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Amylase activity of a starch degrading bacteria isolated from soil

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ABSTRACT

The enzymes from microbial sources are more stable and obtained cheaply. Amylases are among the most important enzymes and are of great significance in present day industry. Starch degrading bacteria are most important for industries such as food, fermentation, textile and paper. Thus isolating and manipulating pure culture from various soil and waste materials has manifold importance for various biotechnology industries. In the present investigation bacterial strains were isolated from soil sample and growth pattern as well as optimum growth condition was determined. Characteristic feature of the strains indicates them as Bacillus subtilis, Pseudomonas florescens, Escherichia coli and Serratia marscens. The optimum temperature for production was 35-40 ^{0}C, whereas maximum growth was observed at 1% dextrose concentration but increases with increase in substrate concentration. The pH range was found to be 7 and incubation time 48hrs with 1ml as inoculum for optimum growth. Other optimum parameters include Yeast extract as Nitrogen source, Calcium chloride as chloride and Manganese sulphate as sulphate source for amylase production.

Key words: Amylase, *Bacillus*, *Pseudomonas*, *Escherichia*, *Serratia*, Starch degrading.

INTRODUCTION

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper [1, 2]. Amylases are obtained from various origins like plant, animal, bacterial and fungal. Several researchers produces amylase enzyme using *Bacillus* sp. [3, 4, 5, 6].

There are about 3000 enzymes known today only few are industrially exploited. These are mainly extracellular hydrolytic enzymes, which degrade naturally occurring polymers such as starch, proteins, pectin's and cellulose [7]. In the production of glucose syrup the α-amylase is used in the first step of enzymatic degradation yielding a mixture of glucose and fructose with high fructose content. The amylases can be derived from several sources such as plants, animals and microbes. The microbial amylases meet industrial demands because it is economical when produced in large quantities [8]. Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors.

The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. Microbial amylase has almost surpassed the synthetic sources in different industries [1]. Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized in to exo-acting, endo-acting and debranching enzymes. Unusual bacterial amylases are found in acidophilic, alkalophilic and thermo acidophilic bacteria [9].

Nowadays amylase from these sources is vastly used in amylase production under extreme conditions of pH and temperature.

There are various reports on starch degrading micro-organisms from different sources and respective amylase activity [10, 11 and 12]. Soil receiving the kitchen wastes is one of the rich sources of starch degrading microorganism as it contains mostly starchy substrate. The two bacterial strains *Bacillus amyloliquefaciens* and *Bacillus licheniformis* has been exploited on the industrial scale. The present study describes the identification and isolation of amylase producing bacteria from soil. We also studied the influence of different production parameters such as pH, temperature, carbon source and incubation period required for the maximum production of amylase in liquid culture fermentation process.

In the present study, we report the isolation and optimization of novel amylase producing bacteria from the soil samples collected from different field conditions of Jaipur, India. Production conditions were optimized (temperature, pH, metal ions etc.) to achieve high enzyme production and better enzyme activity.

MATERIALS AND METHODS

Isolation of Amylase Producing Microorganisms:
Soil samples were collected from different environment sources. Serial dilution was made and was plated on nutrient agar by spreading 0.1ml of the diluted sample. Then the plates were kept for incubation at 37°C for overnight.

Screening for Amylase Activity (Starch Iodine Test)
Isolated colonies were picked up from each plate containing pure culture and streaked in straight lines in starch agar plates with starch as the only carbon source. After incubation at 37ºC for 24-48 hrs., individual plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5gm potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue colour forms, which is the basis of the detection and screening of an amylolytic strain. The colonies which were showing zone of clearance in starch agar plates were maintained on to nutrient agar slants.

Morphological and Biochemical Characteristics:
Gram staining, motility, indole production, methyl red, Vogues Proskauer's, citrate utilization, triple sugar iron, nitrate reduction, catalase, oxidase, gelatin liquefaction, urease, hydrolysis of casein, hydrolysis of starch were carried out.

Enzyme production medium
Production medium contained (g/l) Trypticase 10gm, peptone 5gm, $(NH_4)_2$ SO_4 3gm, K_2HPO_4 2gm, L-Cysteine HCl 0.5gm, MgSO4 0.2gm .10 ml of medium was taken in a 100 ml conical flask. The flasks were sterilized in autoclave at 121 ^oC for 15 min and after cooling the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37 ^oC in shaker incubator for 24 hr. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Amylase Assay
The enzyme activity was assayed following the method of Bernfeld (1955) [13] using 3, 5- dinitrosalicylic acid.

Process Optimization for Amylase Production
Carbon Source: The effect of various carbon sources such as Glucose, Maltose, Lactose, Fructose and Sucrose at the concentration of 1 to 5% was examined in the production medium. [14].

Organic and Inorganic Nitrogen Sources: The amylase production by the selected bacterium was also optimized by supplementing different inorganic and inorganic nitrogen sources individually at the concentration of 0.5% such as potassium nitrate, ammonium sulphate, sodium nitrate, ammonium nitrate, ammonium chloride, casein, malt extract, peptone, urea, gelatin and yeast extract. The enzyme assay was carried out after 24 hours of incubation [15].

pH: The effect of pH for amylase production was determined by culturing the bacterium in the production media with different pH. The experiment was carried out individually at various pH 5, 6, 7, 8 and 9. The enzyme assay was carried out after 24 hours of incubation [16]

Temperature: Temperature is an important role for the production of amylase. The effect of temperature on amylase production was studied by the incubating the culture media at various temperatures 35, 40, 45, 50, 55 and 60°C along with arbitrary control at 37°C. The enzyme assay was carried out after 24 hours of incubation [17].

Incubation Period: The amylase production by the selected experimental microorganisms was determined by optimizing the media by adding different bacteria in the production media. The experiment was carried out individually at various incubation periods such as 24, 48, 72 and 96h. The enzyme assay was carried out after 24 hours of incubation [18].

Agrobased Waste Material: To find out the suitability of Agro based waste as substrate for enzyme production, different substrates i.e. Groundnut cake, Coconut cake, Soy cake, Wheat bran were taken in the growth medium under submerged condition. The enzyme activity is measured after 24h of incubation.

Inoculum Concentration: The amylase production by the selected experimental microorganisms was determined by adding bacterium at different inoculum concentration such as 100 µl, 200 µl, 400 µl, 600 µl, 800 µland 1ml to test its ability to induce amylase production in the production medium. The enzyme activity is measured after 24h of incubation.

Ammonium salts: The amylase production by the selected experimental microorganisms was determined by adding bacterium at different salts such as Ammonium chloride, ammonium sulphate and sodium nitrate to test its ability to induce amylase production in the production medium. The enzyme activity is measured after 24h of incubation.

EDTA Concentration- Influence of various concentration of EDTA on amylase production was determined by incubating the medium with different concentration from 0.05% to 0.5%. The enzyme activity is measured after 24h of incubation.

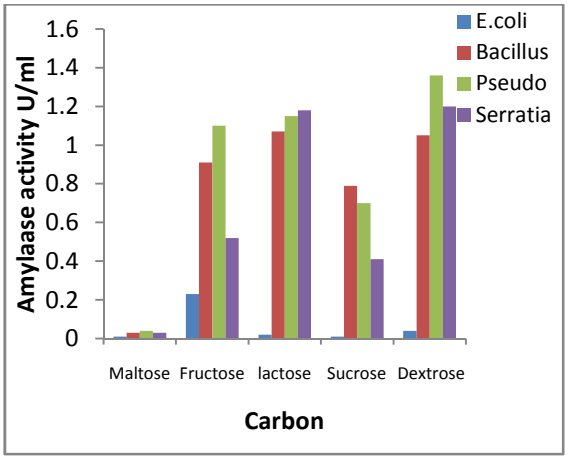
Chlorides: The amylase production by the selected experimental microorganisms was determined by adding bacterium at different chlorides such as Potassium chloride, Sodium chloride and Magnesium chloride, Barium chloride, Ammonium chloride, ferric chloride and Calcium chloride to test its ability to induce amylase production in the production medium. The enzyme activity is measured after 24h of incubation.

Sulphates: The amylase production by the selected experimental microorganisms was determined by adding bacterium at different Sulphates such as Ammonium sulphate, ferrous sulphate Magnesium sulphate, Manganese sulphate, Potassium sulphate and Sodium sulphate to test its ability to induce amylase production in the production medium. The enzyme activity is measured after 24h of incubation.

RESULTS AND DISCUSSION

Screening of Amylase Producing Bacteria: The bacteria isolated from soil were screened for amylase production on starch agar medium. From the soil samples 7 bacterial strains were isolated. But later during screening it was found that only 4 strains showed amylase activity. The four potential isolates were identified by standard morphological and biochemical characterization as *Pseudomonas fluorescens, Bacillus subtilus, E.coli and Serratia marscens.*

Carbon source: Various sources of Carbon such as Starch, Fructose, Maltose and Sucrose were used to replace Glucose which was the original carbon source in growth media. Results obtained showed that, Glucose brought the highest amylase production compared to other carbon sources at 24 hr incubation in *Pseudomonas sp* (Fig 1). Rao and Sathyanarayana. [19] reported that the different carbon sources have varied influence on the extracellular enzymes especially amylase strains. Results obtained showed that increase in concentration of various substrates increases the amylase production more in Glucose (Fig 2, 3, 4, 5 and 6)



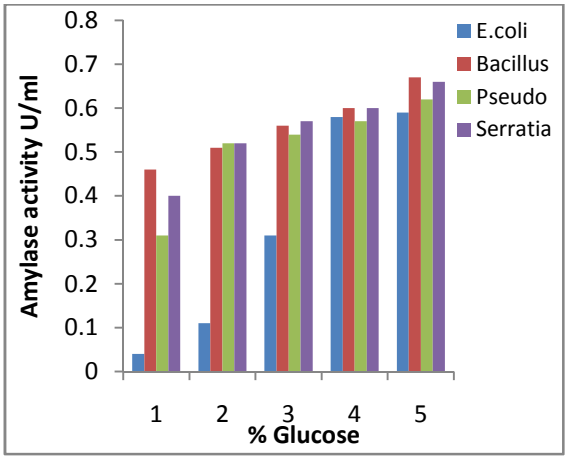
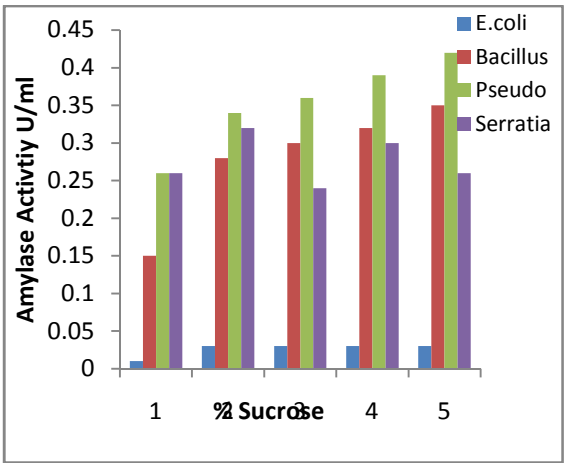


Fig1: Effect of Different Carbon sources **Fig2: Effect of % C (Glucose) sources**



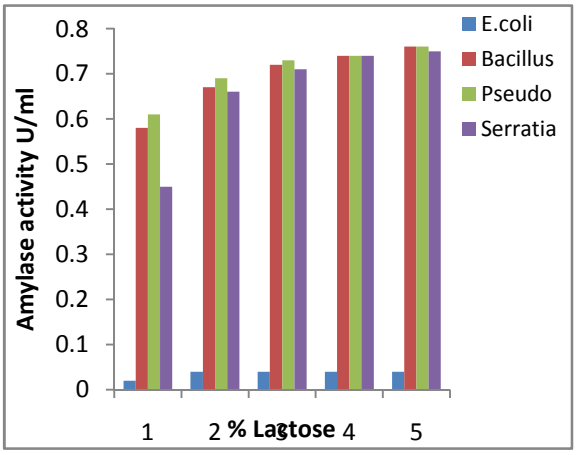
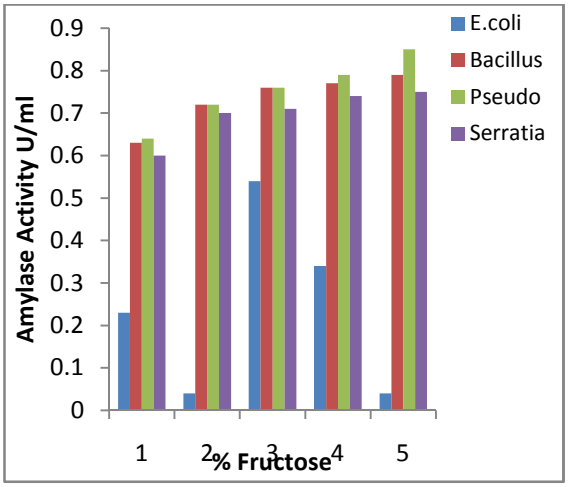


Fig3: Effect of % C (Sucrose) sources **Fig4: Effect of % C (Lactose) sources**



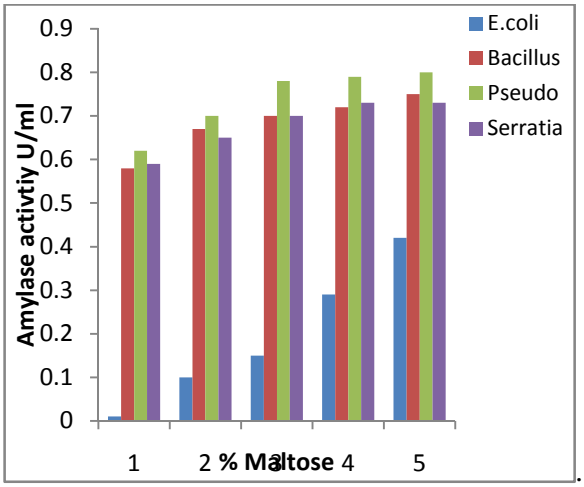


Fig5: Effect of % C (Fructose) sources **Fig 6: Effect of % C (Maltose) sources**

Organic and Inorganic Nitrogen Sources: Various sources of Nitrogen such as Urea, Ammonium sulphate, Peptone and Yeast Extract were used as nitrogen source in growth media. Results obtained showed that, Yeast Extract brought the highest amylase production compared to other nitrogen sources at 24 hr incubation in all bacterial strains except E.coli (Fig 7). Similar results were reported for *B. Thermooleovorans* [20].

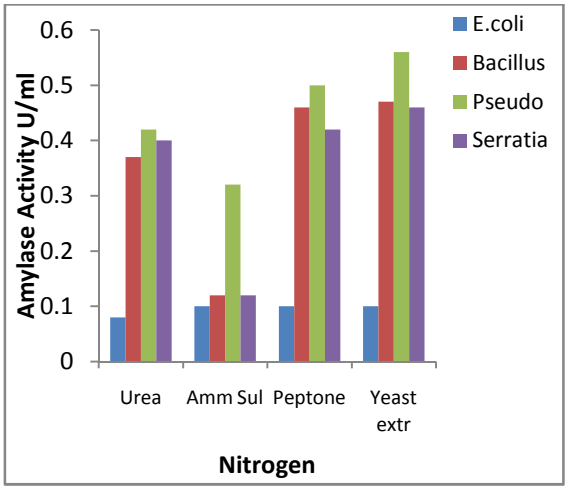


Fig7: Effect of Nitrogen sources

Effect of pH: All the four isolates were allowed to grow in media of different pH ranging from 6.0 to 11.0. Maximum enzyme activity was observed in medium of pH 7.0 in case of *E.coli*, *Pseudomonas fluorescens*, *Bacillus subtillis* and *Serratia marscens* (Fig 8). However majority of microorganisms producing alkaline amylases show growth and enzyme production under alkaline condition. Behal *et al* [21] studied thermostable amylase producing *Bacillus* sp that revealed an optimum enzyme activity at pH 8.0 whereas in other species the optimum activity was at pH 7.0 [22].

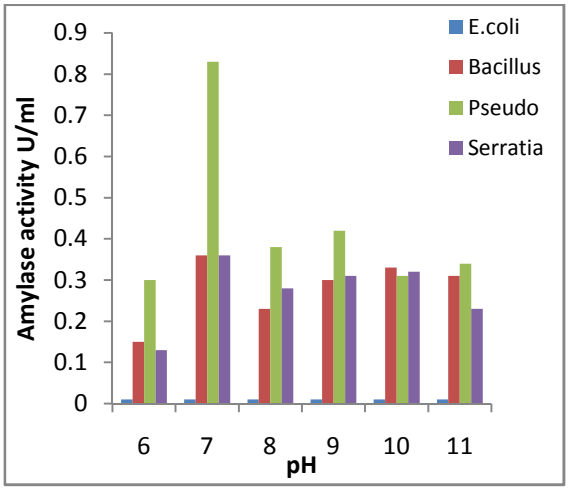


Fig8: Effect of pH

Temperature: Enzyme activity recorded at different temperatures revealed that the all the four bacteria yielded maximum amylase production at 35 - 40^{o}C (Fig 9). Mohamed *et al*, [23] reported that the thermal stability of some wheat α -amylases were stable up to 50°C and some at 40°C after incubation for 15 min whereas in *P. erosus* tubers α -amylase was stable at temperature up to 40°C for 30 min incubation followed by rapid inactivation above 40°C [24].

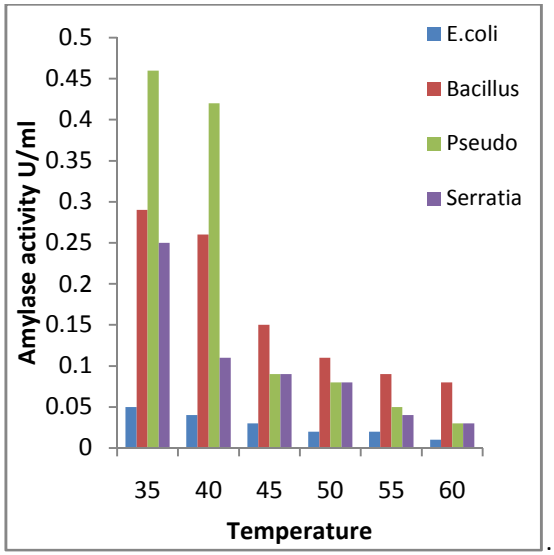


Fig 9: Effect of Temperature

Incubation period: Effect of incubation period on amylase production showed that 48 hours was the optimum duration for maximum amylase enzyme activity (Fig 10). Above this period the amylase enzyme activity started to decrease. This is because, the cells may reach the decline phase and displayed low amylase synthesis [25]. *Bacillus* sp. shows that the amylase production was detected from 48-72 hours and reached maximum activity at 48 hours by Prabakaran and Hewitt [26].

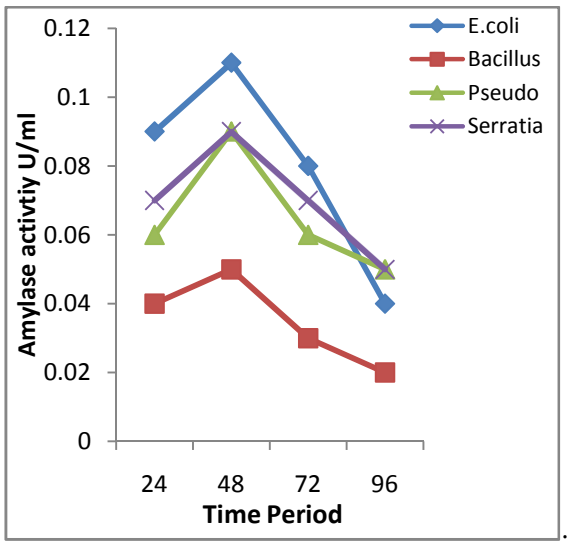


Fig10: Effect of IncubationTime period

Agrobased waste: Commercially most of the production of α-amylases is carried out in submerged fermentation, but solid-state fermentation is being looked at as a potential tool for its production, especially applying agro industrial residues as substrate. In present study, we observed that addition of Wheat Bran as substrate showed the maximum production (Fig 11). Similar results were reported by Swain *et al*, [27].

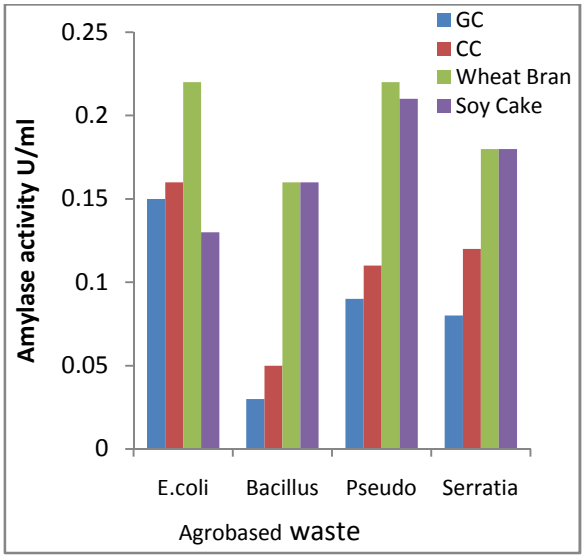


Fig11: Effect of Agro based waste material

Inoculum concentration: The size of inoculum plays an important role in the fermentation of enzymes [28]. Optimum inoculum size of 1000 µl gave highest yield of enzyme production respectively (Fig 12). The production of enzyme was increased with increase in the size of inoculum and found to be optimal at 8% inoculum level. As, the inoculum level was further increased, the production of the enzyme was gradually inhibited. It might be due to the fact that inoculum level at high concentration, the bacteria grows rapidly and the nutrients essential for the growth of bacteria were consumed at the initial stages that resulted in the accumulation of other by products in the fermentation medium. Thus, the production of alpha amylase was affected at higher concentration of inoculum. The insignificance of the result at low level of the inoculum might be due to the fact that the organism grew slowly and the time period for the bacteria to reach in the stationary phase was increased [29].

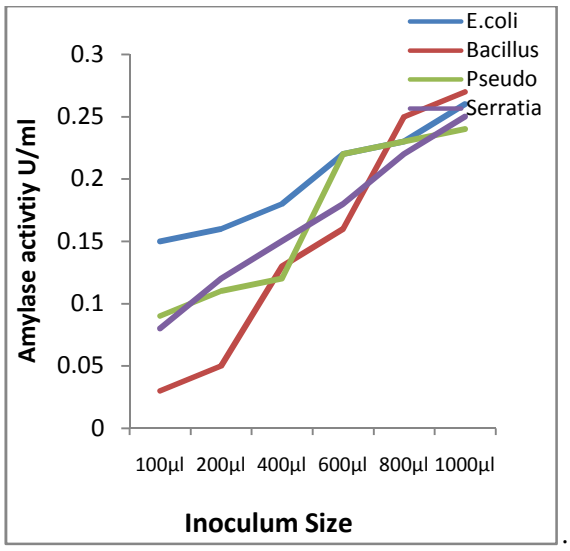


Fig12: Effect of Inoculum Size

Ammonium salts: In the present study, ammonium sulphate was found to be the most suitable inorganic nitrogen source for *E.coli and Pseudomonas florescens* and in sodium nitrate supplied medium in case of *Bacillus subtilis and Serratia marscens* (Fig 13). Ramachandran [30] reported that ammonium salts enhanced the enzyme activity. Sodium nitrate showed a negative influence, showing a steep decrease in "-amylase activity. Pederson and Nielson [31] also reported that nitrate was inferior to ammonia in and ammonium nitrate (inorganic nitrogen sources) inhibited the enzyme production by *P. chrysogenum* under SSF.

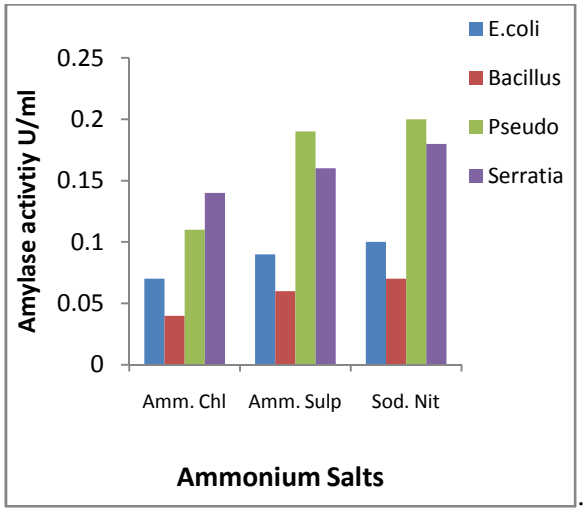


Fig13: Effect of Ammonium Salts

EDTA Concentration- Influence of various concentration of EDTA on amylase production was determined by incubating the medium with different concentration from 0.05% to 0.5% (Fig 14). Surfactants in the fermentation medium are known to increase the secretion of proteins by increasing cell membrane permeability. Therefore, addition of these surfactants is used for the production of extracellular enzymes [32].

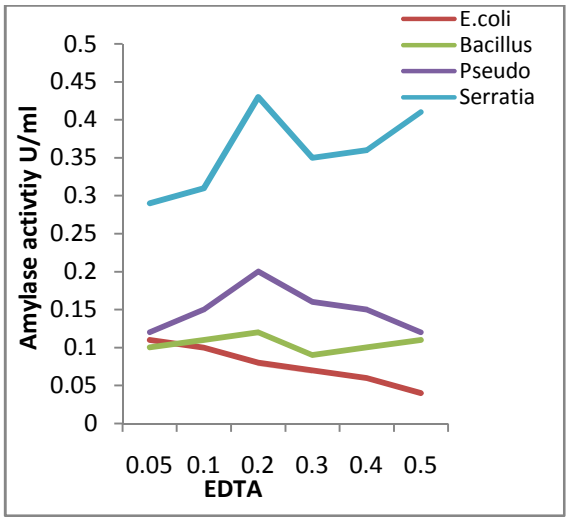


Fig14: Effect of EDTA concentration

Chloride salts: In the present study, Potassium chloride was found to be the most suitable chloride source for all bacterial sp (Fig 15). Supplementation of salts of certain metal ions provided good growth of microorganisms and thereby better enzyme production (as most a-amylases are known to be metalloenzymes). Ca^{2+} ions are reported to be present in majority of these enzymes. Addition of $CaCl_2$ to the fermentation media increased the enzyme production [33, 34].

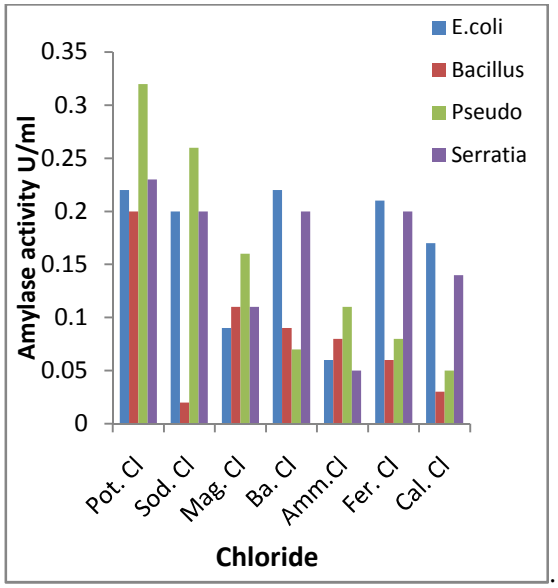


Fig15: Effect of Chloride

Sulphate salts: In the present study, Manganese sulphate was found to be the most suitable sulphate source for all bacterial sp (Fig 16). $LiSO_4$ (20 mM) and $MnSO_4$ (1 mM) increased a-amylase production by *Bacillus* sp. I-3 [35], but $FeCl_3$ and $MgSO_4$ exhibited negative influence on a-amylase production [36].

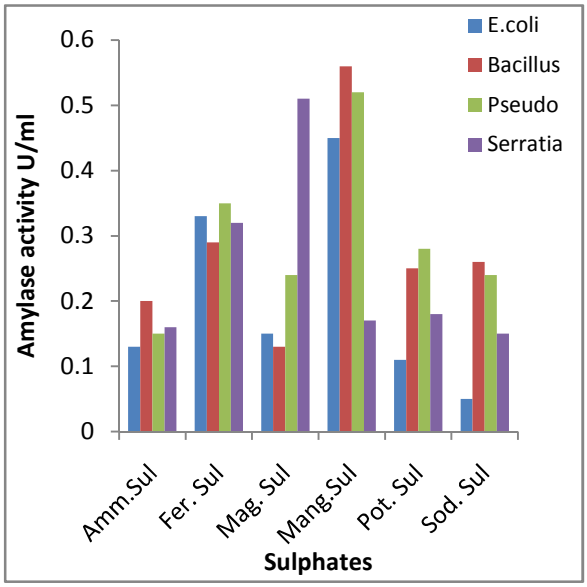


Fig16: Effect of Sulphates

CONCLUSION

a-Amylases are one of the most widely used enzymes required for the preparation of fermented foods. Apart from food and starch industries, in which demand for them is increasing continuously, they are also used in various other industries such as paper and pulp, textile, etc. [37]. With increase in its application spectrum, the demand is for the enzyme with specificity. From the present study it can be concluded that the bacterial isolate produce amylases at alkaline culture conditions and different factors greatly regulates the growth and production of amylases. The results in this study on different factors will be useful during further production of amylase by these microorganisrns. Of all the isolates evaluated, the highest protease activity was obtained from *Pseudomonas florescens* followed by *Bacillus subtilis* and *E.coli*. Commercially most of the production of a-amylase is carried out in submerged fermentation, but solid-state fermentation is being looked at as a potential tool for its production, especially applying agrobased waste residues such as Wheat bran as substrate.

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