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STUDIES ON A MALTOHEXAOSE (G6) PRODUCING ALKALINE AMYLASE FROM A NOVEL ALKALOPHILIC STREPTOMYCES SPECIES

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ABSTRACT

The present paper describes the production and characterization of alkaline amylases from a novel obligate alkalophilic Streptomyces sp. This strain was isolated from the alkaline soil of the meteorite Lonar lake, situated in Maharashtra, India. This is the first report of a maltohexaose producing alkaline amylase from an alkalophilic Streptomyces sp and the fourth report of an alkalophilic Streptomyces sp producing an alkaline amylase. The optimum growth of the culture occurred in the range of pH 8 to 10 at 28^{o} C. No visible growth occurred at neutral or acidic pH. Optimum enzyme production occurred only at alkaline pH, the optimum being pH 10.5. Soluble starch as well as raw starch induced the enzyme, induction being higher with raw starch. Substantial quantities of the enzyme was also produced in a non-starch medium containing malt extract, yeast extract and glucose. The enzyme was active in the range of pH 7-10, the optimum pH being 9.0 at 45^{o}C. Starch electrophoresis of the culture filtrate revealed the presence of two amylolytic bands. The molecular weights of the two bands as determined by native electrophoresis were 36,920 and 36,300 daltons respectively. The enzyme was found to be endo in action, the predominant products being maltohexaose (48%) followed by maltotetrose, maltotriose and maltose, at the initial stages of hydrolysis as well as after 24 hours and hence would have a potential application in the food and pharmaceutical industry. The enzyme preparation was also effective in removing starch based stains. Hence, the alkaline amylases from the Streptomyces sp would have a potential application in the food and detergent industries.

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KEY WORDS

Alkalophilic Streptomyces sp; alkaline amylase; maltohexaose; application in food and detergent industries;

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[I] INTRODUCTION

Amylases are ubiquitous and have been isolated, purified and characterized from a number of sources. Most of these amylases are active at acidic or neutral pH. In contrast, alkaline amylases have been reported mainly from the genus Bacillus [1], with a few reports from other genera i.e. Natronococcus [2], Myxococcus [3], and Micrococcus [4]. The alkalophilic *Streptomyces sp*. as well as its amylases is the fourth report, besides the three available reports of alkalophilic *Streptomyces* strains producing alkaline alpha-amylases [5-7].

An important characteristic of the amylase is that it hydrolyses starch to produce maltohexaose (G6) as the major product along with maltotriose (G3), maltotetraose (G4) and maltose (G2) and hence would have a potential application in the food and pharmaceutical industry. To date, in the genus *Streptomyces* there are two reports of maltotriose producing amylases [7,8] and a single report each of maltotetraose [9] and maltohexaose [10] producing strains. However, this is the first report of a maltohexaose producing alkaline amylase from an alkalophilic *Streptomyces* strain. In recent years, there has been an increasing interest in the use of malto-oligosaccharides as biopreservatives [11, 12]. Since these oligosaccharides are natural components of foods such as fruit, vegetables, milk and honey, they are increasingly being favoured in lieu of chemical additives which are becoming less welcome by the consumers [13-15]. Amongst the malto-oligosaccharides, G3 ie maltotriose, G4 ie maltotetraose, G5 i.e. maltopentaose and G6 ie maltohexaose have attracted attention because they not only provide useful modifications to flavour and physiocochemical characteristics of the food, but also have properties that are beneficial to human health [11,12,14, 16,17]. Major uses of maltooligosaccharides are in beverages, infant milk powders, confectionery, bakery products, yoghurts and dairy desserts. The price of pure maltooligosaccharides is extremely high because the chemical manufacture of malto-oligosaccharides larger than maltotriose has been very difficult. However, the discovery of microbial enzymes that specifically act on starch substrate to produce specific malto-oligosaccharides has made it possible to produce syrups containing various malto-oligosaccharides [16]. As compared to the vast number of amylases from different sources

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that have been isolated, purified and characterized, there are very few amylases which act on starch to specifically produce malto-oligosaccharides with DP of 3, 4, 5 and 6.

Our experiments have also shown that the alkaline amylases from the *Streptomyces sp*. could effectively remove starchy stains. This paper thus describes the production and characterisation of the alkaline amylases which could have potential application not only in the food and pharmaceutical industry but also in the detergent industry.

[II] MATERIALS AND METHODS

2.1 Materials

Malt extract, yeast extract, peptone, tryptone, casaminoacids, glucose, potato starch, corn starch were from Hi- media chemicals, India. DNSA, Maltotetraose, maltose, Brilliant Blue G 250, bis acrylamide and TEMED were from Sigma Chemicals, USA. Pre-coated TLC silica gel 60 sheets were from Merck KgaA, Germany. Acrylamide was from Sisco Research Laboratories, India. All other chemicals used were of analytical grade. Tapioca starch, rice flour, wheat flour and soyabean flour were purchased from the local market.

2.2. Isolation of the organism

The soil sample used for isolation was collected from the vicinity of the Lonar Lake, an alkaline salt lake situated in Buldhana district of Maharashtra, India. The protocol and media for isolation of alkalophilic actinomycetes was as described by Mikami et al [18]. The pH of the medium was adjusted to 10.5 by addition of sterile Na_2CO_3, prior to inoculation. Purification of the isolated cultures was carried out by the dilution technique repeatedly to overcome bacterial contamination. No antibiotics were used. The cultures were screened using the plate assay method as described below.

2.3. Plate Assay

Screening of cultures for amylase activity was carried out on agar media in petri plates containing 1% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.05% K_2HPO_4, 0.1% $MgSO_4$ and agar 2.4% (PSA). The pH of the medium was adjusted to 10 by the addition of sterile Na_2CO_3 to a final concentration of 1% prior to pouring the plates. The culture was inoculated at the center of the plates and incubated for 96 h at 28^{0}C. The plates were then flooded with 0.1% iodine in 2% KI. Amylolytic cultures were identified by a zone of clearance against a dark blue background. The amylolytic strain used in the present study was identified as a *Streptomyces sp*. based on morphological characteristics and rDNA analysis (Unpublished results).

2.4. Medium composition and culture conditions

Amylase production was carried out in three steps as follows:

Step1: The organisim was subcultured on an alkaline potato starch-agar plate (PSA) as described above. A five day old agar plate was used to prepare the inoculum. The plates were inoculated and incubated at 28^{0}C.

Step 2: The medium had the following composition: 0.5% yeast extract, 0.5% peptone, 0.5% raw starch, 0.05% K_2HPO_4. 0.01% of $MgSO_4$. The starch was dry heated at 110^{0}C for 20 min and dry sterilized separately for 20 min at 15 lbs. The pH of the medium was adjusted to 10.5 by the addition of sterile 10% Na_2CO_3 to a final concentration of 1%. The raw starch and Na_2CO_3 were added to the medium prior to inoculation. Growth equivalent to one cm was inoculated after maceration.. The flasks were incubated on a rotary shaker at 28^{0}C for 48 h.

Step 3: Production of the enzyme was carried out in 250 ml Erlenmeyer flasks containing one-fifth volume of medium. The fermentation medium consisted of 0.5% yeast extract, 0.5% peptone, 0.1% K_2HPO_4, 0.02% $MgSO_4$ and 3% potato raw starch. Raw starch was sterilized and added as described for the inoculum. The pH of the medium was adjusted to 10 by Na_2CO_3 as described above. Vegetative inoculum 10% (v/v) was transferred to the experimental flasks which were then incubated at 28^{0}C. with shaking at 200 rpm. The cell-free supernatant liquid obtained by centrifugation at 8000 rpm for 30 min at 4^{0}C, was used for determining alkaline amylase activity.

2.5. Enzyme Assay

a) Saccharifying activity

The amylase activity of the culture was estimated using soluble starch as the substrate by the dinitrosalicylic method as described by Bernfeld [19]. The reaction mixture of one ml contained 0.5 ml of suitably diluted enzyme in 50 mM glycine NaoH buffer pH 9 and 0.5 ml of 1% starch solution. The reaction mixture was incubated at 45^{0}C for 30 min and the reaction was terminated by the addition of one ml of DNSA. The tubes were heated in a boiling water bath and the color intensity was read at 540 nm after dilution with 10 ml of distilled water. In the case of assays carried out at other pH values, the corresponding amount of soluble starch prepared in water was used and the final concentration of buffer was 50 mM. A standard maltose curve was used for calculating enzyme activities. One International unit (IU) of amylase activity was defined as the amount of enzyme required to produce one micromole of maltose or equivalent in one min under the assay conditions.

b) Dextrinizing activity

This was estimated as described by Krishnan and Chandra [20]. The reaction mixture contained 0.5 ml of 1% soluble starch, 0.4 ml of 0.125 M glycine NaOH buffer pH 9 and 0.1 ml of suitably diluted enzyme. In the control tube, the enzyme was replaced by an equivalent amount of buffer. The reaction mixture was incubated at 45^{0}C for 5 min and was terminated by the addition of 0.5 ml of 1N HCl. A volume of 0.1 ml of this reaction mixture was diluted with 14.3 ml of distilled water. To this 0.5 ml of 1N HCl was added, followed by the addition of 0.1 ml of 0.3% Iodine in 3% KI. The blue color was read at 600 nm. One dextrinizing unit is defined as the amount of enzyme which hydrolysed 1 mg of starch in 5 min.

2.6. Protein estimation

Amount of protein was determined according to Bradford et al [21] using bovine serum albumin as the standard.

2.7. Electrophoresis

Native PAGE in 10% slab gels was carried out as described by Davis [22]. The protein bands were visualized by staining the gels by silver staining [23].After the run, the gels were sliced into two halves and one half of the gel was stained with silver stain to visualize the bands while the second half was processed for detecting amylase activity. For activity staining, 0.1 IU of enzyme was used. The gel was sandwiched between two 10% PAGE gels containing 0.3 % starch and incubated at 37^{0}C for 2 h. The sandwiched gel was then flooded with 0.1% Iodine in 2% KI. The amylase bands appeared as white bands on a dark blue background

2.8. Molecular weight determination

Molecular weight of the amylases was determined by the method of Hedrick and Smith [24] using the native gel system containing starch. The standard proteins used were bovine serum albumin (66,000 daltons),

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carbonic anhydrase (29,000) daltons), soybean trypsin inhibitor (20,000 daltons) and myoglobin (18,800 daltons). The proteins were detected by Brilliant Blue G-250 [23]. Each of the standard proteins and the partially purified sample was loaded on native PAGE gels of different acrylamide concentration (5%, 7.5%, 10%, 12% and 16%). The slopes of the standard proteins and the sample were determined by plotting log Rm versus gel concentration. A plot of the slopes versus the molecular weight of standard proteins was drawn and the molecular weights of the amylases were determined from this graph by co-relating the slopes.

2.9. Determination of isoelectric pH

This was determined in polyacrylamide tube gels as described by Vesterberg [25].

2.10. HPLC

To determine the mode of action of the enzyme, the degree of polymerization (DP) of the products was determined by HPLC as described by Nirmala [26].Hydrolysis of starch was carried out as follows: 2% soluble starch was hydrolysed in a total volume of 2 ml containing 2 IU of enzyme in 50 mM sodium phosphate buffer (pH 8.0), for different intervals of time ranging from 1 h to 24 h. At the end of the reaction time, the mixture was neutralized by the addition of 1 N HCL followed by boiling for 5 min. 3 vol. of absolute ethanol was added to the hydrolysate and the mixture was kept at 4^0C for 6 h for precipitation after which it was centrifuged at 10,000 rpm. The supernatant was concentrated in a rotavap and the product was dissolved in ultra pure water and filtered through a 0.22 µM membrane and analyzed by HPLC. 20 µL sample was injected and analyzed by HPLC. The amounts of oligosaccharides (DP1-DP7) were quantified by peak integration, with standards detected using a refractive index detector. HPLC was carried out in a Shimadzu system equipped with a RI detector with the following specification: Column: µBondapak NH2 (3.9 x 300 mm, 10 µm)

Solvent system used was acetonitrile: water (70 : 30); Temperature : 25^0C; Flow rate : 1 ml/ min, isocratic; Injected volume : 20 µL. Standards used:–maltose to heptaose

2.11. Raw starch adsorption

Amylase adsorption on raw starch was measured as described by Gashaw et al with slight modifications [27]. The reaction mixture (1 ml) contained 1.2 IU of enzyme in 50 mM phosphate buffer (pH 7.0) and 0.5 g of corn / potato starch. The reaction mixture was gently stirred for 15 min. at 30^0C. The suspension was then microfuged for 5 min. at 10,000 rpm and the amylase activity in the supernatant was determined. The percentage adsorption of the enzyme was calculated with respect to the original activity.

2.12. Test for checking efficacy of the enzyme preparation in removing starchy stains

The application of the amylase preparation as a detergent additive was evaluated as described by Kamal Kumar et al [28]. The application of amylase as a detergent additive was evaluated on pieces of white cloth (2x2 cm) stained with 0.3 % starch solution. The stained cloth pieces were dried at 50 ^{0}C for 30 min. The soiled cloth pieces were washed with 10 ml of water containing 2 mg and 7 mg/ml of surf Excel and 50 U/ml of crude amylase enzyme respectively. The flasks were incubated for 30 min, 1 h and 4 h respectively. The controls consisted of soiled cloth pieces without enzyme treatment. The treated and untreated samples were compared visually, to evaluate the efficacy of the enzyme treatment.

[III] RESULTS

3.1. Isolation and characterisation of the organism

The obligate alkalophilic *Streptomyces sp* was isolated from the alkaline soil of the meteorite Lonar Lake, situated in the state of Maharashtra, India. The colony showed the typical dry powdery characteristic of an actinomycete colony. The aerial mycelial formation was extensive. This actinomycete strain was found to grow only at alkaline pH indicating the strain to be alkalophilic in nature. The optimum growth of the culture occurred in the range of pH 8 to 10 at 28^oC. No visible growth occurred at neutral or acidic pH **[Figure–1a and 1b]**. The strain was identified to be a *Streptomyces* species based on its morophological characteristics and 16s rDNA (unpublished results).

3.2. Plate assay

The *Streptomyces sp* gave a distinct zone of clearance on starch agar medium when flooded with iodine solution in KI indicating the strain to be amylolytic.

3.3. Alkaline Amylase production inoculum

Enzyme production was studied in media containing soluble starch and raw starch as an inducer. The optimum concentration of raw starch in the inoculum was found to be 0.5%. Equivalent amounts of soluble starch gave 56% of the activity as that obtained with raw starch (data not shown). Hence, raw starch was used in all further studies, unless otherwise mentioned.

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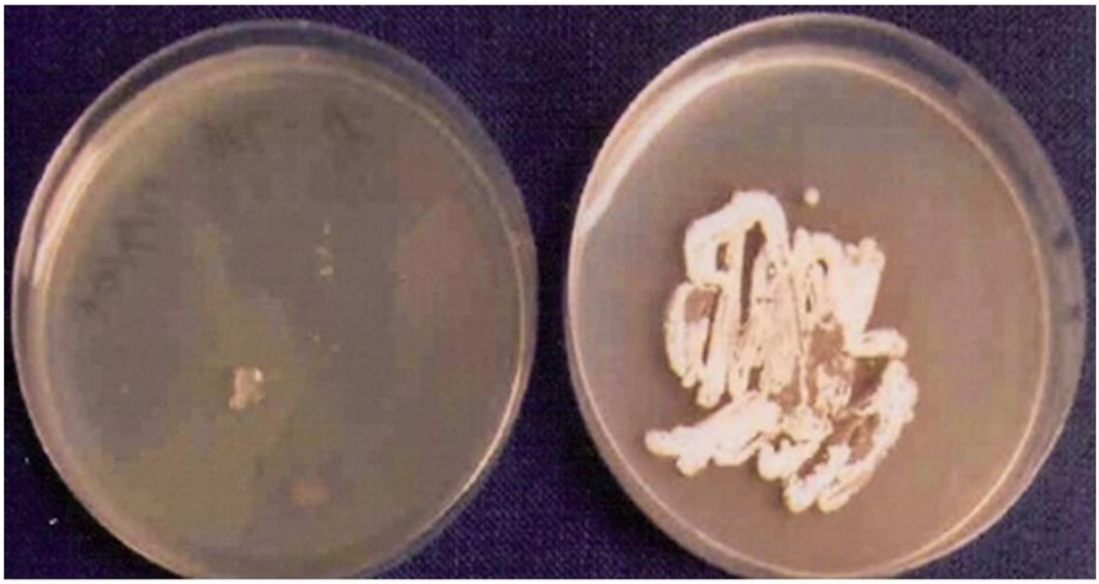

Fig. 1a

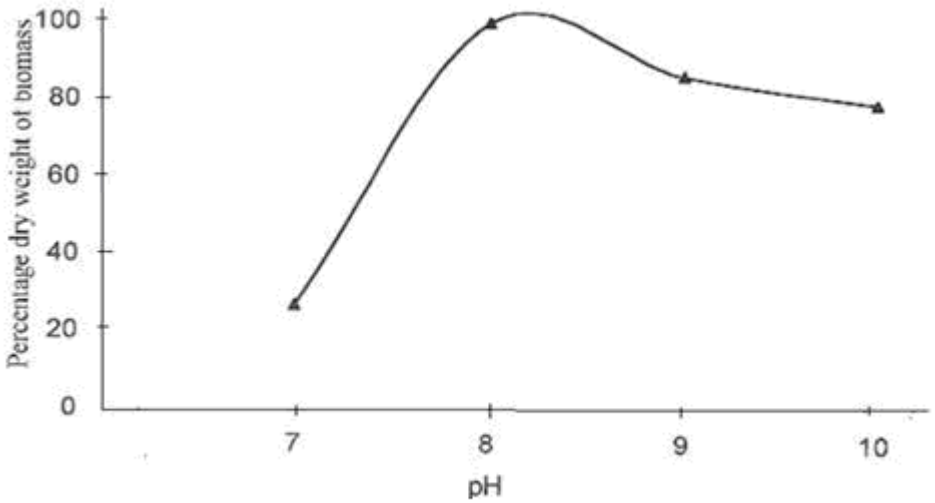

Fig. 1b

Fig: 1. (a) Growth characteristics of the alkalophilic *Streptomyces sp* at neutral and alkaline pH **(b)** Effect of pH of the medium on growth of the culture.

3.4. Requirement of Na^+ and K^+

A number of alkalophilic bacteria are known to have a stringent requirement for Na+ and which cannot be replaced by K+ [1, 29]. The growth of the *Streptomyces sp* was not affected when Na+ ions was replaced with K+ ions in alkaline medium **[Figure– 2a]**. However, amylase production was only 50% when Na_2CO_3 was replaced by K_2CO_3 **[Figure– 2b]** The organism was not halotolerant as only 20% growth was observed in 5% NaCl as compared to the control.

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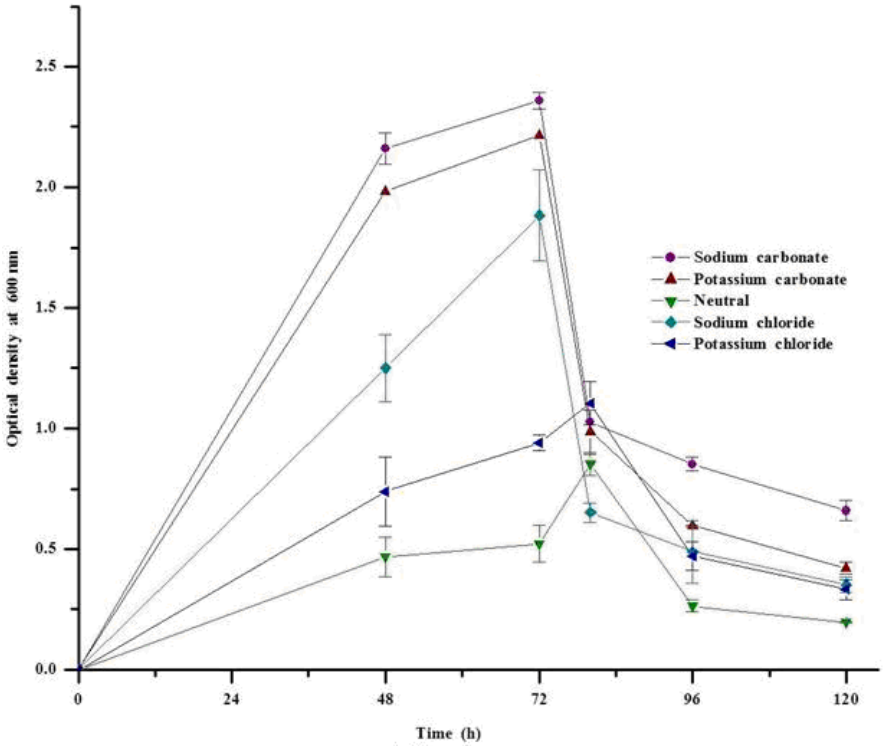

Fig. 2a

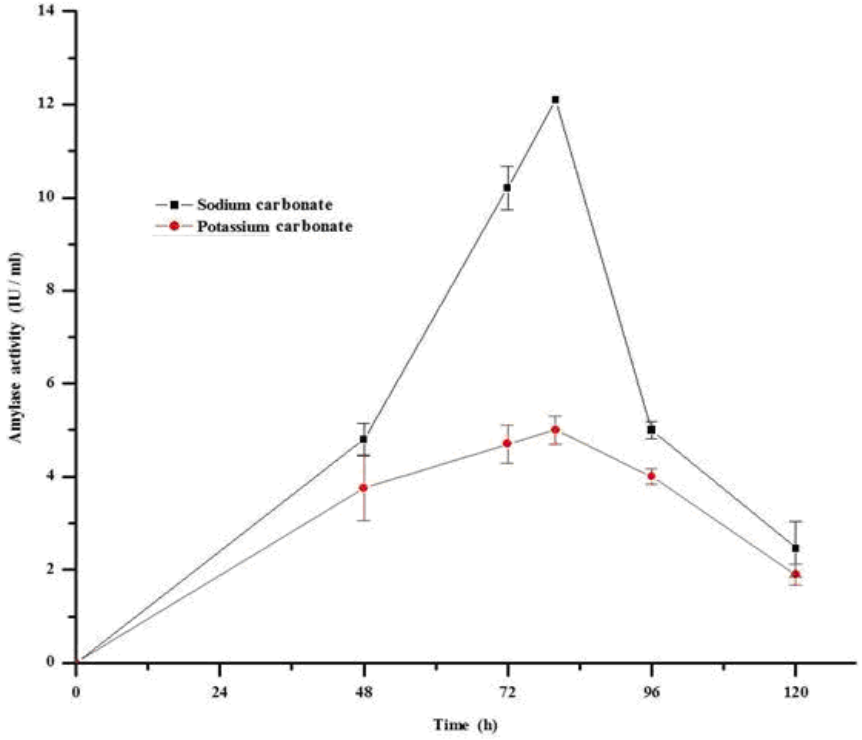

Fig. 2b

Fig: 2. Effect of Na^+ and K^+ on **(a)** growth **(b)** alkaline amylase production

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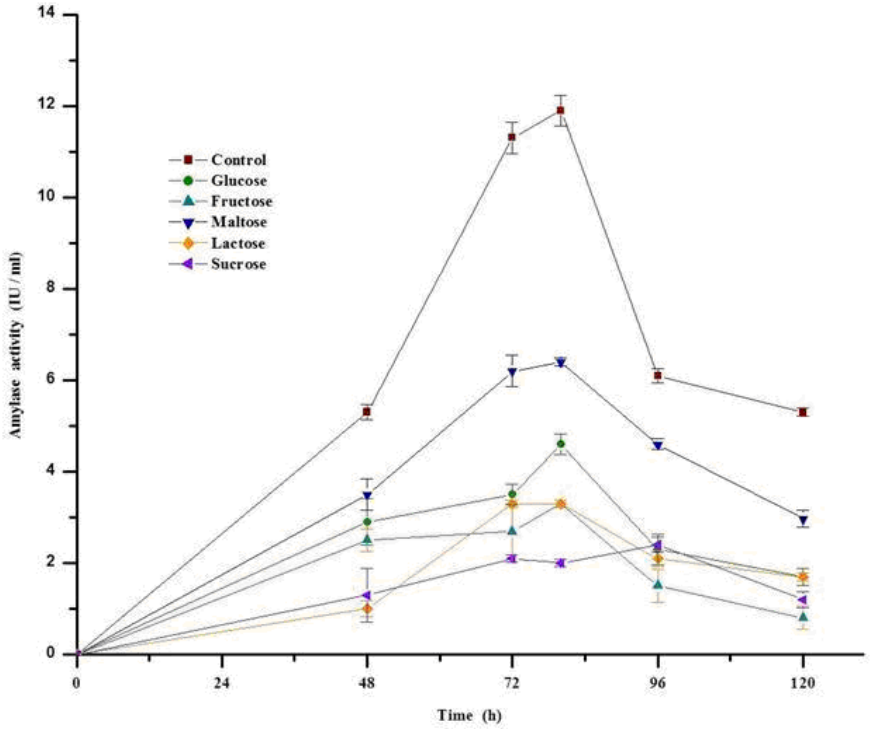

Fig. 3a

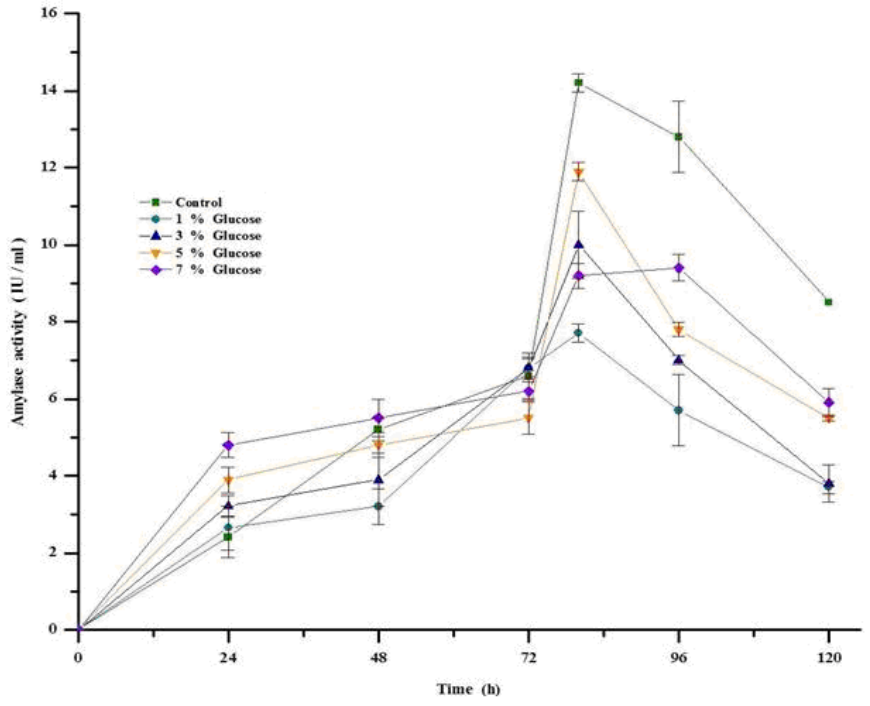

Fig. 3b

Fig: 3. Effect of carbon sources on alkaline amylase production. **(a)** different inducers (sugar inducers 3 %) yeast extract 0.5% ,peptone 0.5 %,$MgS0_4$ – 0.02 % K_2HPO_4 - 0.1 %, sodium carbonate 1 %.) n = 4, significance 1% (ANOVA). **(b)** Alkaline amylase production in MGYP medium, n= 4, significance 1% (ANOVA)

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3.5. Optimization of constituents in the fermentation medium

3.5.1. Starch concentration

The concentration of starch was varied between 1-7% in the fermentation medium, keeping the other constituents constant. It was observed that a maximum activity of 13.2 IU/ml was obtained in presence of 3% raw starch at 80 h. **[Supplementary Table– 1]**. Soluble starch when added at the same concentration gave only 70% of the maximal activity as compared to raw starch. Higher concentrations of soluble starch or incremental addition of soluble starch gave lower activities. Hence, in all further experiments raw starch was added to a final concentration of 3%.

3.5.2. Effect of various starch sources on alkaline amylase production

Starch from various natural sources was studied for their effect on amylase production. It was observed that starch from corn and potato gave maximum and equivalent activities followed by wheat flour **[Supplementary Table – 2]**. Enzyme production was only 36 to 40% of maximal activity with rice and refined wheat flour.

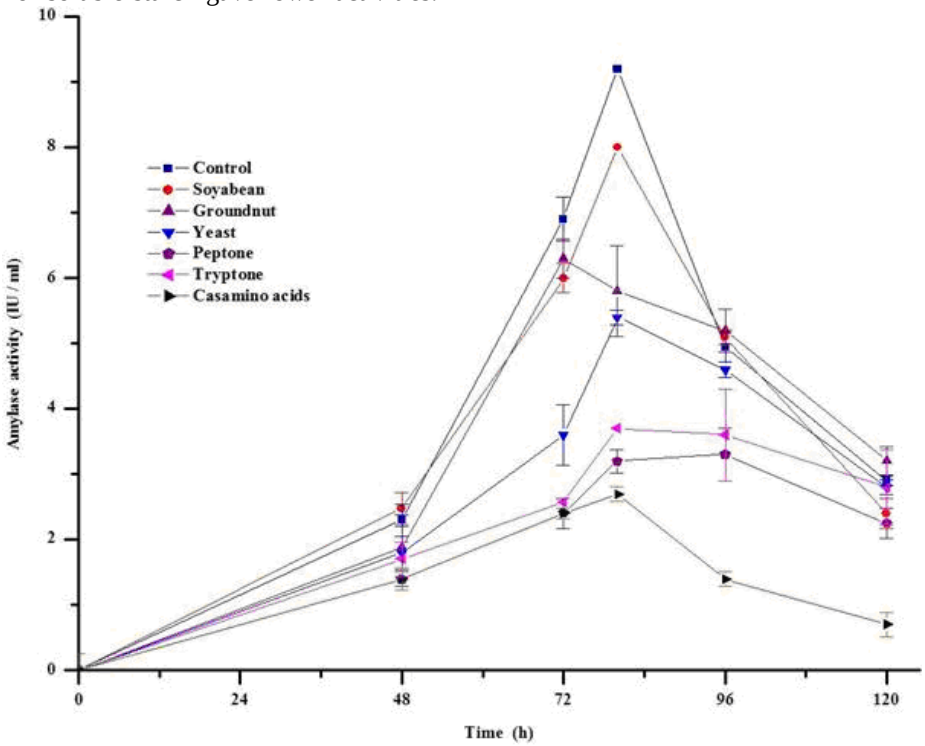

Fig. 4

Fig 4: Effect of organic nitrogen sources on amylase production. (n= 4, significance 1% (ANOVA))

3.5.3. Effect of carbon sources

The effect of different carbon sources was evaluated on amylase production **[Figure– 3a]** Amylase synthesis is known to be induced by starch and maltose. The only report of a constitutive production of an amylase has been from *Pseudomonas sp* IMD 353 where a higher amylase activity was observed in a non-starch medium [30]. In our studies it was observed that 3% maltose gave 54% of the control activity (potato starch), while glucose gave 30% of the control activity indicating that there is a substantial basal level of constitutive production of the enzyme. In a MGYP medium (1% glucose, 0.3% malt extract, 0.5% yeast extract, 0.5% peptone) where the effective concentration of maltose from malt extract was approximately one tenth of that added in the 3% maltose experiment, 65% of the control activity was obtained indicating that a factor in addition to maltose from malt extract is inducing the amylase **[Figure–3b]** When the concentration of glucose was increased from 1% to 5% there was a gradual increase in activity and at 5% glucose concentration, 83% of the control activity was obtained. Further increase in glucose concentration resulted in a decrease in amylase activity. These results indicate that the repressive effect of glucose could be overcome partially by the addition of malt extract. Similar observations were made by Diaz et al [31] in the case of ß amylase production from *Xanthophyllomyces dendrorhous*. Eighty eight percent of ß amylase activity was obtained in a MGYP medium when compared to a control medium containing starch and glucose. It

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has been shown that the repressive effect of glucose was reduced in solid state fermentation [32]. A number of amino acids are known to increase the production of various enzymes such as amylases [33] and xylanases [34]. Chandra et al [35] showed that in the presence of a mixture of amino acids the repressive effect of monosaccharides, especially glucose at low concentrations did not effect amylase synthesis. It is therefore likely that amino acids present in the malt extract could have played a role in enhancing the activity besides induction by maltose.

3.5.4. Effect of nitrogen sources on amylase production

Various nitrogen sources individually or in combination was evaluated using an optimum starch concentration of 3%. **[Supplementary Table– 3, Figure– 4]** Maximum activity was obtained in control flasks containing a combination of yeast extract and peptone followed by soybean. Yeast extract and peptone when used singly gave lower activities. Tryptone gave poor yields. A combination of tryptone and yeast extract gave only 57% of the control activity. Similar results were obtained by Hayashi et al [36] in the case of alkaline amylase production in *Bacillus sp H-167*. A review of the literature showed that the requirement of an optimal nitrogen source varied with the organism. Tigue et al [37] have shown that yeast extract alone gave maximum yields as against a combination of yeast extract and peptone. Narang and Satyanarayana [38] have shown that tryptone or yeast extract individually or in combination gave optimal levels of amylase production in *Bacillus thermooleovorans*. In a few cases inorganic nitrogen sources such as ammonium hydrogen phosphate, ammonium sulphate and ammonium nitrate in combination with an organic nitrogen source has given enhanced amylase production [39]. In the case of most organisms yeast extract was found to be essential for optimal production of amylases.

The quality of the protein and the carbon –nitrogen balance appears to play an important role in optimal production of the enzyme.

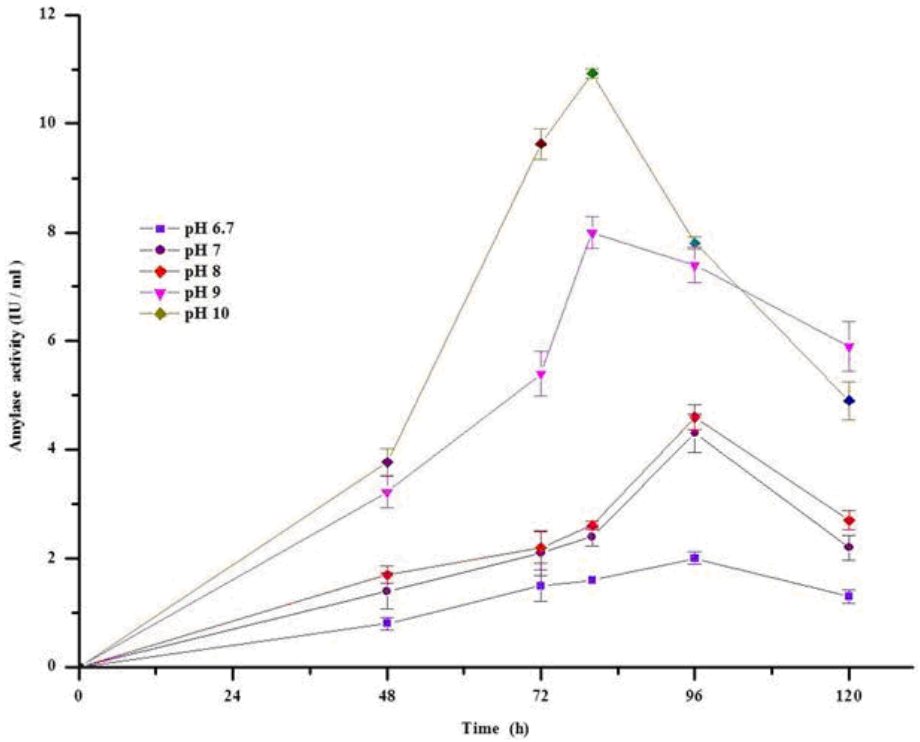

Fig. 5

Fig: 5. Effect of initial pH of the medium on alkaline amylase production n= 4, significance 1% (ANOVA)

3.5.5. Effect of metal ions on amylase production

Various metal ions were evaluated for enzyme production, keeping the concentration of the nitrogen and carbon source constant. The metal ions K_2HPO_4, $MgSO_4$, and $CaCl_2$ when added individually gave approximately 30 to 50 % of the maximal activity. Maximum amylase production occurred with a

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combination of K_2HPO_4 and $MgSO_4$. A combination of K_2HPO_4 and $CaCl_2$ gave only 65 % of the control activity indicating that calcium ions were not essential for amylase production **[Supplementary Table– 4]**. Malhotra et al [40] too showed that amylase production was calcium independent and Mg ions enhanced enzyme production. Similar observations were made by Chandra et al [35].

3.6. Effect of initial pH on amylase production

Since the growth of the *Streptomyces* culture occurred at pH 8 and above, fermentation was carried out at different pH in order to determine the optimum pH for amylase production. The pH of the medium was varied by the addition of 10% sodium carbonate which was autoclaved separately and added prior to inoculation. Other constituents of the fermentation medium were kept constant. To determine the extent of amylase production at various pH values, equal amounts of inoculum (10% v/v) grown at alkaline pH was added to all the flasks at different pH values. The flasks were incubated at 28^{0}C at 180-200 rpm. It was earlier seen that the growth of the culture at pH 7.0 in a MGYP medium was 20 % when compared with the growth at pH 10 indicating that the culture was truly alkalophilic in nature **[Figure– 1b]**. Concomitantly at neutral pH only 20 % of amylase production was observed even though the pH of the medium rose to a pH of 8. **[Figure– 5])** At 96 h the pH of the medium rose further to a pH of 9.45, but the corresponding amylase activity was only 35 % of the control activity. Thereafter the activity declined.

These results indicated that the initial pH of the medium was critical for maximum amylase production which was preferably above a pH of 9.0.

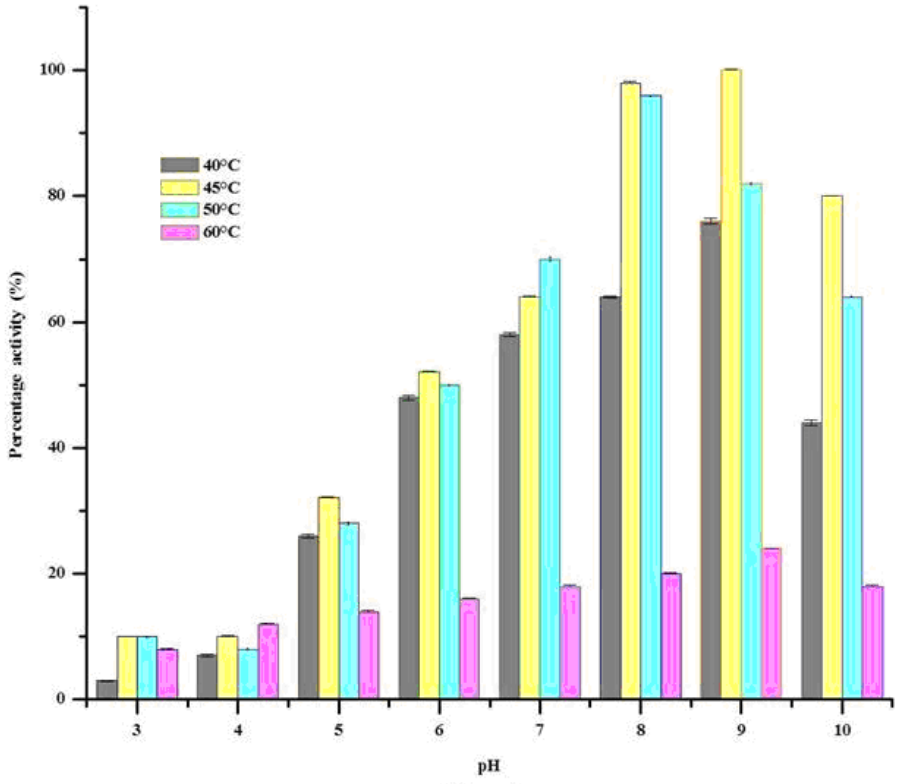

Fig. 6

Fig: 6. Profile of optimum pH and temperature of alkaline amylases from alkalophilic *Streptomyces sp*. Buffer system: sodium acetate buffer 50 mM (pH4.0-5.0); Potassium phosphate buffer, 50 mM (pH 6.0- 8.0); Glycine- NaOH buffer, 50 mM (pH 9.0 – 10.0). Temperature 45^{0}C, n= 3, significance 1% (ANOVA)

3.7. Characterisation of the amylases from the Streptomyces sp

3.7.1. Optimum pH and temperature

The optimum pH of amylase activity was determined at various pH values ranging from pH 4 to 10 as a function of temperature. From **Figure– 6** it can be seen that the amylase is active in the alkaline range. Maximum activity was obtained at pH 9 and at 45^{o}C. The enzyme was found to be active over a temperature range of 40^{o}C to 50^{o}C at pH 9 and 10. The enzyme was stable over a pH range of 7-10, maximum being at pH 7. The above pH

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and temperature parameters make the enzyme suitable for detergent, leather and textile applications where enzymes active at alkaline pH are desirable.

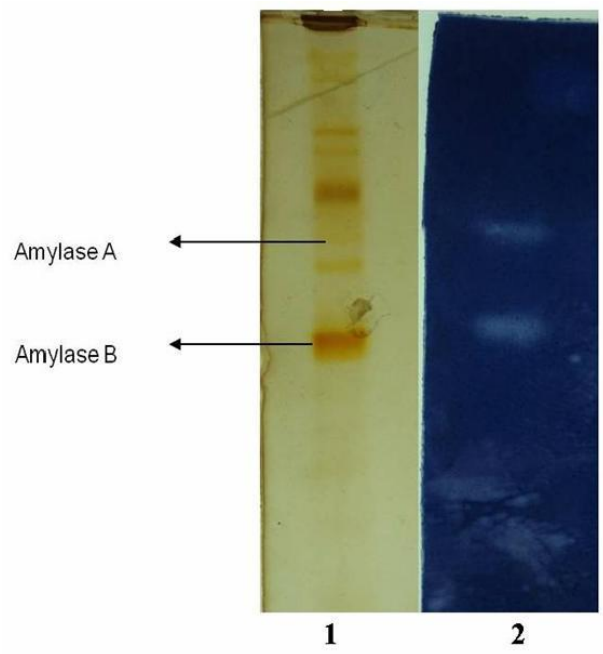

Fig. 7

Fig: 7. Silver stained PAGE gel electrophoresis of the crude extract of alkalophilic *Streptomyces sp* (Lane 1). Starch gel electrophoresis of the crude extract of *Streptomyces sp* (Lane 2).

3.7.2. Effect of additives on amylase activity

Alkaline amylases were found to vary in their response to the chelating agent EDTA [41]. In our studies the effect of EDTA was found to be interesting **[Supplementary Table–5]**. When EDTA was added at a concentration of 2 mM approximately 50% of the amylase activity was lost. However, with increasing concentrations of EDTA there was a progressive increase in activity and at 20 mM, 80% of the original activity was restored. These results were further corroborated by dialysis of the enzyme against EDTA. When the enzyme was dialysed overnight against 10 mM Tris-HCl, pH 8 containing 2 mM and 20 mM EDTA, the activities obtained were 52 and 80% respectively confirming that Ca^{+2} was essential for activity. Similar observations were made Bernhardsdotter et al [42]. The authors reported 30% enhancement of activity with 10mM EDTA as compared to 5 mM. Vishnu et al [43] too reported an enhancement of activity with EDTA at a higher concentration in the case of the amylases from *Lactobacillus amylophilus GV6*. Presently there is no explanation that can be given for these observations and must await the determination of the crystal structure. It has been observed that generally the saccharifying amylases are stable in their response to EDTA / EGTA as compared to the liquefying amylases [44]. The amylase activity decreased progressively with increasing concentrations of denaturing agents such as SDS and urea. The 6M urea treated enzyme when dialysed against buffer did not regain its activity but when dialysed against buffer containing 5 mM $CaCl_2$, 55% of the original activity was restored indicating that Ca^{+2} ions was essential for proper folding of the enzyme. The enzyme lost 15% activity as compared to the control when incubated with 0.2% Surf a commonly used detergent for 15 min at 40 to 50^0C, indicating that the amylases from this strain could have a potential application in laundry detergents. A progressive increase in activity was observed with increasing concentrations of reducing agents such as ß-mercaptoethanol and cysteine hydrochloride. The increase was threefold approximately with cysteine hydrochloride. Similar observations were made in case of xylanases where 10 mM cysteine and β-mercaptoethanol led to an increase in activity ranging from 47% to 116% [45-47].

3.7.3. Electrophoresis Pattern

The starch gel electrophoretic pattern is shown in **Figure–7**. Both, anodic as well as cathodic runs were carried out. In the anodic run, two bands of amylase activity were detected. No activity bands were detected in the cathodic run, suggesting that the amylases are anionic in nature.

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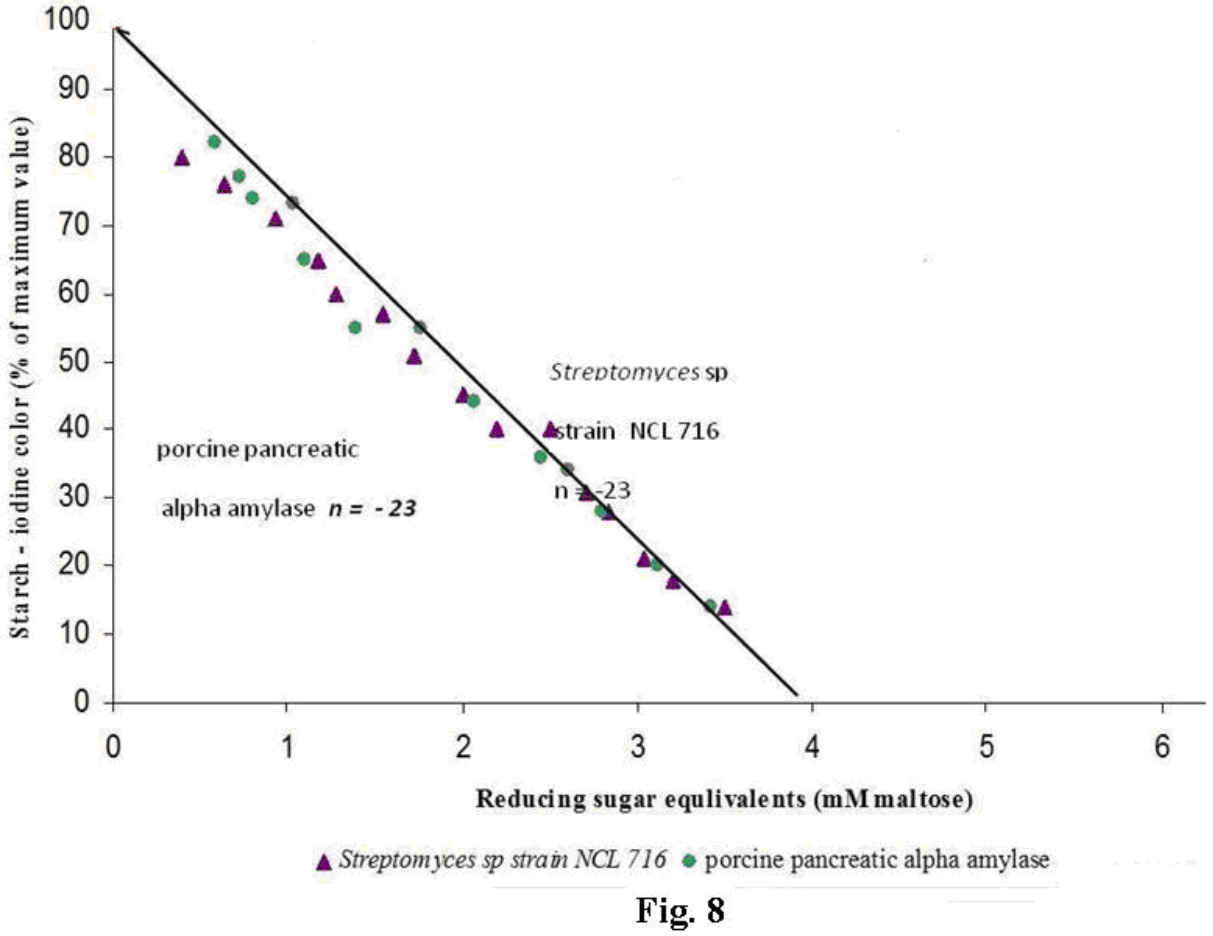

Fig. 8

Fig: 8. Determination of mode of action of the amylase. The values for the starch-iodine blue color are plotted against the production of reducing sugar equivalents in terms of maltose. The slope of the linear curve determines the endo- or exo-action of the enzyme.

3.7.4. Molecular weight and pI

Molecular weights of the amylases as determined by the mobilities in native gels of different concentration were found to be 36,920 and 36,300 daltons respectively. The pI of the major amylase was found to be 6.26 confirming the anodic nature of enzyme.

3.7.5. End product analysis

The slope of a plot of reducing sugar versus starch-iodine color could predict the mode of action of the amylase. A steep slope accounts for a fast reduction of the starch-iodine blue color due to random endo activity, while a comparatively flat slope indicates a prevalence of exo activity [48]. A slope of -26 was obtained for the endo α-amylase from *Bacillus amyloliquefaciens* while the maltohydrolase enzyme from *Pseudomonas stutzeri NRRLB 3389* showed a slope of -12. The *Bacillus stearothermophilus* exo-maltogenase showed a slope of -2. A comparative study of porcine pancreatic amylase with the amylases from *Streptomyces sp*. was carried out. The well characterized porcine pancreatic amylase gave a plot of –24 confirming that it is endo in action **[Figure– 8]**. The alkaline amylases from the *Streptomyces sp*. also gave a value of –24 indicating it to be also endo in action The HPLC profile of the starch hydrolysate for the *Streptomyces sp*. amylase showed the presence of maltohexaose (~ 48%) as the predominant end-product followed by almost equivalent amounts (~ 10%) of maltotetrose, maltotriose and maltose, even at the early stages of hydrolysis indicating it to be different from porcine pancreatic amylase **[Figure– 9]** The first report of amylases producing specific sugars was reported by Robyt et al from the culture filtrates of *Pseudomonas stutzerii* [49]. Subsequently, there have been several reports of amylases producing specific sugars ranging from G2 to G6, mainly from the genus Bacillus and

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Pseudomonas, most of which are active at neutral or alkaline pH. To date there are only two reports of maltotriose being produced by neutrophilic *Streptomyces* strains [7,8] and one report each of maltotetrose and maltohexaose being produced by *Streptomyces* strains [9,10].

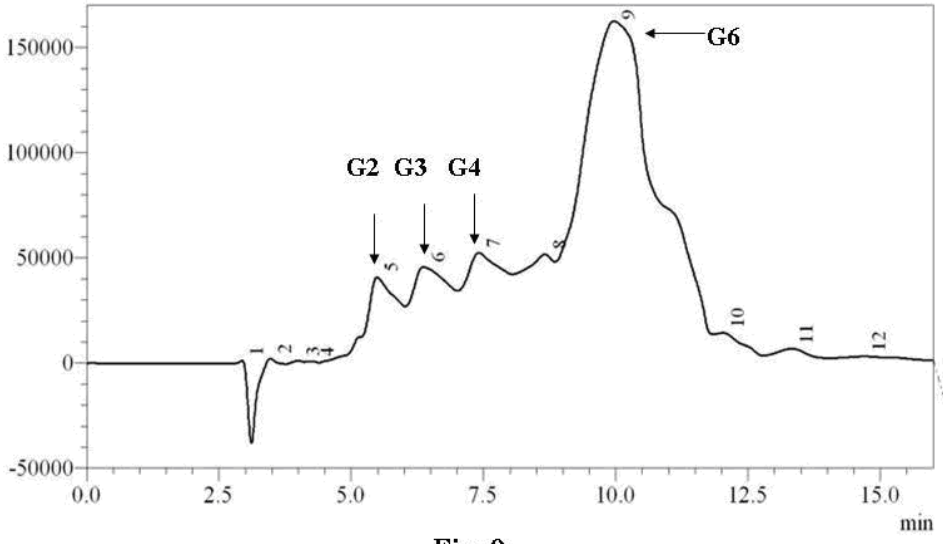

Fig. 9

Fig 9: HPLC analysis of the end products of hydrolysis of soluble starch. G2 – Maltose; G3 – Maltotriose; G4 – Maltotetraose; G6 : Maltohexaose

3.7.6. Raw starch determination

Amylases can be classified as endo–adsorbant and non-adsorbant [50]. The alkaline amylases from *Streptomyces sp* were unabsorbed at pH 7.0 indicating them to be of the non-adsorbant type.

3.7.7. Efficiency of the enzyme preparation in removing starchy stains

To increase the efficiency of detergents, a combination of proteases, amylases, cellulases and lipases are incorporated in the detergents. Alkaline amylases are especially used as additives in detergent formulations to remove starch based stains. The amylase from the *Streptomyces sp* lost 15% activity as compared to the control when incubated for 15 min at 40^0C to 50^0C with 0.2% Surf. From **Figure– 10** it is seen that the addition of the enzyme as an additive to the detergent Surf improved the washing and efficiency of removal of the starch based stain.

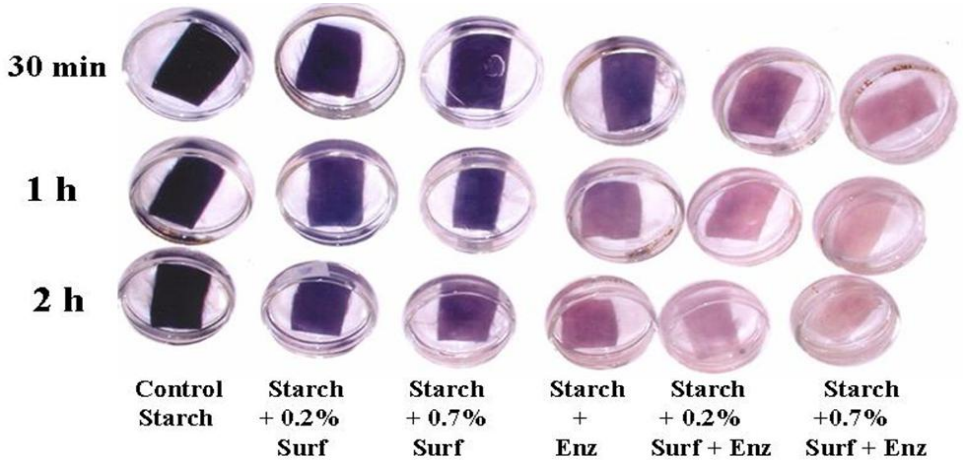

Fig: 10. Evaluation of the washing performance of the alkaline amylase preparation from the *Streptomyces sp* when added as an additive to the detergent Surf.

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[IV] CONCLUSION

The alkaline amylases from the alkalophilic *Streptomyces sp*. is the first report of a maltohexaose (G6) producing alkaline amylase from the genus *Streptomyces*. The distinguishing properties of the amylases of this strain are:

(i) The amylases break down starch to give maltohexaose (G6) as the predominant product. Malto-oligosaccharides with DP in the range of 3-6 are produced by only few amylases and such maltooligosaccharides are increasingly being used in food and pharmaceutical products due to their properties such as low sweetness, high water holding capacity and anti-staling capacity. The amylase activity from the *Streptomyces sp* is comparable with the activity of maltohexaose producing amylases from other organisms [10, 36, 44, 51-54] and hence would have a potential application in the food and pharmaceutical industry for the production of maltohexaose (G6).

(ii) Our results obtained using the amylase preparation as an additive with detergent is promising suggesting that the alkaline amylase preparation from this *Streptomyces sp* could also have an application in the detergent industry.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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SUPPLEMENTARY TABLES (As supplied by authors)

Supplementary Table: 1. Determination of optimum starch concentration on alkaline amylase production

Raw starch (%)	Maximum amylase activity* (IU/ml)/80h
1	4.0 ± 0.52
3	13.2 ± 1.42
4	10.7 ± 0.77
5	9.3 ± 0.49
7	8.7 ± 1.06

Control - yeast extract 0.5 %, peptone 0.5 %, potato raw starch 3 %, $MgS0_4$ – 0.02 %, K_2HPO_4 - 0.1 % , sodium carbonate 1 %. *n = 4
Significance 1% (ANOVA)

Supplementary Table: 2. Effect of various starch sources on alkaline amylase production

Raw starch (3%)	Maximum amylase activity* (IU/ml)/80h
Potato(control)	13.6 ± 0.84
Corn	13.5 ± 1.27
Wheat	12.6 ± 0.14
Tapioca	10.8 ± 0.28
Rice	8.7 ± 0.14
Refined wheat flour	7.5 ± 0.424

Control- yeast extract 0.5 %, peptone 0.5 %, potato raw starch 3 %, $MgS0_4$ – 0.02 %, K_2HPO_4 - 0.1 % , sodium carbonate 1 %
*n= 4, Signficance 1% (ANOVA)

Supplementary Table 3: Effect of combination of different nitrogen sources on alkaline amylase production

Nitrogen sources (%)	Maximum amylase activity * (IU/ml) / 80h
Control	9.9 ± 2.18
Soyabean (1%)	7.3 ± 0.69
Soyabean (2%)	6.5 ± 0.41
YE (0.5%) + Tryptone (0.5%)	5.9 ± 0.29
YE (0.5%) + Soyabean (1%)	5.3 ± 0.52
YE (1%) + Soyabean (1%)	4.3 ± 0.24

Control - yeast extract 0.5 %, peptone 0.5 %, potato raw starch 3 %, $MgS0_4$ – 0.02 %, K_2HPO_4 - 0.1 %, sodium carbonate 1 % *n = 4
Significance 1% (ANOVA)

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Supplementary Table: 4. Effect of metal ions on alkaline amylase production

Metals (%)	Maximum amylase activity*(IU/ml) / 80h
$MgS0_4$+ K_2HPO_4 (Control)	10.9 ± 0.98
$MgS0_4$	3.8 ± 0.29
K_2HPO_4	4.1 ± 0.18
$CaCl_2$	4.0 ± 0.81
K_2HPO_4+ CaCl2	7.4 ± 0.51
$MgS0_4$ + K_2HPO_4 +$CaCl_2$	7.7 ± 0.25

Control- yeast extract 0.5 %, peptone 0.5 %, potato raw starch 3 %, $MgS0_4$ – 0.02 %, K_2HPO_4 - 0.1 % , sodium carbonate 1 %. *n = 4
Significance 1% (ANOVA)

Supplementary Table: 5. Effect of various additives on the activity of alkaline amylases from alkalophilic *Streptomyces sp*.

Effectors	Concentration mM)	Retained activity(%)
Control	0	100 ± 2.9
$CaCl_2$ [a]	1	103 ± 1.8
$CaCl_2$ [a]	5	138 ± 2.3
EDTA [b]	2	48 ± 3.3
EDTA [b]	5	60 ± 1.6
EDTA [b]	10	70 ± 3.3
EDTA [b]	20	80 ± 1.6
SDS [b]	1 %	60 ± 2.9
SDS [b]	2 %	43 ± 2.6
SDS [b]	5 %	17 ± 1.8
Urea [b]	6 M	18 ± 1.8
Dithiothreitol [b]	10	132 ± 1.8
Cystine HCl [b]	10	343 ± 1.1
Surf [b]	0.2 %	48 ± 3.4 (30 min)
Surf [b]	0.2%	38 ± 1.8 (45 min)

[a] -Tris HCl buffer (50 mM), [b] -Glycine NaOH (50 mM) *n= 3 ,Signficance 1% (ANOVA)