90°C for 30 min. The buffers used were as follows: Citrate-phosphate (pH :3.0-5.5), Na-phosphate buffer (pH 6.0-7.5), Tris-HCl buffer (pH: 8.0-9.0) and Borax-NaOH (pH: 9.5-11.5). Temperature stability was performed by pre-incubating the enzyme at temperatures between 4 and 90°C for 30 min. in optimum pH. For the pH stability, the enzyme was also pre-incubated at different pH ranged 4.0 to 11.0 at optimum temperature for 15 and 30 min. Then the remaining acitivity was determined under the standart assay conditions. The effect of NaCl on the activity was tested by adding the enzyme into the substrate containing different NaCl, while the stability of enzyme was tested by pre-incubating the enzyme in different NaCl concentration (0.1-4.0 M) at optimum pH and temperature for 30 min, All the experiments were conducted three times and mean values were taken.

Effect of some chemicals and inhibitors on enzyme activity

The effect of chemicals suchs as metal ions, chelaters, detergents and inhibitors on enzyme activity were tested by pre-incubating the

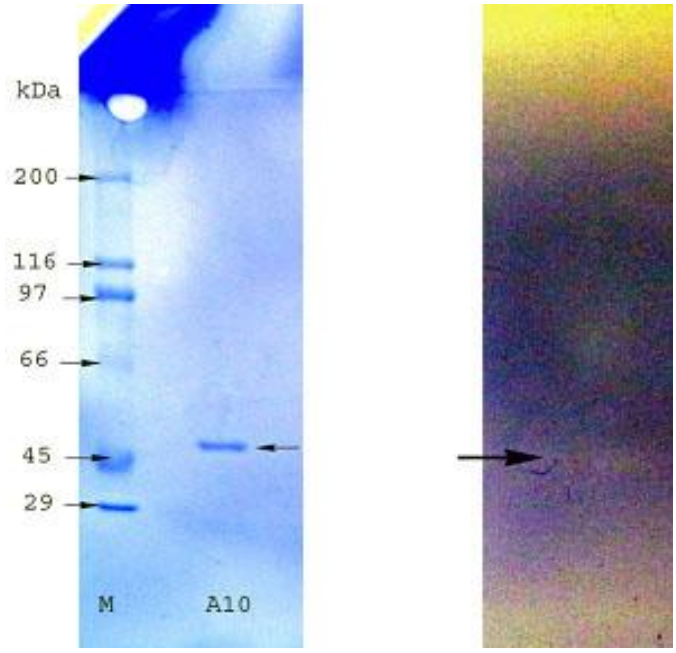

Figure 1. SDS-Page and Zymogram Analysis of α-amlyase A10. M: Protein mixture SDS6H2 (SIGMA) Porcine myosin (200 kDa), *E. coli* β-Galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa), Bovine albumin (66 kDa), Ovalbumin (45 kDa), Bovine erythrocytes Carbonic Anhydrase (29 kDa).

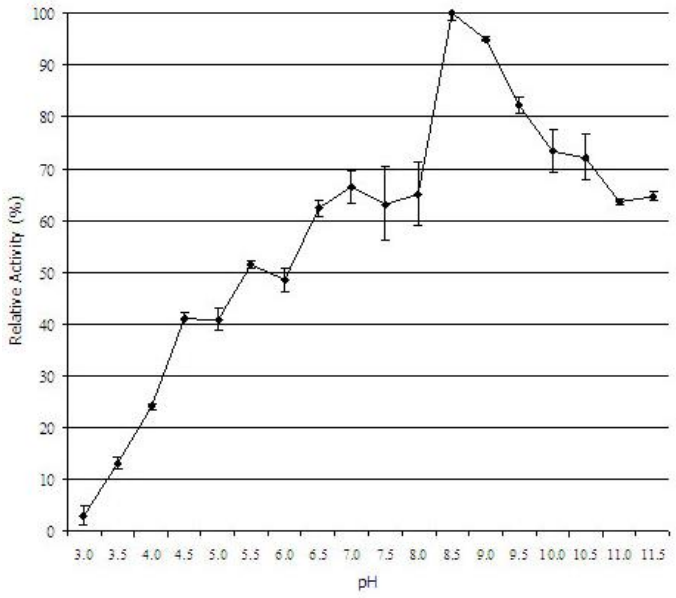

Figure 2. Effect of pH on the activity of *Bacillus subtilis* A10 α-amylase.

amylase in different concentration of the material at optimum pH and temperature for 30 min. The remaining activity was determined according to the control tubes which were not containing any additives.

Analysis of the end products

For the determination of amylase action on soluble starch thin layer chromatography analysis was performed on 'Aluminium oxide 60 F_{254} neutral' TLC plates (Merck). Firstly, soluble starch was digested with amylase at 45°C for 60 min of incubation. Cold ethanol was added to the mixture to stop the reaction after incubation. Development was carried out with a solvent system of butanol-acetic acid-water (3:1:1,by volume). Then the spots were visualized by spraying 20% sulphuric acid in ethanol and keeping the plates in an oven at 120°C for 30 min.

RESULTS

Total of 247 isolates were tested for α-amylase production on agar plates containing soluble starch (Shibuya et al., 1986). Among them, total 231 amylase positive isolates were selected after application of iodine vapour. The highest amylolitic potential showing strain A10 was chosen for enzyme production (Bernhardsdotter et al., 2005). The strain was aerobic, Gram positive, rod shaped, motility and catalase positive and spore forming. Although the enzyme synthesis by *Bacillus subtilis* A10 was observed between pH 7.0-11.0, with an optimum 37°C, the maximum amylolitic potential was at pH 9.0 on agar plate.

The nucleotide sequence of 16S rDNA gene of the selected strain A10 was determined and the sequence analysis showed that the strain A10 shared more than 98% of its identidy with diferent *Bacillus subtilis*. Then the organism was named as *Bacillus subtilis* A10.

Determination of molecular weight and zymography

SDS-PAGE analysis revealed a single band for the purified α-amylase, indicating this enzyme has been purified to near homogeneity by the fractional ammonium sulfate precipitation and Sephadex G-100 chromatography (Figure 1). Its molecular weight was estimated as 46.9 kDa. Zymogram analysis was accomplished by SDS-PAGE including soluble starch. The gel was subjected to the renaturation solution as described by Saul et al. (1990). Activity band was observed after soaking the gel in iodine solution (Hashim et al., 2004).

Properties of the α-Amylase

The optimum pH was determined with four different buffer systems. Although the organism showed maximum enzyme production at pH 9.0, the α-amylase showed the maximal relative activity at pH 8.5 (Figure 2). The α-amylase also presented a mean activity around 73.4% in between pH 6.5 and 11.5. Optimum temperature of the α-amylase was observed at 45°C with an average 71% activity between 25 and 55°C (Figure 3).

The pH stability of the α-amylase was determined by pre-incubating the enzyme at 45°C for 15 and 30 min. The remaining activity was surveyed by standart assay method. The highest stability was in between pH 7.0 and

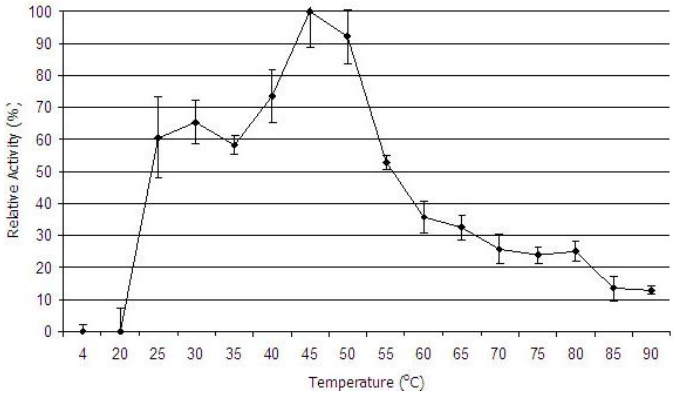

Figure 3. Effect of temperature on the activity of *Bacillus subtilis* A10 α-amylase.

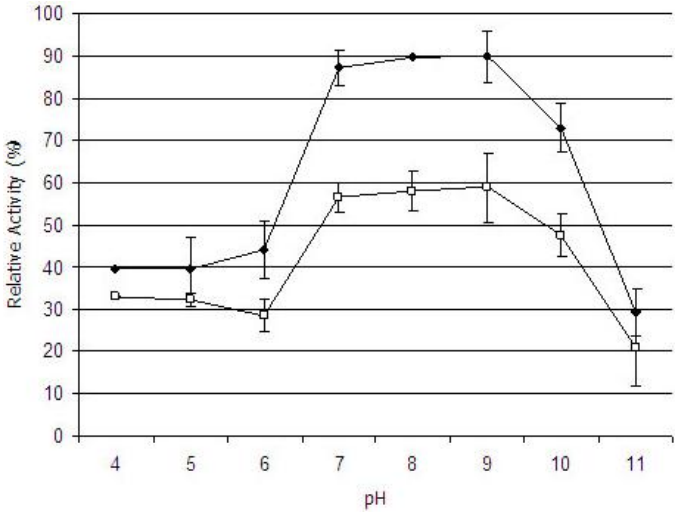

Figure 4. Effect of pH on the stability of *B. subtilis* A10 α-amylase. The enzyme was preincubated in different buffers for 15 (♦) and 30 (□) min. The buffers used were 100 mM Citrate-phosphate (pH :4.0-5.0), Na-phosphate buffer (pH 6.0-7.0), Tris-HCl buffer (pH: 8.0-9.0) and Borax-NaOH (pH: 10.0-11.0).

10.0 with an activity average 84.9% for 15 min. The enzyme was also stable over 50% remaining activity for 30 min (Figure 4). In both preincubation period, the maximal remaining activity was at pH 8.0 and 9.0. For thermal stability estimation, the enzyme was preincubated at diferent temperatures (4-90°C) for 30 min at pH 8.5. The enzyme was highly stable in between 4 and 50°C with an average 91.5% remaining activity (Figure 5).

To detect the effect of NaCl on α-amylase activity and stability, different NaCl concentration ranging from 0.1 to 4.0M were used. The maximal activity and stability were obtained in the presence of 1M (5.85%) NaCl (Figure 6) .

Effect of some chemicals on enzyme activity

The α-amylase was preincubated at 45°C for 30 min in

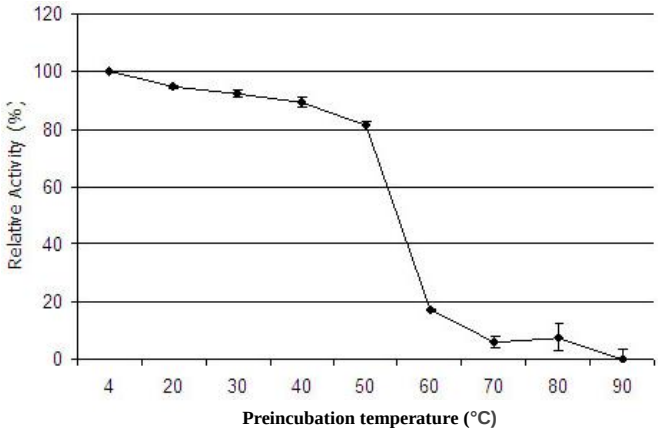

Figure 5. Effect of temperature on the stability of *B. subtilis* A10 α-amylase. The enzyme was pre-incubated at temperatures from 4 to 90ºC for 30 min at optimum pH. Remaining activity (%) was determined under standard assay condition.

the presence of chemicals prior to standart assay reactions. The enzyme activity was inhibited in the presence of $ZnCl_2$, urea, KCl, EDTA, $CaCl_2$, and SDS to 35.2, 24.3, 35, 51.6, 46.35 and 24.1%, respectively. Among the substances tested, Na_2SO_3 and β-Mercaptoethanol did not effect the enzyme activity indeed (Figure 7).

Analysis of the end product of enzyme action

Enzymatic hydrolysis product from soluble starch were analysed by TLC using aluminium oxide plate. After 60 min incubation of reaction mixture at 45°C, glucose, maltose and other longer oligosaccharides were the main products produced (Figure 8).

DISCUSSION

Among the microorganisms, the genus *Bacillus* are one of the extracellular enzyme producing bacteria and they have taken an important place in various industrial application. Amylases are one their significant hydrolytic enzymes for industries. This study reports that isolation of microorganism producing amylase, production, purification, and characterization of α-amylase.

The isolated strain *Bacillus subtilis* A10 for α-amylase production showed a growth mainly in the alkaline range between pH 6.5 to 11.0 giving the largest colony at pH 10.0 as in the findings of Johnvesly and Naik (2001). Alkaliphilics grow best above pH 8.0 and cannot grow or grows poorly around neutral pH (Horikoshi, 1999), therefore, we called the organism A10 is an alkaliphilic.

Bacillus subtilis A10 α-amylase enzyme was calculated as 46.9 kDa with SDS-PAGE analysis. Similar results for alkaliphilic α-amylase between 42 to 70 kDa have been

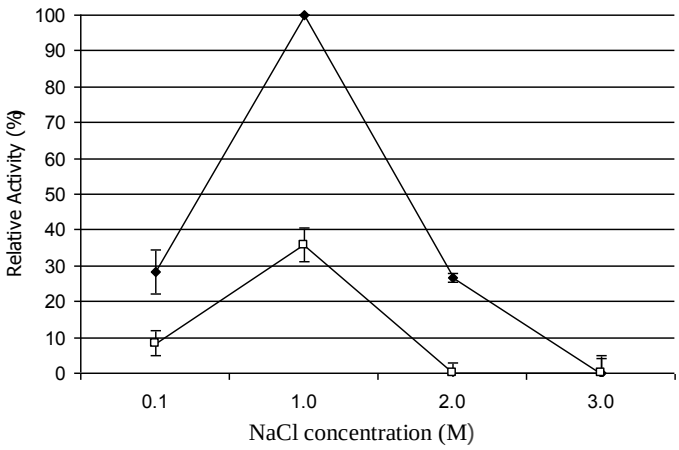

Figure 6. Effect of salt concentration on enzyme activity (♦)and stability (□) of *Bacillus subtilis* A10 α amylase.

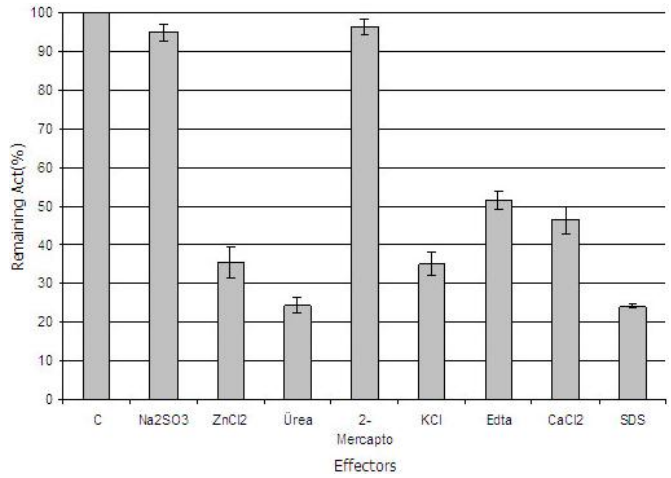

Figure 7. Effect of Some Chemicals on the activity of of *B. subtilis* A10 α-amylase.

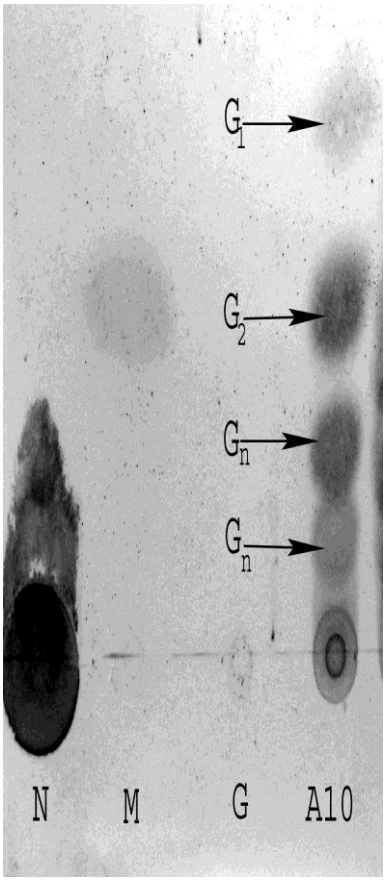

Figure 8. TLC of enzyme products from *Bacillus subtilis* A10. N: Untreated soluble starch; G: Glucose M: Maltose, A10: Enzyme substrate mixture (G_1: Glucose, G_2: Maltose, G_n: oligosaccharides).

reported by Horikoshi (1971); Igarashi et al. (1998); Ben et al. (2001); Das et al. (2004) and Annamalai et al. (2011). Many alkaline α-amylases from different *Bacillus* sp. were reported that optimal temperatures were around 40 to 70°C. The amylase from *Bacillus subtilis* A10 presented also an optimum temperature at 45°C as in the previously reported alkaline amylases (Igarashi et al., 1998; Lin et al., 1998; Cordeiro et al., 2002; Bernhardsdotter et al., 2005).

Amylase A10 is alkaline, but not thermostable as most of amylases reported earlier. Its thermostability at pH 8.5 up to 50°C for 30 min is also sufficient with a remaining activity over 80% for most of the industrial applications using amylases. Das et al. (2004) and Saxena et al. (2007), reported that urea highly denatured the amylase and their findings support our results. Although the denaturation action of urea (8M), uneffected amylase from *Thermus* sp. was also reported by Shaw et al. (1995).

The enzyme A10 was also presented a similar results to *Thermus* sp. amylase (Shaw et al., 1995) in presence of Ca^{2+} and EDTA. There was no Ca^{2+} activation was observed, on the other hand, sensitivity to EDTA was about 50%. Although the inhibiton with EDTA indicates that the enzyme requires metal ions, but enzyme may not need Ca^{2+} for activity or stability due to the adaptation to environment deficient in Ca^{2+} ions since carbonates production. The effect of Zn^{2+} on amylase activity is variable. It could be an effective inhibitor or ineffective at al (Kim et al., 1995; Mamo and Gessesse, 1999; Demirkan et al., 2005). The inhibition with Zn^{2+} was explicated as the competition in between exogenous and protein associated cations (Lin et al., 1998). Sodium sulfite is a chemical agent used for deinking processes in wastepaper industries. Uninhibitory effect of sodyum sulfite makes the enzyme rewarding for paper industries. This findings were in agreement with Krishnan and Chandra's (1983) findings too. Additionally, β-mekaptoethanol did not affect the enzyme substantially (Ozcan et al., 2010). According to the TLC plate, that the glucose band observed pale in

comparison with maltose and longer oligosaccharides. This is probably due to liberation of longer fragments at the beginning of hydrolysis of starch (Das et al., 2004).

Conclusion

Amylases are one of the most important enzymes for industrial applications. Microorganisms are the most efficient sources for enzyme production. The enzyme A10 α-amylase was produced alkaliphilic *Bacillus subtilis* A10 those are accepted as GRAS status. The enzyme is alkaline with an optimum pH 8.5. Its stability up to 50°C and activity in the presence of sodyum sulfite makes the enzyme merit in waste-paper industries for brightening processes. Its amylolytic action makes it worthy in starch industries as well as bioethanol production.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Authors thank KSU BAP for financial support of this study (2009/2-12M).

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