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Research Article

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Identification and Biochemical characterization of an acidophilic, detergent stable amylase from *Klebsiella* species isolated from industrial soil of Punjab region

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ABSTRACT

Amylases are starch saccharifying enzymes, which find numerous applications in industry. In this study, amylase producers were isolated from acidic industrial soil of Phagwara region of Punjab, India. Among the amylase producing isolates, one strain Ssta2 showed significant amylase activity in the plate assay. The enzyme was the most active at pH 4.0 and temperature of 40°C. $MgCl_2$ and $CaCl_2$ elicited enzyme activity whereas $HgCl_2$, $CuSO_4$, $FeCl_3$ and $CoCl_2$ inhibited the enzyme. Further analysis revealed that the Ssta2 amylase was stable in presence of 2% SDS, 2% CTAB and 2% Tween-80. The enzyme bears high promise at an industrial scale because of its robust activity.

Keywords: Amylases, Phagwara, Punjab, Ssta2, Tween-80

INTRODUCTION

Soil is one of the major habitats for vast range of prokaryotes. These prokaryotes build up the decomposition system of soil to decompose the plant and animal wastes deposited over billions of years [1]. In a broad sense, the term “amylases” (or amylolytic enzymes) refers to a variety of starch hydrolases and other related enzymes that are active in the hydrolysis, trans glycosylation and isomerization of starch molecules with respect to the α-glucosidic bonds present in them and other related polysaccharides and oligosaccharides [2].The rationale to look for newer and better amylases is quite simple. Starch is a highly abundant and economical source of fermentable sugars. The only challenge being faced is a cost-efficient saccharification method for breakdown of starch. The applications of such amylases are endless and have been listed previously in many reports. The most key uses have been reported in food industry [3], Biofuel production [4] detergents [5], paper industry [5], textiles [6] and pharmaceutical industry [7]. In this study, an acidophilic and detergent-stable amylase was characterised. The amylase producing strain called *Klebsiella ssta2* was characterized for saccharification parameters such as the optimum temperature, optimum pH and metal ions.

EXPERIMENTAL SECTION

Isolation of Amylase producing microorganisms: Amylase producers were isolated from the soil samples using an enrichment method. The soil samples were serially diluted in peptone water and then inoculated in to the Amylase Enrichment Broth (Starch 5g/L; peptone 5g/L; $MgSO_4.7H_2O$ 0.5g/L; $FeSO_4.7H_2O$ 0.01g/L; NaCl 1g/L). The cultures were shaken for 24 hours at 37^oC. Post-incubation, the culture was plated onto Amylase Enrichment Agar (Starch 5g/L; peptone 5g/L; $MgSO_4.7H_2O$ 0.5g/L; $FeSO_4.7H_2O$ 0.01g/L; NaCl 1g/L; Agar 20g/L) in

duplicates. The plates were grown overnight at 37^{o}C and analyzed by staining with starch staining solution (0.5% I_2; 0.05% KI).

Identification of Amylase producing strains: The amylase producing strain ssta2 was cultured and genomic DNA was isolated using the modified method of Zhou et. al. (1996) [9]. The genomic DNA was used in a PCR reaction to amplify the 16S rRNA gene using the primers 518F (5'- CCAGCAGCCGCGGTAATACG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The expected size of the product was 1050bp. The amplified 16S r-RNA gene was sequenced at Yaazh Xenomics, Chennai, India.

Biochemical Characterization of Amylase ssta2

Amylase assay: Amylase activity was measured using the DNS assay [8]. 0.5mL of 1% starch solution was incubated with or without the crude enzyme (enriched media) at 37^{o}C for 30 minutes. The reaction was stopped using DNS reagent and the tubes were heated at 95^{o}C for 5 minutes. The absorbance was measured at 540nm. The enzyme activity was expressed in terms of enzyme units as calculated from a glucose standard curve.

Effect of temperature on enzyme activity: 1% starch was used as a substrate for the amylase assay. The tubes were incubated with or without the enzyme at different temperatures from 10^{o}C to 90^{o}C in intervals of 10^{o}C. The enzyme activity was measured using DNS assay and glucose standard curve.

Effect of pH on enzyme activity: 1% starch was incubated with or without the enzyme in reaction buffers of different pH values ranging from 3 to 10. After 30 minutes of incubation, the reactions were stopped with DNS reagent and the enzyme activity was measured at 540nm with the help of glucose standard curve.

Effect of metal ions on enzyme activity: The crude enzyme was incubated with 1% starch solution in a reaction supplemented with or without $CaCl_2$, $MgCl_2$, $HgCl_2$, $CuSO_4$, $FeCl_3$, $MnCl_2$, $CoCl_2$ and $ZnCl_2$ at concentrations of 2mM and 5mM. The reaction was incubated for 30 minutes and the enzyme activity was estimated using the DNS assay.

Effect of inhibitors on enzyme activity: The crude enzyme was incubated with 1% starch and supplemented with various inhibitors *viz.* SDS (Sodium Dodecyl Sulphate), CTAB (Cetyl Trimethyl Ammonium Bromide), Triton X-100, Tween-20, Tween-80, Phenylmethane sulphonyl fluoride (PMSF), Urea and Gn-HCl (Guanidine hydrochloride). The reaction was carried out for 30 minutes and stopped by adding DNS reagent. The enzyme activity was obtained at 540nm with the help of glucose standard curve.

Sequencing of the ssta2 amylase gene: For sequencing the amylase gene, genomic DNA was isolated from an overnight grown culture of Ssta2 using the method of Zhou et. al. 1996.[9] The isolated DNA was subjected to PCR amplification using degenerate amylase specific primers. The forward primer was (Amy11F) [5'-GGN TTY ACN GCN ATH TGG ATH -3'] and reverse primer was (Amy11R) [5'-RTA DAT DAT YGG DAT YCC RTC-3']. The reaction protocol was as follows:

Initial Denaturation	Amplification (35 cycles)			Final Extension	Hold
94^{o}C	94^{o}C	58^{o}C	72^{o}C	72^{o}C	4^{o}C
2 Minutes	30 Seconds	45 Seconds	90 Seconds	5 Minutes	---

RESULTS AND DISCUSSION

Isolation of amylase producing microorganisms

Primary Screening: Amylase producers were isolated by the traditional plate-screening method on enrichment agar media. The isolates were grown at 37^{o}C for 24 hours and the plates were then stained with starch staining solution. The amylase activity was confirmed as zone of clearance and was clearly visible (Fig. 1). The zone of clearance indicated absence of starch from the media because of amylase activity of the isolates.

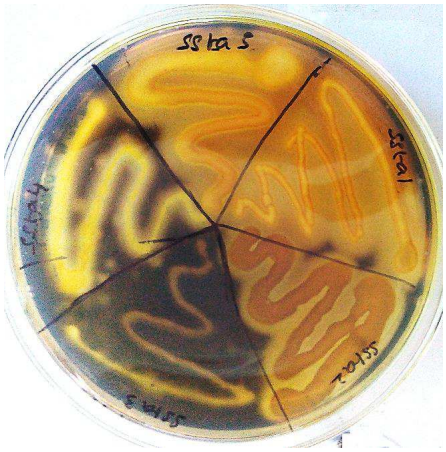

Fig. 1: Photograph of culture plate showing zone of clearance in the amylase enrichment isolates

Identification of amylase producing isolates: The genomic DNA was amplified by PCR using 16S rDNA primers. Figure 2 shows the gel containing the PCR amplified gene product along with 100bp ladder DNA.

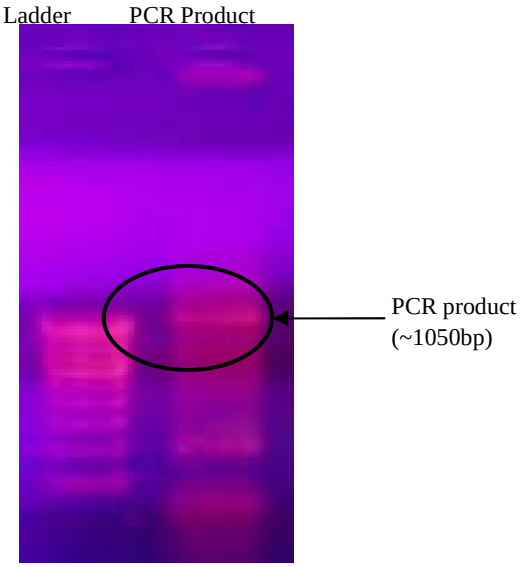

Fig. 2: 1% Agarose gel showing the 16S rRNA Amplicon

The 16S rRNA sequence was as follows:

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGT
GACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAAC
GGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGT
GCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAG
AGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGC
ACTTTCAGCGGGGAGGAAGGCGTAAGGTTAATAACCTCTCGATTGACGTTACCCGCAGAAGAAGCACC
GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGA
AACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT
CTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCT
TGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA
CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAG
AACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTG

The sequence was analyzed using the BLASTn program of NCBI. BLASTn results showed that the amylase producer was a strain of *Klebsiella pneumoniae*.

Biochemical Characterization of ssta2 Amylase

Effect of temperature on enzyme activity: Effect of temperature was measured by incubating the enzyme and substrate at different temperatures. The activity was measured by DNS assay. Ssta2 showed maximum activity at 40°C and retained almost 80% of its activity till 60°C, which indicates thermostability of the enzyme. The enzyme activity vs. temperature plot is as shown in figure 3.

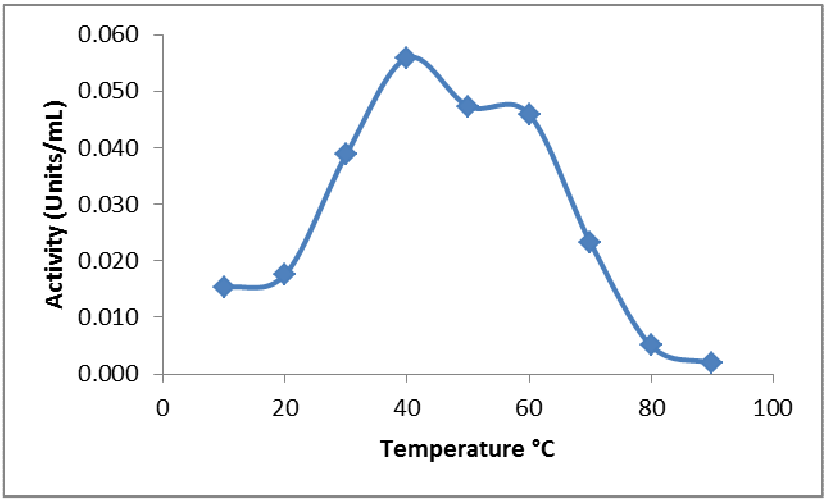

Fig. 3: Graph showing the effect of temperature on ssta2 activity

Effect of pH on ssta2 activity: Effect of pH on ssta2 was measured by incubating the enzyme and substrate in solutions of different pH values ranging from 3 to 10. The activity was quantified using the DNS assay. It was found that the highest enzyme activity was at pH 4.0 and the enzyme retained around 50% of its activity at pH 8.0. The enzyme was thus found to be acidophilic. Previously, N. Kumari et. al. [10] have reported an acidophilic amylase from *Bacillus cereus*, which showed optimum activity at pH 5.5. Similarly, Antrim et. al. [11] in 1991 isolated a thermostable α-amylase with optimum activity at pH 5.5. Uguru et. al. [12] in 1997 isolated a strain of *Thermoactinomyces thalpophilus* which produced an extracellular amylase with optimum pH of 5.0.

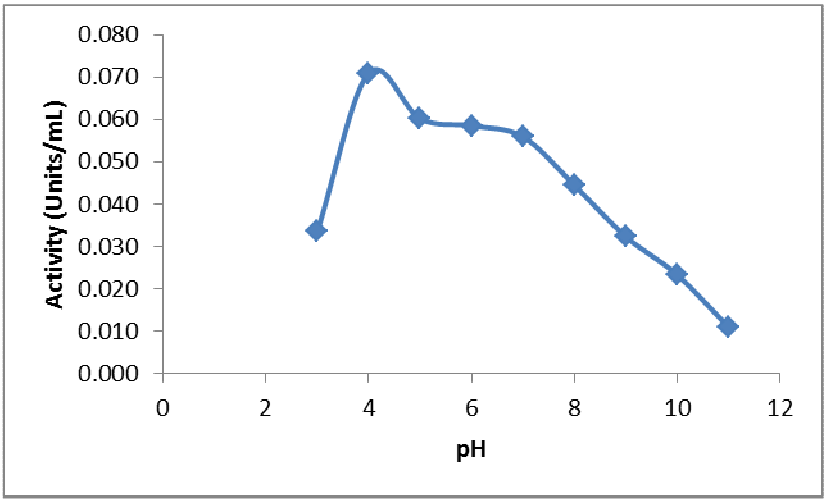

Fig. 4: Graph showing the effect of pH on ssta2 activity

Effect of Metal Ions on ssta2 activity: Metal ions have been known to affect enzyme activity through co-factor functions. So for ssta2, the optimal metal ions were identified using the standard DNS assay with or without metal ions at concentrations of 2mM and 5mM. It was found that Magnesium (Mg^{2+}) ions enhanced the enzyme activity by 280%, whereas Calcium (Ca^{2+}) ions increased the activity by 150%. On the other hand, Mercuric (Hg^{2+}), Cupric (Cu^{2+}), Ferric (Fe^{3+}) and Cobalt (Co^{2+}) inhibited the enzyme activity. Zn^{2+} does not affect the activity much at 2mM concentration and decreased the activity drastically at 5mM concentration. Fig. 5 shows the bar-graph depicting the

effect of metal ions on ssta2 activity. Contrastingly, N. Kumari et. al. [10] reported enhanced activity of *B. cereus* amylase in presence of K^+ and Zn^{2+}.

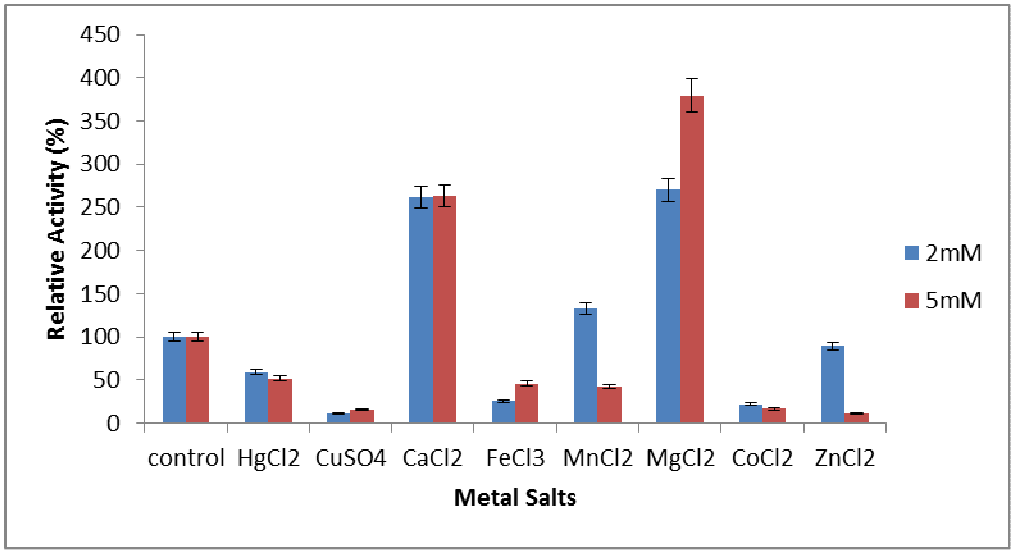

Fig. 5: Bar-graph showing the effect of metal ions on ssta2 activity

Effect of Inhibitors on ssta2 activity: Effect of inhibitors on the enzyme activity of amylase produced by ssta2 colony was analysed using DNS assay with the control in which there was no inhibitor added. Figure 6 shows the effect of inhibitors on the enzyme activity keeping the temperature and pH optimum. The results showed that SDS, CTAB, T-80 increased slightly the enzyme activity at 2% concentration and decreased the activity at 5% concentration. T-20 and H_2O_2 decreased the enzyme activity slightly at 2% and 5% concentrations respectively and decreased the activity at 5% and 10% concentrations respectively. T-X-100, PMSF, Urea and Gn-HCl decreased the activity drastically with increasing concentrations. The immediate conclusion from these results was that the enzyme ssta2 is detergent stable as well as oxidant stable. Previous reports by various groups such as CAF Yasemin et. al. [13] and Roohi et. al. [14] has reported detergent stable amylases. While the former reported SDS-stable amylase from *Bacillus sp. Calp12-7*, the latter reported a SDS, Urea and EDTA-stable amylase from *B. cereus sp. GA6*.

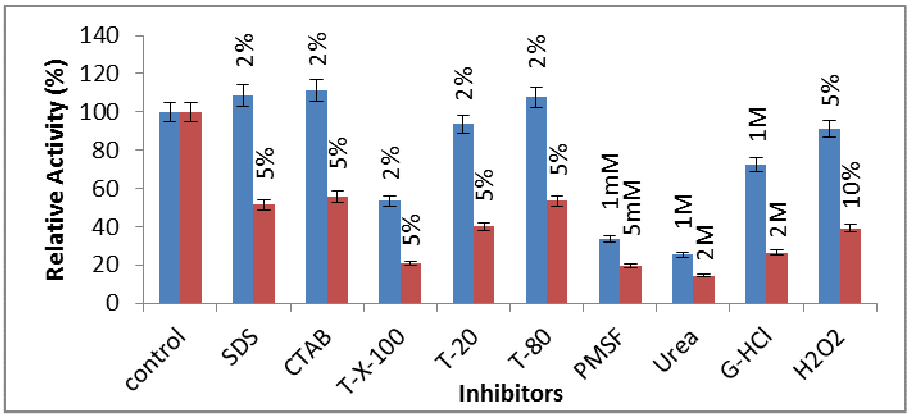

Fig 6: Bar-graph showing the effect of inhibitors on ssta2 activity

Sequencing of ssta2 Amylase Gene: The genomic DNA was isolated from ssta2 culture using the method of Zhou et. al. [9]. The amylase gene was amplified using degenerate primers for amylase gene. Following sequence was obtained:

GAATTTGATCAGAAAGGCAGCGTGGCGACCAAATATGGCGATAAAGCGCAGCTGCTGGCGGCGATTA
ACGCGCTGAAAGAACATAACATTGCGGTGCTGCTGGATGTGGTGGTGAACCATCGCATGGGCGCGGAT
GAAAAAGAAGCGCTGAAAGTGCAGCGCGTGGATGAACAGGATCGCACCCAGATTGATGAAGAAATTA
TTGAATGCGAAGCGTGGACCCGCTATACCTTTCCGGTGCGCGCGGGCCAGTATAGCCAGTTTGTGTGG
GATTATAAATGCTTTAGCGGCATTGATCATATTGAAAACCCGACCGAAGATGGCGTGTTTAAAATTGT
GAACGATTATACCGGCGAAGGCTGGAACGAACAGGTGGATGAAGAACTGGGCAACTTTGATTATCTG

ATGGGCGCGAACATTGATTTTCGCAACCATGCGGTGACCGAAGATATTAAATATTGGGCGCGCTGGGT
GATGGAACAGACCGGCTGCGATGGCTTTCGCCTGGATGCGGTGAAACATATTCCGGCGTGGTTTTATA
AAGCGTGGATTGAACATGTGCAGGAAGTGGTGCCGCAGCCGCTGTTTATTGTGGCGGAATATTGGAGC
CATGAAGTGGAAAAACTGCAGCAGTATATTGATCTGGTGGAAGCGAAAAGCATGCTGTTTGATGCGCC
GCTGAACATGAAATTTCATGAAGCGAGCCGCCAGGGCCGCGATTATGATATGAGCCAGATTTTTAGCG
GCACCCTGGTGGAAGCGGATCCGTTTCATGGCGTGACCCTGGTGACCAACCATGAAACCCAGCCGCTG
CAGGCGCTGGATGCGCCGGTGGAACCGTGGTTTAAACCGCTGGCGTATGCGCTGCTGCTGCTGCGCGA
AAACGGCGTGCCGAGCGTGTTTTATGCGGATCTGTTTGCGGCG

The obtained sequence was translated using the Translate Tool of EXPASY and the following result was obtained:

MGADEKEALKVQRVDEQDRTQIDEEIIECEAWTRYTFPVRAGQYSQFVWDYKCFSGIDHIENPTEDGVFKI
VNDYTGEGWNEQVDEELGNFDYLMGANIDFRNHAVTEDIKYWARWVMEQTGCDGFRLDAVKHIPAWFY
KAWIEHVQEVVPQPLFIVAEYWSHEVEKLQQYIDLVEAKSMLFDAPLNMKFHEASRQGRDYDMSQIFSGT
LVEADPFHGVTLVTNHETQPLQALDAPVEPWFKPLAYALLLLRENGVPSVFYADLFAA.

The amino acid sequence was analyzed through BLASTp program of NCBI. The BLASTp results showed a match with the cytoplasmic alpha amylase of *Klebsiella pneumoniae* strain *IS43*.

CONCLUSION

In conclusion, this study describes the identification and biochemical characterization of an amylase isolated from *Klebsiella species* from industrial soil of Punjab region. The study and enzyme assay strongly suggested that the amylase is acidophilic and detergent stable.

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Conflict of interest: The authors declare that they have no conflict of interest.

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