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ISOLATION OF BACTERIA FOR COMMERCIAL PRODUCTION OF AMYLASE USING FLASK FERMENTATION

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ABSTRACT: Amylases are produced from different sources, of which microbial amylases are the most produced and used in industry, due to their productivity and thermo stability. Soil is the potential source for isolation of microorganism strains producing amylases. To accomplish this, a brief study was carried out to know the presence of different bacteria in soil that produce amylases. An efficient isolate was selected and studied further for amylase production in flask fermentation. The effect of various physiological parameters such as temperature, aeration, inoculum size, pH of the medium and substrate concentration on the production of amylase by the best isolate W4-3 was studied. In this study it was observed that amylolytic bacteria can be isolated from different soil samples and efficient bacterial isolates can be developed for commercial production of bacterial amylase.
Key words: Amylase, amylolytic bacteria, fermentation, physiological parameters

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INTRODUCTION

Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1, 4-*O*--glycosidic bonds in polysaccharides. The hydrolysis of starch with amylase result first in the production of short chain polymers called dextrins [1-3], then the disaccharide maltose and finally glucose units. Amylase belongs to the class of hydrolases and sub group glycosides as it brings about hydrolysis of true glycosides. The first commercial method for production of amylase used strains of *Bacillus subtilis, Bacillus mesentericus [4, 5]*. The enzymatic hydrolysis of starch, an easier and efficient process that has a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps can be described as follows [6].

Starch molecules are glucose polymers linked together by the α-1, 4 and α-1, 6 glycosidic bonds. In order to make use of the carbon and energy stored in starch, the human digestive system, with the help of amylases, must first break down the polymer to smaller assimilable sugars, which is eventually converted to the individual basic glucose units [7]. Due to the existence of two types of linkages, the α-1, 4 and the α-1, 6, different structures are possible for starch molecules. An unbranched, single chain polymer of 500 to 2000 glucose subunits with only the α-1, 4 glycosidic bonds is called *amylase [8-10]*. On the other hand, the presence of α-1, 6 glycosidic linkages results in a branched glucose polymer called *amylopectin*. The degree of branching and the side chain length vary from source to source, but in general more the chains are branched, more the starch is soluble [11].

Starch is generally insoluble in water at room temperature. Because of this, starch in nature is stored in cells as small granules which can be seen under a microscope. Starch granules are quite resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the same molecule and with other neighboring molecules [12-14]. However, these inter- and intra-hydrogen bonds can become weak as the temperature of the suspension is raised. When an aqueous suspension of starch is heated, the hydrogen bonds weaken, water is absorbed, and the starch granules swell [15]. This process is commonly called *gelatinization* because the solution formed has a gelatinous, highly viscous consistency. The same process has long been employed to thicken broth in food preparation [16].

Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose, maltose, and glucose, etc. Dextrins are shorter, broken starch segments that form as the result of the random hydrolysis of internal glycosidic bonds. A molecule of maltotriose is formed if the third bond from the end of a starch molecule is cleaved; a molecule of maltose is formed if the point of attack is the second bond; a molecule of glucose results if the bond being cleaved is the terminal one; and so on. The initial step in random depolymerization is the splitting of large chains into various smaller sized segments [17, 18]. The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called *liquefaction* because of the thinning of the solution. The final stages of depolymerization are mainly the formation of mono-, di-, and tri-saccharides. This process is called *saccharification*, due to the formation of saccharides.

MATERIALS AND METHODS

All general chemicals used were analytical grade and were obtained from Qualigens (India). Peptone, Beef Extract and other dehydrated media were obtained from Himedia (India). The reconstituted media were sterilized by autoclaving at 121^{o}C for 15min. For the isolation of microorganisms commonly found in soil the following media was used.

Isolation of amylolytic organisms

Sample collection

Soil samples were collected near rice mills, starch industries from Hyderabad, Karimnagar, Warangal and Medak for screening of micro flora exhibiting amylolytic activity.

Sample dilution and plating

The collected samples were diluted by performing serial dilution up to 10^{-8} in sterile saline and 0.1ml of the 10^{-7} and 10^{-8} diluted samples were plated onto a starch agar plate by spread plate technique and incubated at 37^{o}C for 24 hours.

Selection

After incubation, the plates were flooded with iodine solution and the colonies exhibiting the zones of starch hydrolysis were selected as amylolytic micro organisms diametere of zone was measured and used for further study.

Purification of the amylolytic isolates

The selected amylolytic isolates were then streaked onto a starch agar plate by the standard quadrant streak technique and were incubated at 37^{o}C for 24hours to ensure the formation of purified colonies.

Preservation of the obtained cultures

The purified cultures were streaked onto starch agar slants, incubated at 37^{o}C for 24hours for growth and preserved in a refrigerator for further study.

Characterization of the pure culture isolates

Staining and microscopic observation

Gram's staining: A smear of active culture grown in nutrient broth was prepared and heat fixed. To this, crystal violet was added and kept for 2 minutes and then washed with drop of water. Iodine solution was added and allowed to stand for 1minute, then washed with drops water and allowed to dry. To this 95% alcohol was added to decolorize and kept for 30 seconds and then washed with drops water. Counter stain saffranine was added and kept for 1-2 minutes and then washed with tap water and allowed to dry. Thus the stained slides were observed under microscope at 100x and the morphological characteristics were studied.

Growth characteristics

To study the growth characteristics, individual isolate was inoculated into the nutrient broth tubes and incubated at 37^{o}C for 24hours for growth and the growth pattern was observed.

Amylase production

Flask fermentation

30ml of starch broth was transferred into a 100ml conical flask and was sterilized. Into each of these, individual isolates were aseptically inoculated and incubated at 37^{o}C for 24hours with 200rpm agitation in an Orbital Shaker. (All incubations were performed at the above mentioned conditions unless and until specified.)

Amylase enzyme preparation

After incubation, 2ml of the broth was taken into an appendorff tube and centrifuged at 8000rpm for 15minutes. The supernatant thus obtained was used as a crude enzyme.

Amylase assay

To 1ml of the 1% starch solution prepared in phosphate buffer of pH 7.0, 0.5ml of the crude enzyme was mixed and incubated at 37^{o}C for 30 minutes. After incubation, the reducing sugars formed were estimated by DNS method.

Blanks were also prepared in the same manner with DNS inactivated enzyme.

Estimation of the reducing sugars

1ml of the sugar sample was transferred into test tubes containing 1ml of DNS reagent and was placed in a water bath at 100^{o}C for 10 minutes. After incubation, the volume in each tube was made up to 5ml with distilled water and allowed to cool down to room temperature and absorbance was read at 540nm.

Effect of different substrates

Different starchy substrates (wheat flour, rice flour, tapioca flour, corn starch, cassava starch, soluble starch, potato starch, maida) were employed at a concentration of 1% to study their effect on the production of amylases. To 30ml of sterilized starch broth containing the above mentioned substrates, 1% (v/v) active inoculum was added aseptically and incubated. The most suitable substrate for the production of amylase was thus determined by performing amylase assay.

Effect of substrate concentration

To find the optimum concentration of substrate for production of amylases, 30ml of starch broth with varying concentration (1- 5% w/v) of soluble starch was prepared and sterilized, to these 1% active inoculum was added and incubated. Optimum concentration of the substrate that can be employed for the production of amylase was then determined by performing the amylase assay of fermented broth.

Effect of temperature

Optimum temperature required for production of amylase was determined as follows. 30ml of starch broth was prepared in flasks and sterilized. The flasks were incubated at different temperatures with 1% inoculum.

Effect of pH

Optimum pH required for production of amylases was determined as follows. 30ml of the starch broth was prepared and sterilized. The pH of the media was adjusted to 3-8 using 1N NaOH and 0.5N H_2SO_4. To these, 1% active inoculum was added aseptically and incubated. Amylase assay of fermented broth was then performed.

Effect of aeration

Optimum aeration required for amylase production was determined as follows.30ml of starch broth was prepared and sterilized. To this 1% inoculum is added and incubated in Orbital shaker at different shaking speeds ranging from 50-200 rpm.

Effect of varying inoculum size

Active inoculum of varying size (1-5% v/v) was added into 30ml of sterilized starch broth and incubated. Optimum inoculum size required for the production of amylase was then determined by performing the amylase assay of fermented broth.

RESULTS AND DISCUSSION

Amylases are the most important enzymes used industrially, particularly in the processes involving starch hydrolysis. Though amylases originate from different sources (plants, animals and micro organisms), the microbial amylases are the most produced and used in industry, due to their productivity and thermo stability. Soil is the good source for isolation of microorganism strains producing amylases.

Soil samples were collected from different areas and processed for the isolation of amylolytic organisms. Out of 9 soil samples screened, 29 amylolytic isolates were obtained (Table-1, Fig-1).

Table 1: Amylolytic bacterial isolates obtained from different soil samples

S.No.	Soil sample	Area	No.amylolytic org. obtained	Amylolytic strain
1	W1	Warangal	4	W1-1
				W1-5
				W1-4
				W1-6
2	W2	Warangal	0	NIL
3	W3	Warangal	5	W3-1
				W3-2
				W3-6
				W3-7
				W3-8
4	W4	Warangal	4	W4-1
				W4-2
				W4-3
				W4-4
5	W5	Warangal	4	W5-1
				W5-2
				W5-5
				W5-6
6	M	Medak	1	M
7	M1	Medak	2	M1-1
				M1-2
8	M2	Medak	4	M2-1
				M2-3
				M2-5
				M2-6
9	RC	Hyderabad	3	RC-1
				RC-2
10	HM	Karimnagar	2	HM-1
				HM-2

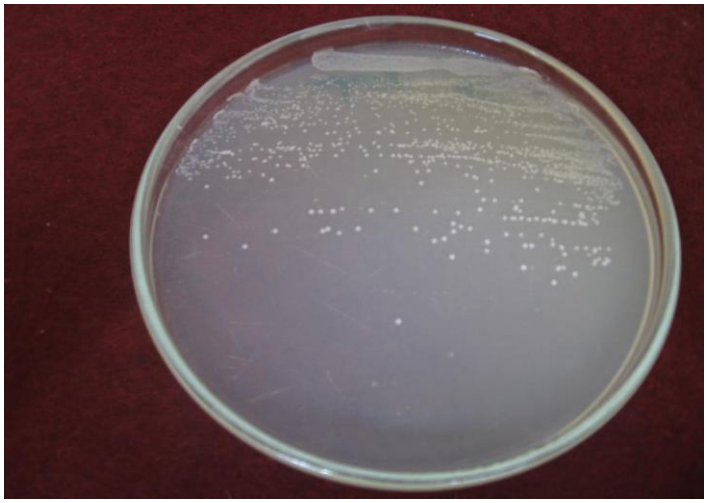

Fig 1: Colonies of amylolytic bacterial isolates grown on starch agar medium by streak plate method.

The samples showed an existence of more than one amylolytic organism. A study made on the characteristic growth pattern of the purified isolates using agar and broth media revealed that different isolates showed colonies of different sizes where in the size of a smooth, large colony was 3-4 mm while a small pinpointed colony being 1-2 mm indicating that they belong to different groups. In support to this, the growth pattern in broth of isolates W3-1 & W3-7, showed a pellicular growth indicating an excess O_2 requirement in comparison to the other isolates showing uniform growth which possess a moderate O_2 requirement. (Table 2, Fig 2).

Morphological characteristics of the organisms were studied by Gram's staining followed by microscopic observation. It was observed that all the isolates obtained were Gram positive (Table 2). The isolates were 15 bacilli & 14 Streptobacilli (Table 2).

Table 2: Culture characteristics of the purified amylolytic bacterial isolates.

Strain	Growth in nutrient broth	Gram's reaction	Microscopic Morphology
W1-1	Uniform growth	positive	Streptobacilli
W1-5	Uniform growth	positive	Streptobacilli
W1-4	Uniform growth	positive	Streptobacilli
W1-6	Uniform growth	positive	Bacilli
W3-1	Uniform growth	positive	Streptobacilli
W3-2	Uniform growth	positive	Streptobacilli
W3-6	pellicle	positive	Bacilli
W3-7	pellicle	positive	Bacilli
W3-8	pellicle	positive	Streptobacilli
W4-1	flocculent	positive	Bacilli
W4-2	Uniform growth	positive	Bacilli
W4-3	Uniform growth	positive	Bacilli
W4-4	Uniform growth	positive	Bacilli
W5-1	Uniform growth	positive	Streptobacilli
W5-2	Uniform growth	positive	Bacilli
W5-5	Pellicle	positive	Streptobacilli
W5-6	pellicle	positive	Streptobacilli
M	Uniform growth	positive	Bacilli
M1-1	Uniform growth	positive	Strept bacilli
M1-2	Uniform growth	positive	Streptobacilli
M2-1	flocculent	positive	Streptobacilli
M2-3	Uniform growth	positive	Bacilli
M2-5	flocculent	positive	Streptobacilli
M2-6	flocculent	positive	Bacilli
RC-1	Uniform growth	positive	Bacilli
RC-2	flocculent	positive	Bacilli
RC-3	Uniform growth	positive	Streptobacilli
HM-1	Uniform growth	positive	Bacilli
HM-2	Uniform growth	positive	Bacilli

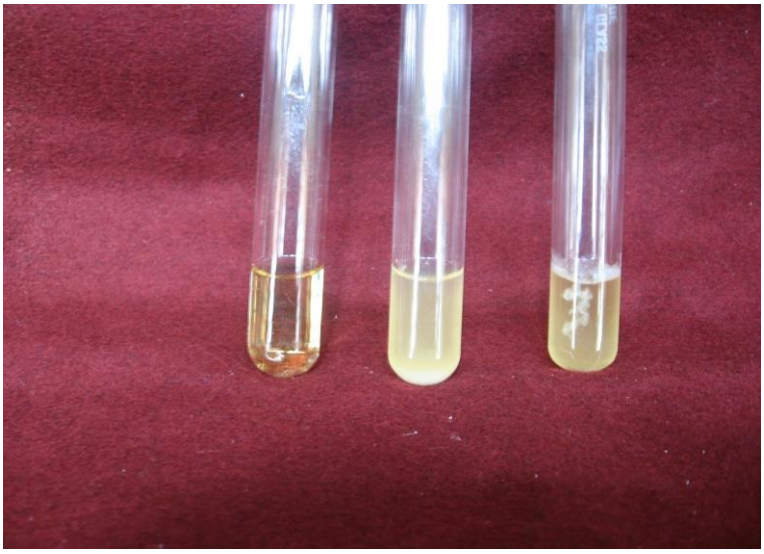

Fig 2: Amylolytic bacterial growth in Starch broth.A-Sterile broth without bacterial growth,B-Uniform growth,C-Pllicular/Superficial growth.

Purified amylolytic isolates were tested for qualitative amylolytic pattern by placing individual isolates on starch agar plates followed by staining with Iodine (I_2) solution. Starch hydrolysis zones that represent the potency of an amylolytic organism were then measured for each of them. Results indicated that the isolate W4-3 is a potential amylase producer with a starch hydrolysis zone diameter of 23mm while the isolate M2-1,HM-1 stand in the next position with a starch hydrolysis zone diameter of 21mm followed by other isolates showing zones of lesser diameter (Table 3 and Fig 3).

Amylolytic bacteria obtained were further tested for quantitative amylase activity using soluble starch as a substrate in flask level fermentation. The extra cellular enzyme was collected by centrifugation, amylase activity was performed. Correlating with the results obtained in primary screening, isolate W4-3 showed maximum amylase activity in fermented broth after 24hrs where the amount of enzyme produced was found to be 69U/ml, followed by M2-1(60U/ml) and HM-1(58U/ml) (Fig-4).

Fig 3: Fermentation broth containing 1% soluble starch used for amylase production in flask fermentation .A-Sterile broth broth before inoculation, B-Fermented broth with bacterial growth after incubation

Table 3: Amylolytic activity of pure culture isolates obtained from soil

Isolate	Starch hydrolysis Zone Diameter (mm)
W1-1	11
W1-5	13
W1-4	20
W1-6	16
W3-1	12
W3-2	14
W3-6	17
W3-7	21
W3-8	21
W4-1	20
W4-2	17
W4-3	23
W4-4	21
W5-1	20
W5-2	21
W5-5	11
W5-6	10
M	20
M1-1	19
M1-2	16
M2-1	21
M2-3	16
M2-5	15
M2-6	17
RC-1	14
RC-2	18
RC-3	19
HM-1	21
HM-2	13

Determination of the best substrate is a prerequisite for production of amylases which was done by employing different crude starchy substrates like rice flour (RF), corn flour (CF), cassava starch(CS), tapioca flour(TF), maida flour(MF), soluble starch(SS), potato starch(PS), wheat flour(WF) and studied for amylase production by selected amylolytic bacterial isolate W4-3. It was found that the highest amount of enzyme (78.74U/ml) was produced using soluble starch as a substrate followed by potato starch with (50U/ml) while the remaining showed less production relatively (Fig. 5) indicating that soluble starch is best substrate for the production of amylase. Hence soluble starch was used as the substrate for further study.

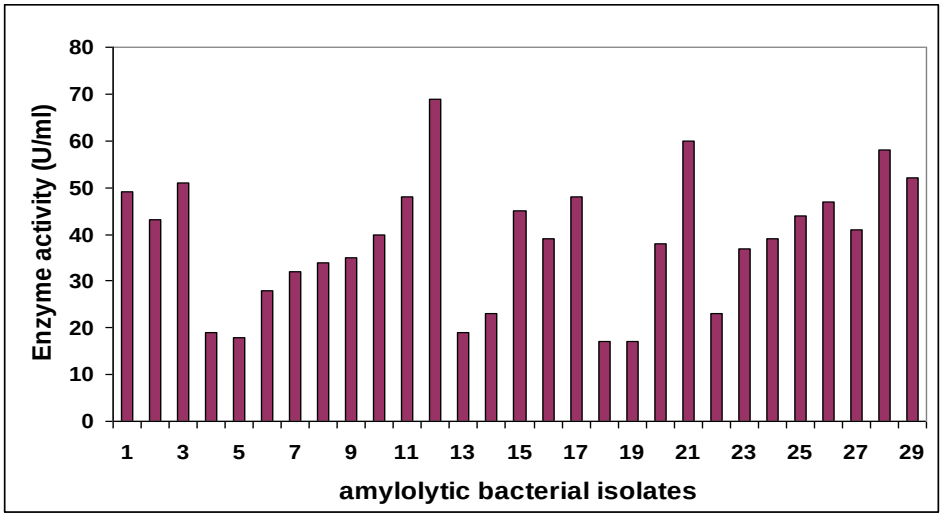

Fig 4: Production of amylase by different bacterial isolates

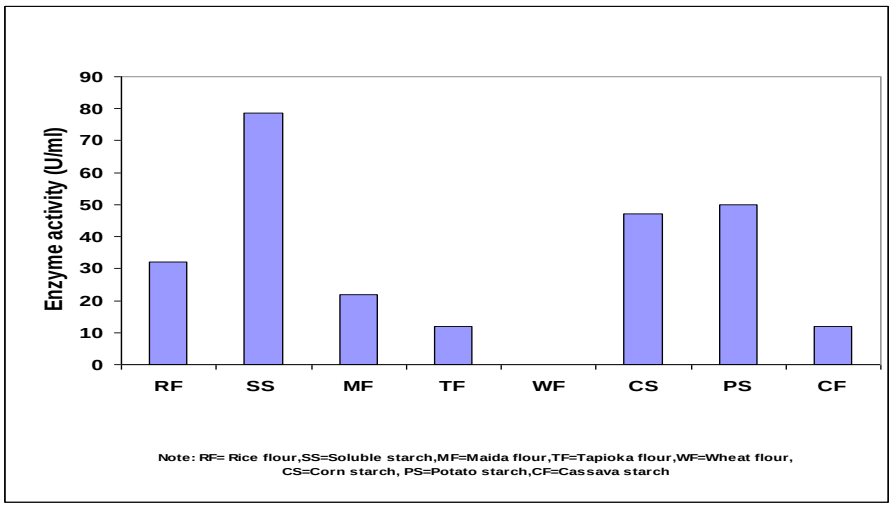

Fig 5: Amylase production by selected bacterial isolate (W4-3) using different substrates

Soluble starch of increasing concentration (1-5%) was used to study its effect on production of amylase. It was found that the highest production of amylase was found at 3% concentration of soluble starch (92U/ml) followed by a small decrease at 4% (80U/ml), 5% (76U/ml) while there was a slight at 1% (69U/ml) and 2% (73U/ml) indicating that the production of amylases is optimum at 3% concentration of soluble starch (Fig 6).

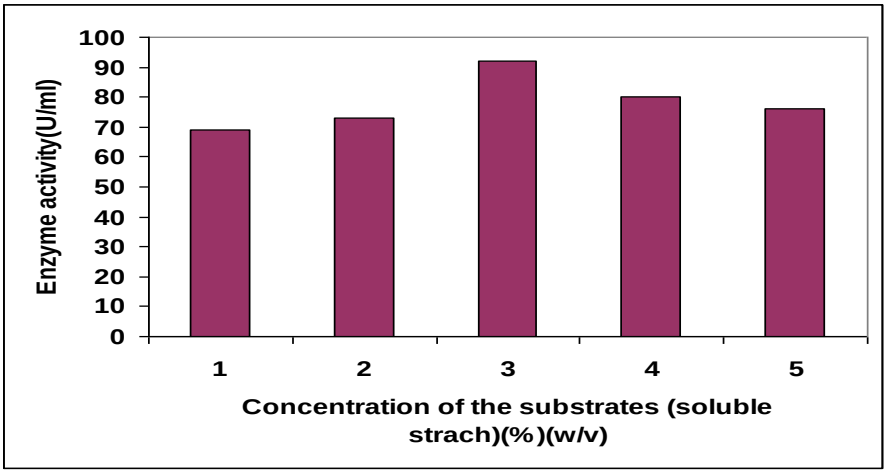

Fig 6: Effect of substrate (soluble starch) concentration on amylase production by bacterial isolate W4-3

Optimization of different fermentation parameters like inoculum size, temperature, aeration and pH of the media is necessary for efficient production of amylases. Hence a study was made to know the effect of above parameters on amylase production using the selected isolate with best substrate, soluble starch.

Effect of Temperature on amylase production: Some amylases are thermostable in nature. The enzyme production by related bacterial isolate (W4-3) was highest at 40°C which produced 38U/ml (Fig 7). When tested with variation in temperature effect the enzyme production. The optimum temperature range is 30-40 for highest enzyme production.

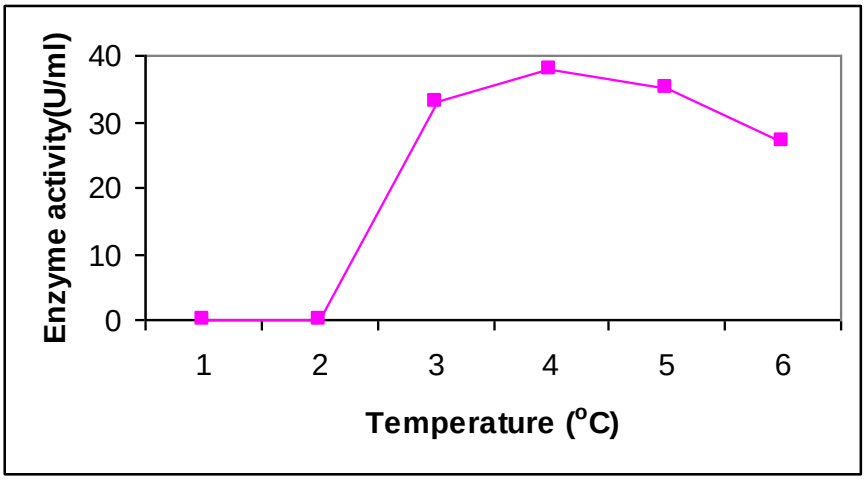

Fig 7: Effect of temperature on amylase production using isolate W4-3

pH of the growth medium plays an important role by inducing the morphological changes in the organism and in enzyme secretion. The production of amylase is very sensitive to pH of fermentation medium. The enzyme production was highest at pH 7.0, which yielded 87 U/ml (Fig 8). When tested with variation in pH result from substrate consumption (e.g. .protein hydrolysis) and/or metabolite production (e.g. organic acids). Results indicate that enzyme production was generally stable at pH range from 6-8 which indicates good buffering property of the substrates used for fermentation.

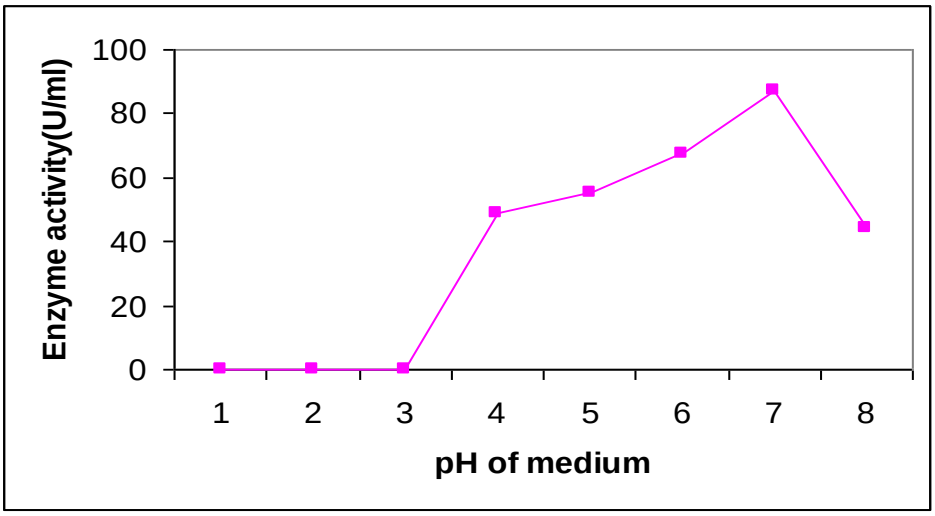

Fig 8: Effect of pH medium on amylase production by bacterial isolate W4-3

Agitation effects the growth of microorganisms, in which air is supplied to the organisms and leads to production of enzyme in more quantity. High enzyme was produced at agitation speed of 100 rpm (92U/ml). (fig. 9)

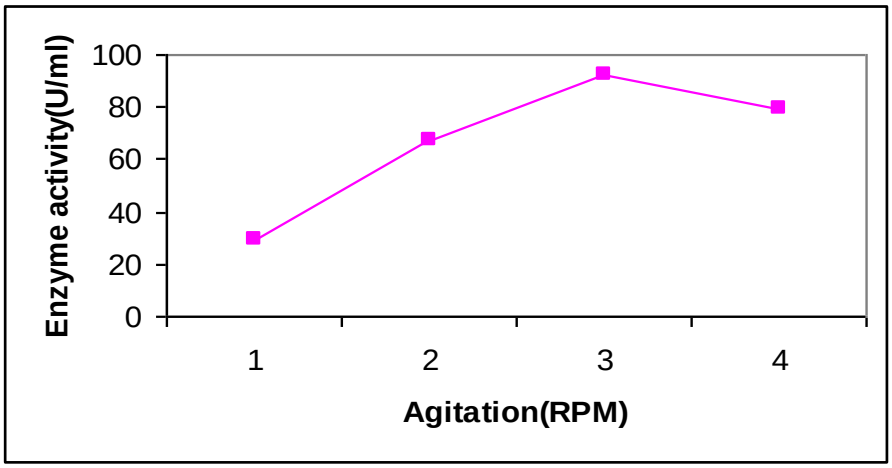

Fig 9: Effect of agitation on amylase production by bacterial isolate W4-3

Inoculum of increasing size (1-5%) was used for the study of its effect on amylase production. Results indicated a gradual increase in the enzyme production as the size of inoculum increased from 1 to 5% while maximum production was found at 5% (122units/ml) inoculum size followed by 4% (102units/ml). This could be due to an increase in the number of microbial cells in inoculum, which show an active participation in amylase production. Thus 5% was used as optimum inoculum size for further studies (Fig 10).

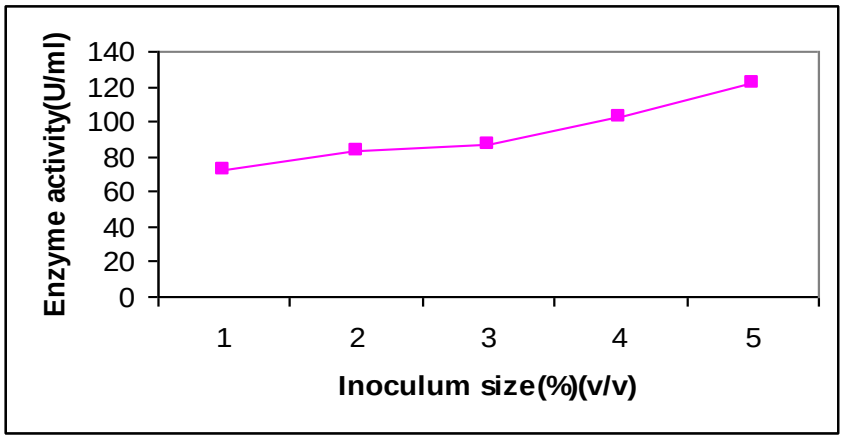

Fig 10: Effect of inoculum size on amylase production by bacterial isolate W4-3

CONCLUSION

Amylases are the most important enzymes used in biotechnology with a wide variety of applications. Amylases are produced from different sources (plants, animals and micro organisms), of which microbial amylases are the most produced and used in industry, due to their productivity and thermo stability. Soil is the potential source for isolation of microorganism strains producing amylases. To accomplish this, a brief study was carried out to know the presence of different bacteria in soil that produce amylases. An efficient isolate was selected and studied further for amylase production in flask fermentation. The effect of various physiological parameters such as temperature, aeration, inoculum size, pH of the medium and substrate concentration on the production of amylase by the best isolate W4-3 was studied. Based on the observation made in this brief study, the following conclusions are drawn.

- Majority bacteria present in soil studied were amylolytic.
- Varying amylolytic activity was present among the bacteria present in soil.
- Among the different starchy substrates employed, soluble starch was the best substrate for production of amylase using isolate W4-3.
- Optimum pH required for production of amylase using isolate W4-3 was 7.0.
- Optimum temperature for amylase production was 40^{o}C.
- Production of amylase was highest at 100 rpm of agitation.
- Production of amylases using isolate W4-3 was found to be maximum at an inoculum size of 5% (v/v).

In this brief study, it was observed that amylolytic bacteria can be isolated from different soil samples and efficient bacterial isolates can be developed for commercial production of bacterial amylase.

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