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RESEARCH ARTICLE

Purification and characterization of maltose forming thermostable alkaline α-Amylase from *Bacillus gibsonii* S213

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Abstract

Bacillus gibsonii S213 is a Gram +ve, alkalophilic, endospore forming bacterium that is capable of producing maltose and maltotriose forming thermostable α-amylase that has an optimum activity at pH 8.5 and 60°C. The enzyme was purified 21 fold with the yield of 18% using ion exchange and gel filtration chromatography. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified enzyme revealed a single band of molecular weight 48 KDa. End products of hydrolysis as estimated by high pressure liquid chromatography and thin layer chromatography were mainly maltose and less concentration of maltotriose. The enzyme was stable at broad range of pH (7-9.5) and temperature (50-80°C) and was able to completely hydrolyze raw wheat and soluble starch suggesting its application in starch industry.

Introduction:-

Amylum or starch, an important nutritional element is naturally available polysaccharide consisting of glucose units joined by glycosidic bonds and is synthesized chiefly in plants (Wang and Copland, 2015). Many food industries use starch and starch products as raw materials; but before its consumption it has to undergo processing. The hydrolysis of starch for its applications in various industries can be either chemical based or enzyme based, though in today's scenario enzyme based saccharification of starch is preferred (Abbas et al., 2010). Owing to high demands of processed and convenient low calorie food, food companies these days have started replacing high fat food with enzymatically processed low fat modified starches (Miyazaki et al., 2006). According to market researcher, Global Industry Analysts, Inc. it is expected that by 2018, the starch consumption can reach 133.5 million metric tons globally. To reach the high demands of processed starch, development in biotechnological applications and search of novel bacteria producing starch hydrolyzing enzymes have emerged in the last few decades.

Among all the enzymes hydrolyzing starch, α-amylases from microorganisms have attained considerable interests for researchers (Xian et al., 2015). Since microbes can be handled and easily altered to get desirable results, microbial source is more preferred to obtain amylases than plants and animals (Kohli et al., 2014). These α-amylases have been extensively used in industries since time immemorial to hydrolyze starch into smaller polymers of glucose units. Amylases function by cleaving the glycosidic bond of the starch chain which is the principal function of the class hydrolases among the enzymes. Many industrially important enzymes are generally synthesized from *Bacillus* spp. since their growth rate is high and are generally regarded as safe by FDA. They also secrete important proteins extracellularly (Liu and Xu, 2007). Knowing the importance of *Bacillus* spp., they have been manipulated and used in industry for their commercial usage of amylase. Till today, very few bacteria are reported that are able to hydrolyze high levels of raw starch. Therefore, research focused on finding new bacteria that were able to hydrolyze raw wheat and soluble starch, thus simplifying the starch conversion process.

High activity and stability of the enzyme is also a matter of concern for industries. Many physiological (temperature, pH) and nutritional (metal ion requirement, carbon and nitrogen sources etc.) factors play crucial role in the stability

of the enzyme (Gupta et al., 2003). Research has been carried out globally at gene and molecular level to improve the characteristics of these amylases so that they can be exploited for diverse industrial applications.

Materials and methods:-

Materials

Soluble starch, Maltotriose, Sephadex G75 (34 cm x 1.4 cm) and Hi Trap Capto Q (5 x 5 ml) column were purchased from Sigma-Aldrich (St. Louis, United States). Ammonium sulfate was purchased from Merck (Darmstadt, Germany). All the other chemicals used were of analytical grade unless specified.

Bacteria

Bacillus gibsonii S213 was isolated from the sample of alkaline soil collected from 3 to 5 cm depth from the regions of uncultivated areas of south-west Punjab, India. For selective growth of amylase producing bacteria, 1 g of the soil sample was dried and suspended in enrichment medium (2 g of starch as sole source of carbon, 0.5 g of ammonium sulphate, 0.5 g of di-potassium-hydrogen phosphate, 0.1 g magnesium sulphate, and 0.5 g calcium chloride in 100 ml of distilled water) for 24 hours in rotary shaker. Amylase production was confirmed by inoculating serially diluted samples (0.1ml) from the enriched culture on starch agar plate (spread plate method) and incubating at 30° C for 2 days. The plates were flooded with Lugol's iodine solution. The isolates forming clear zones around their margins were confirmed as starch hydrolyzing bacteria. The organism showing maximum zone of hydrolysis (*Bacillus gibsonii* S213) was selected for further study and was propagated at different temperatures ranging from 20-55°C and pH values from 5.0-10.0. Isolated strain was characterized according to Bergy's Manual of Systematic Bacteriology (Sneath, 1986). Identification of the isolate was done by 16S rDNA gene sequence analysis. The isolated organism was routinely revived after period of 15 days on starch agar slants. The culture was also stored at 4°C and also preserved in glycerol stock at -20°C.

16S rRNA gene sequencing

Genomic DNA of the selected bacteria was isolated and 16S rDNA sequence was amplified using 27F (5'- AGA GTT TGA TCC TGG CTC AG- 3') and 1492R (5'- ACG GCT ACC TTG TTA CGA CTT- 3') universal primers. The gel slice containing relevant DNA fragments was excised and sequenced. Sequences were aligned using CodonCode aligner and the gaps were removed from analyses. Neighbor-joining method was used for the construction of phylogenetic tree using boot-strap value of Mega 6 (http://www.megasoftware.net/). Gene sequence was submitted to GenBank (NCBI), and the accession number was obtained.

Amylase production

The organism was revived every time prior to use in nutrient broth and was then proliferated at its optimum temperature of enzyme synthesis (40°C) for 24 hours in minimal medium (1% starch, 1% peptone, 0.02% magnesium sulphate, 0.3% potassium dihydrogen phosphate in Erlenmeyer flasks, at 180 rpm on a rotary shaker. Crude enzyme was obtained at 4°C by centrifugation (12 000 x g, 15 min) and was used for purification.

Purification of amylase

The complete process was carried out at 4°C and 50 mM phosphate buffer (pH 8) was used unless specified. Crude enzyme extract was partially purified by adding ammonium sulfate to 75% saturation. The pellet was then recovered after 24 hours by centrifugation (12 000 x g, 10 min). The precipitates were dissolved in minimum volume of buffer and were dialyzed against the same buffer overnight. The dialyzed enzyme was then loaded to HiTrap Capto Q column (5 x 5 ml) previously equilibrated with buffer for ion exchange chromatography using Akta prime plus system (GE Healthcare Life Sciences). Elution was done using the same buffer containing 1 M NaCl at a flow rate of 1 ml min^{-1}. The fractions were pooled and loaded to Sephadex G-75 column (34 cm x 1.4 cm) for gel filtration chromatography. Fractions obtained were checked for amylase activity using qualitative assay. The active fractions were pooled and lyophilized for further investigations.

SDS-PAGE and Zymogram analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with BioRad- Mini PROTEAN tetra system in 12% gel and was stained with commassie brilliant blue G-25. For the analysis of enzyme activity, zymogram was performed using same conditions of PAGE. Starch zymography was done by incubating the gel containing enzyme bands with 1% starch solution for half an hour in an incubator. The enzyme bands will hydrolyze starch molecules which will form clear bands against the dark background formed when stained with Lugol's iodine solution [3% (w/v) KI and 1% (w/v) I_2].

Amylase assay
Estimation of sugars released by the action of amylase on starch was done by the method of Bernfeld (Bernfeld, 1955). Protein concentration was estimated using the method of Bradford (Bradford, 1976).

Characterization of purified enzyme
Characterization was done by studying optimal temperature and pH for activity and stability of the purified enzyme. DNS enzyme assay using spectrophotometer was done for each experiment. The temperature and pH optima were studied by incubating the reaction mixture at different temperatures ranging from 30- 80°C and pH values ranging from 5.0-10.0 in 50 mM phosphate buffer for 15 min. Temperature stability and pH stability were examined by incubating the enzyme alone for 60 min at differ temperatures (30- 80°C) and pH (5-10), and the residual activity using DNS was calculated after every 15 min.

End products of starch hydrolysis
Amylase (5 mg lyophilized culture) and starch (0.5% w/v) digests were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) to check the end products formed. Glucose, maltose and maltotriose were the standards used. 10 µl of sample and standards (10 mg/ml) were put on TLC Silica gel 60 F_{254} plates (Merck, Germany). Solvent phase used was the mixed solution of butanol : ethanol : water (5:3:2). Aniline-diphenylamine was sprayed for the visualization of sugar spots. For HPLC (Waters 515 Pump, 2414 Refractive index detector), carbohydrate column (3.9 x 300 mm, Waters) was used. The mobile phase used was acetonitrile-water (80:20 v/v) and the flow rate set was 1 ml min^{-1}. Empower 2154 software was used for analysis.

Application of purified enzyme in the hydrolysis of raw wheat flour and soluble starch
To study the application of purified enzyme produced by *Bacillus gibsonii* S213, industrial conditions were followed. 30% each of raw wheat flour and soluble starch were incubated for 1h and 2h with 5 mg of purified lyophilized enzyme. After treatment with enzyme for a particular time period, the wheat and starch granules were recovered and analyzed with scanning electron microscope (for studying the degree of hydrolysis). Raw wheat flour and soluble starch that were not treated with enzyme were taken as control. For SEM studies, both the enzyme treated and untreated samples were washed initially with 50 mM phosphate buffer (pH 8) and the pellet recovered after centrifugation (10, 000 x g, 10 mins) was dehydrated with ascending grades of acetone followed by drying. Samples were then put on carbon tape already fixed on SEM metal stub. Gold coating of the samples were done by JEOL ion sputter, JFC 1600 auto fine coater. Scanning electron microscope used was JEOL; JSM-6610 LV. Operating voltage used was 5kv.

Results:-
Microscopic, biochemical and molecular analysis revealed that the bacterium isolated showed maximum homology with *Bacillus gibsonii* and submitted to GenBank as *Bacillus gibsonii* S213 (accession number KT161959). The rod shaped Gram + ve, endospore forming bacterium showed positive results for protease, lipase and catalase activity tests. Exponential phase marked the initiation of enzyme, while the maximum production (94.56 U) was observed at stationary phase after which there was decline in the production of enzyme, thus signifying the substrate dependent production of the enzyme. The organism grew well at temperatures between 25 and 50°C with optimum growth at 40°C. It was observed that the maximum production of amylase was also observed at 40°C. The optimum pH for the growth of the organism and amylase synthesis was found to be 8. It grew optimally at an alkaline pH and a marked decline in the synthesis was observed on acidic side of pH. HPLC and TLC studies predicted that the enzyme produced was α-amylase.

Purification and molecular weight analysis of amylase
After ammonium sulphate precipitation and dialysis of the crude culture, ion exchange chromatography on Hi-Trap Capto Q was done followed by gel-filtration chromatography on Sephadex G-75. Ammonium sulphate precipitation and dialysis resulted in remarkable increase in specific activity of enzyme from 17.5 U/mg to 81.40 U/mg with no α-amylase activity in supernatant. All the peaks monitored at 280 nm during purification process were tested for enzyme activity. The marked peak in the fig. 1(a) showed amylase activity when assayed while other peaks showed negative results. The active fraction was then loaded on Sephadex G-75 column. The final pooled product [active peak as shown in fig. 1(b)] was loaded on SDS-PAGE that appeared as a single band. The final enzyme yield was

18% with 21 fold purification (Table 1). It was found that the specific activity improved from 17.5 U/mg in crude extract to 355.71 U/mg in the purified product.
The apparent molecular weight of the purified enzyme was 48 KDa as estimated by SDS-PAGE (Fig. 2). The final purified enzyme was traced for amylase activity by running a zymogram. Clear zone on gel when kept in lugol's iodine solution confirms the presence of amylase.

Effect of pH and temperature on enzyme activity and stability
It was investigated that the enzyme showed best activity from neutral to moderate alkaline pH (7-9.5) The purified enzyme was optimally active at pH 8.5 (329.24 U/mg of protein) though 85% of its activity is retained at pH 8-9.5 (Fig. 3a). At pH 5.0 only 13.26% of the activity was possessed after 1 h confirming the decline of activity in acidic range. After 1 h, the enzyme retained around 90% of its activity at pH 8.5 and 50.2% at pH 10 (Fig. 3b).
The optimum temperature and stability profile of the purified enzyme is shown in Fig. 4 (a) and (b) respectively. The enzyme had shown its optimal activity at 60°C which was found to be different from the crude enzyme. The activity of enzyme started declining as the temperature was raised above 60°C though it retained 75% of its initial activity at 80°C after 1 hour of pre-incubation. Approximately 61, 78, 95 and 85% of the activity was retained at 40, 50, 60 and 70°C after 1 hour.

End products of starch hydrolysis
The end products of hydrolysis produced by *Bacillus gibsonii* S213 were analyzed by thin layer chromatography, followed by high pressure liquid chromatography that confirmed maltose (5.429 mg/ml) as the major and maltotriose (1.872 mg/ml) as minor end products of starch hydrolysis confirming that the enzyme is α-amylase.

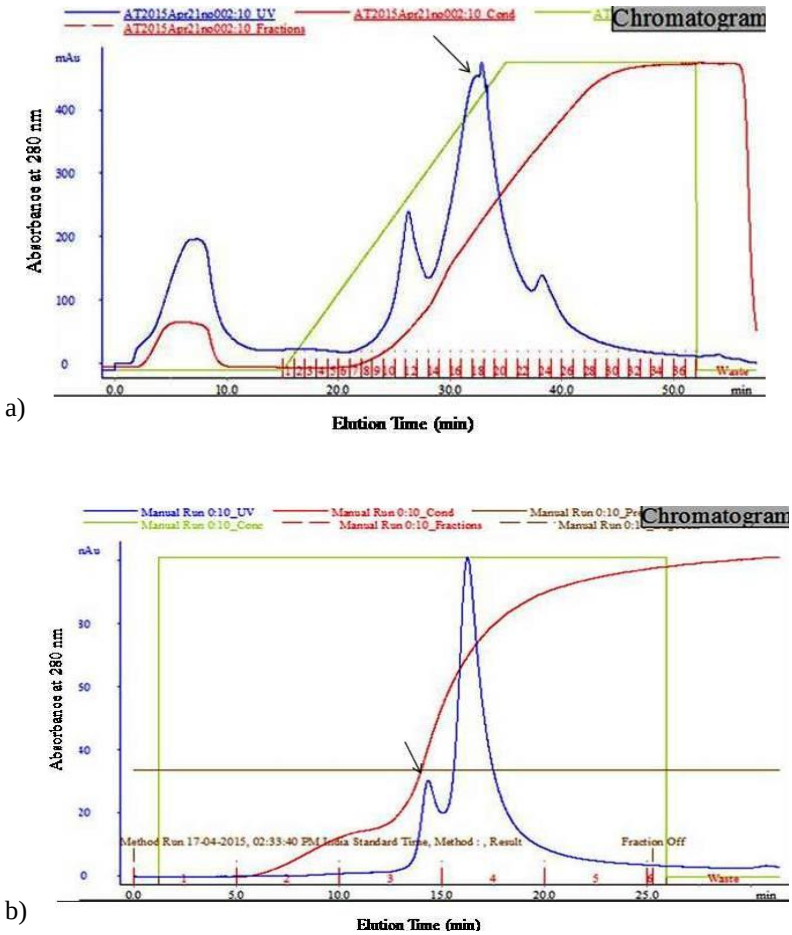
Application of purified enzyme in the hydrolysis of raw wheat flour and soluble starch:
Enzymes capable of hydrolyzing wheat and starch granules form an important component of starch saccharification since their granules are fundamental starch sources worldwide. The application of hydrolysis by the enzyme purified in the study was investigated by estimating the extent of starch and wheat hydrolysis. Scanning Electron Microscopy studies revealed that the raw granules of wheat and starch were almost completely damaged after hydrolysis with the amylase obtained from *Bacillus gibsonii* S213 [Fig. 6(a) and 6(b)]. It was also found that the degree of hydrolysis in soluble starch was more than the wheat starch granules. Another investigation was that with the increase in incubation time from 30 mins to 1 h, the degree of hydrolysis increased and the granules were almost completely hydrolyzed suggesting that the enzyme could be used as a good source for starch sachharification in industries

Discussion:-
The thermostable and well characterized α-amylase (EC 3.2.2.1) is an important starch processing protein enzyme used heavily in industries. *Bacillus gibsonii* sp. S213 analyzed in the present study produced alkaline thermostable enzyme that was purified to 21 fold, using several steps. The optimum temperature for growth and amylase production were found to be similar. Santos and Martins (2003) and Teodoro and Martins (2000) also found that the production of enzyme is related to the growth of the organism. In *Bacillus subtilis* sp. JS-2004, parallel production of amylase and organism growth was reported (Asgher et al., 2006). Saxena et al. (2007) and McMahon et al. (1999) observed that the production of enzyme and optimum growth of organism are not similar in case of *Bacillus* sp. PN5 and *Streptomyces* sp. respectively.
The *Bacillus* strains that are commercially used for the production of amylase have an optimum pH that lie in between 6-9 for the growth and production of enzyme. Alkaline *Bacillus* sp. as reported by Saxena et al. (2007) and Shin et al. (1991) grew well at pH 10. In the present observation, optimum pH for enzyme production was 8.5.
Series of steps (ion exchange chromatography, gel filtration chromatography) were used in this study to purify the enzyme to homogeneity (21 fold purification, 18% yield). SDS predicted the molecular weight of the enzyme purified to be 48KDa. Sodhi et al., (2005) purified the amylase with 12.7 fold purification using gel filtration and hydrophobic interaction chromatography from *Bacillus* sp. PS-7. The authors found 2 bands (55KDa and 71 KDa) when protein enzyme was run on SDS-PAGE. The author explained that one of the bands must be an impurity while other corresponds to the enzyme. Goyal et al. (2005) used starch adsorption-desorption process for purifying amylase from *Bacillus* sp. I-3 rather than using routine processes of ion exchange, gel filtration or hydrophobic interaction chromatography. Many other researchers have reported different molecular weights of the amylase enzyme isolated from different sp. of *Bacillus* (Table 2). Burhan et al. (2003) estimated the molecular weight of amylase from *Bacillus* ANT-6 to be 94KDa as analyzed with SDS-PAGE.
In the present study, it was observed that the temperature and pH optima for the best activity of the purified enzyme were 60°C and 8.5 respectively though in many other species of *Bacillus* secreting alkaline amylase, thermostability

criteria had always been an issue (Horikoshi, 1971; Lo et al., 2001). *Bacillus* sp. GM8901 as reported by Kim et al. (1995) showed optimal activity at pH range of 11.0-12.0, while the enzyme was stable at pH 6.0-13.0. Bekler et al. (2014) investigated alkaline amylase from *Anoxybacillus* sp. KP1 that had an optimum activity at pH 8 and 60°C while the enzyme was stable at maximum temperature range of 80°C. pH and temperature optima of alkaline amylase purified from other *Bacillus* sp. have been given in Table 2.

Purified enzyme from *Bacillus gibsonii* sp. S213 produced maltose as the major end product, as predicted by TLC and HPLC. Goyal et al. (2005) investigated using paper chromatography, that glucose, maltose and maltotriose were the end products of raw potato starch hydrolysis in *Bacillus* sp. I-3. McMahon et al. (1999) investigated that species of *Streptomyces* produced 79% of maltose as an end product. *Bacillus* sp. IMD 434 on hydrolysis of starch produced maltose as a major end product and maltotriose as a minor end product that subsequently decreased forming glucose and increased quantities of maltose (Hamilton et al., 1999). It was also found in the present study that the enzyme was able to hydrolyze raw wheat and soluble starch completely indicating its possible role in starch industries. Mehta and Satyanarayana (2002) found that there was less degree of hydrolysis in corn starch in comparison to wheat granules when treated with amylase produced by *Geobacillus thermoleovorans*. Ring et al. (1988) reported that, with the increase in granule size, the degree of hydrolysis decreased in the same incubation period. He reported the order of hydrolysis in wheat, corn, pea and potato where wheat is hydrolyzed maximum than corn, pea and potato.

Fig 1:

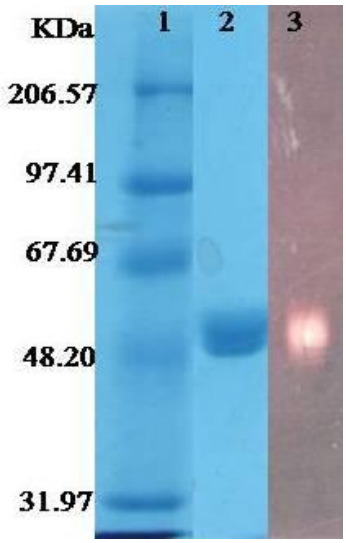


a)

b)

Elution profile of *Bacillus gibsonii* S213 α-amylase on (a) Hi-Trap Capto Q and (b) Sephadex G-75. Active peaks having enzyme activity are indicated

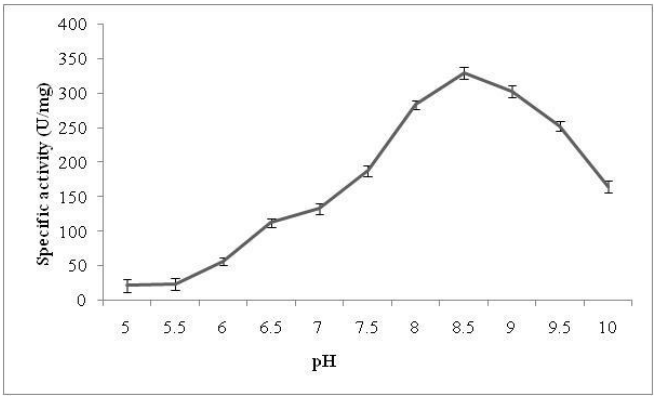
Fig 2:



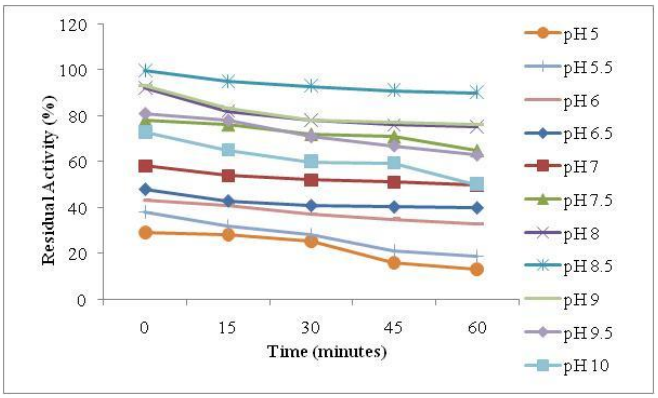
Estimation of molecular weight of amylase produces by *Bacillus gibsonii* S213 by 12% homogenized SDS-PAGE. Lane (1) Molecular weight standard (GE Biosciences, St. Louis, USA, 31.97, 48.20, 67.69, 97.41, 206.57 KDa), (2) Active fraction eluted from Akta Prime (3) zymogram analysis to confirm the presence of amylase on gel.

Fig 3:

a)



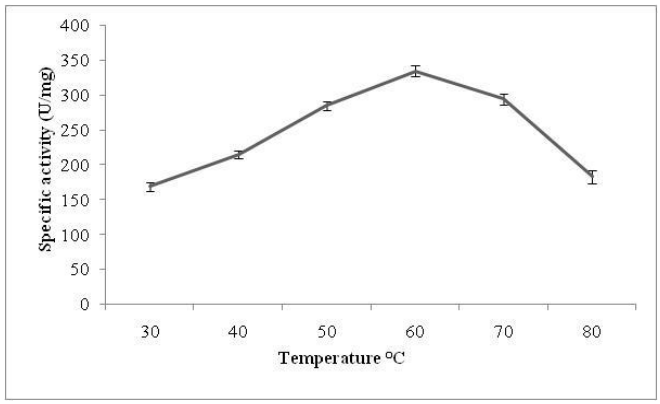
b)



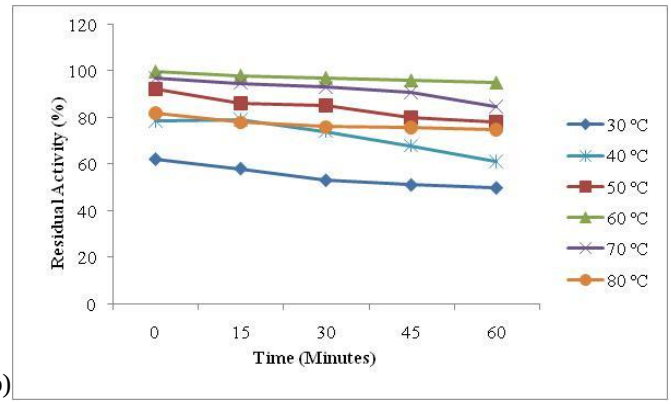
Effect of pH on the activity (a) and stability (b) of *Bacillus gibsonii* S213 amylase

Fig 4:

a)



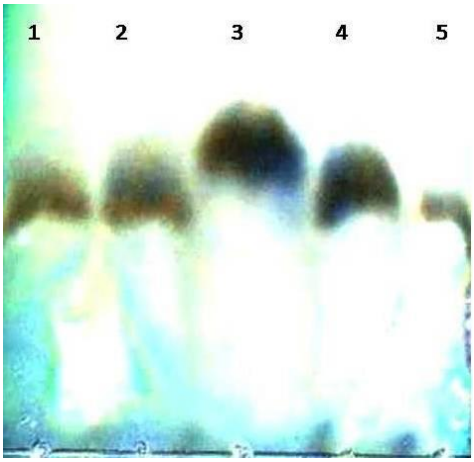
b)



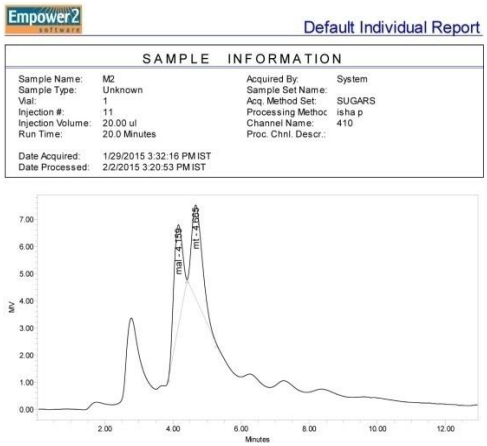
Effect of temperature on the activity (a) and stability (b) of *Bacillus gibsonii* S213 amylase

Fig 5:

a)



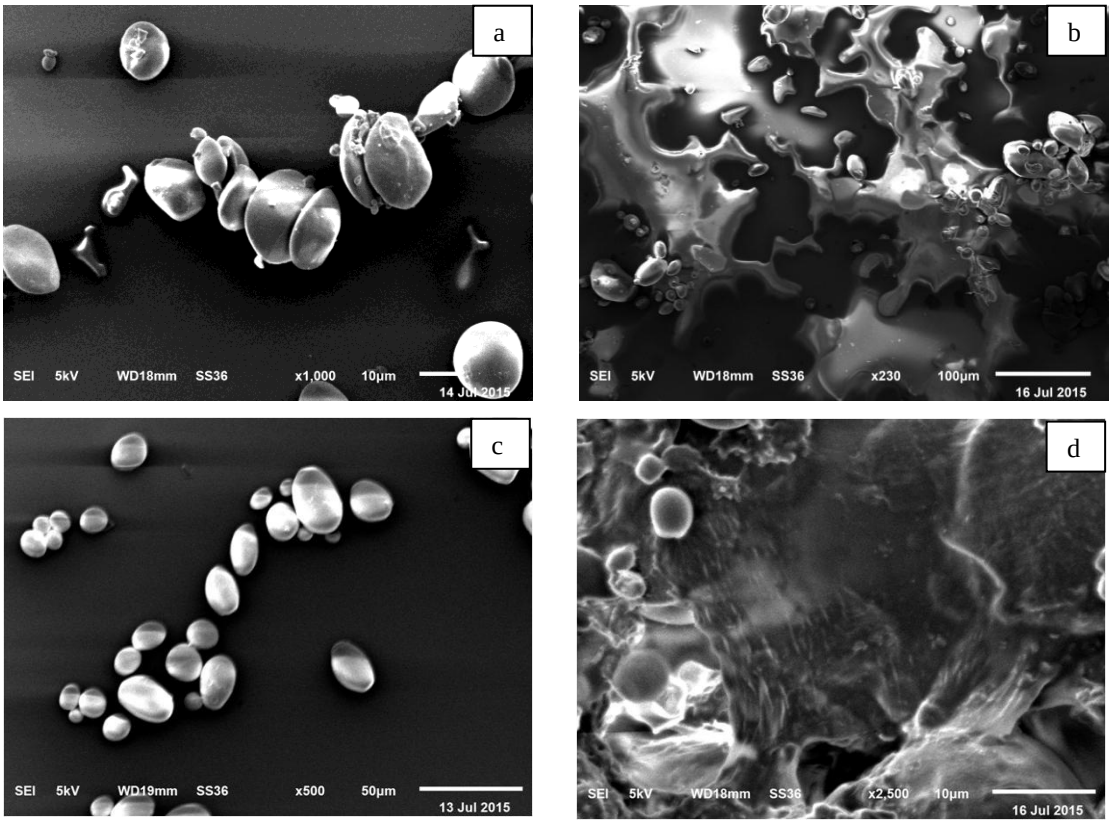
b)



	Peak Name	RT	Area	% Area	Height	Amount	Units
1	glu	3.783					
2	mal	4.159	65345	46.83	3737	5.429	mg/ml
3	mt	4.665	74206	53.17	3435	1.872	mg/ml

End products formed by starch hydrolysis after 1h reaction time as estimated by TLC and HPLC analysis (a) TLC: 1 (5 mg of enzyme reacted with 1% soluble starch), 2 (10 mg of enzyme reacted with 1% soluble starch), 3, 4 and 5 represents 10 mg each of glucose, maltose and maltotriose, respectively: (b) HPLC: The retention time of individual standards (glucose, maltose and maltotriose) were identified and end products of hydrolysis were analyzed

Fig 6:



Scanning electron micrograph showing a) intact structure of wheat; b) deformed structure of wheat after enzyme treatment; c) intact structure of soluble starch; d) deformed structure of soluble structure after enzyme treatment

Table 1:
Purification steps of amylase obtained from *Bacillus gibsonii* S213

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Fold
Crude enzyme	6932	394.12	17.5	100	1
Ammonium sulphate precipitation and dialysis	6024	74.02	81.40	86.9	4.65
Hi-Trap CaptoQ	3246	11.10	292.43	46.86	16.7
Sephadex G-75	1245	3.5	355.71	17.9	20.32

Table 2:
Comparative study of α-amylase characteristics from our study with other *Bacillus* species secreting alkaline amylase

Organism	Mol. weight (kDa)	Optimum Temperature (°C)	Temperature stability (°C)	Optimum pH	pH stability	References
Bacillus sp. ANT-6	94.5	37	80	9	9.5-13	Burhan et al., (2002)
Bacillus sp. PN5	-	60	80-90	7	10	Saxena et al., (2007)
Bacillus sp. CU-48	45	60	-	8	-	Khodayari et al., (2014)
Bacillus subtilis ATCC 6633	66	40	40-60	7	6-11	Maity et al., (2015)
Bacillus gibsonii S213	48	60	50-80	8.5	7.5-9.5	Present study

Conclusion:-

Stability at high temperature (greater than 50°C) and broad pH range on alkaline side mark the possible application of amylase obtained from *Bacillus gibsonii* in detergent and starch sachharification industry. The amylase obtained was able to hydrolyze raw wheat and starch; thus it can be used in bulk starch and wheat processing at industrial scale. Scale up of production of the enzyme obtained in the present study needs to be done for its diverse industrial applications.

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