ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Production, characterization and immobilised dye decolorization of amylase enzyme produced by *Bacillus megaterium* isolated from soil sample

M. Sankareswaran[1]*., K.Kaliselvi[2] ., S. Anbalagan[1]., M.Suganya[1]., K.Abirami[1].

1. Department of Microbiology, Muthayammal College of Arts and Science, Rasipuram.637408, Tamil Nadu, India.

2. Department of Microbiology, PSG College of Arts and Science, Coimbatore-641 014, Tamil Nadu, India.

Manuscript Info

Manuscript History:

Received: 15 June 2015
Final Accepted: 20 July 2015
Published Online: August 2015

Key words:

Bacillus megaterium, Amylase enzyme, Immobilised cells, Dye decolorization

Corresponding Author

M. Sankareswaran

Abstract

Among different types of enzymes obtained from microbial sources, amylases are the most widely used in industries as well as on commercial basis. Amylase production from bacteria is economical as the enzyme production rate is higher in bacteria as compared to other microorganism. The present research work is focus on the production of amylase enzyme from *Bacillus megaterium* soil samples. Various fermentation conditions were checked for the production of amylase enzyme from isolated *Bacillus megaterium*, to improve the enzyme production. The optimum pH of *Bacillus megaterium* grows on pH 7. The optimum incubation time 48 hrs and temperature 37°C for *Bacillus megaterium* given the high yield of amylase enzyme. The extracted amylase enzyme was partially purified. Purification was performed by ammonium sulfate precipitation and DEAE-Cellulose column using ion exchange chromatography. The molecular mass purified amylase was estimated to be approximately 25 KDa by SDS-PAGE. The partially purified amylase enzyme was immobilised. The immobilized enzymes are used for removal of azo dyes by using different parameters. The maximum dye decolorization was found at an alkaline pH8.0 both the 5ml and 10ml enzyme containing 250 and 500 beads showed good adsorption rates.

INTRODUCTION

Enzymes a gorgeous and potential catalyst of biological systems of outstanding molecular devices with high specificity and potency will confirm the pattern of biochemical reactions. (Bunni et al., 2000)

Amylases are enzymes that break down starch or animal starch the key benefits of exploitation microorganisms for production of amylases is in economical bulk production capability and microbes are simple to govern to get enzymes of desired characteristics (Aiyer PV., 2005). A large variety of microorganism amylases are obtainable commercially; and that they have virtually complete replacement for chemical reaction of starch in starch process trade. Starch is that the primary storage sugar in plants. Amylolytic Amylase is a starch degrading enzymes. Amylase enzyme is one amongst the necessary accelerators and of nice significance acceptable in enzyme market (Bernfeld et al., 1955; Myrback and Neumuller, 1950; Ra O et al., 1998).

Amylases enzymes that change the starch molecules into polymers composed of aldohexose units. Alpha-amylases omnipresent in distribution, with plants, microorganism and fungi being the predominant sources. Most of the microorganism α-amylases belong to the family 13 glycosyl hydrolases. (Reddy et al., 2003).The ability of *Bacillus subtilis* to convert starch into reducing sugars was studied by Shafaat et al., (2011).

Amylases enzyme produced by several microorganism and fungi. Amylase enzyme producing organisms are *Bacillus subtilis, B. cereus, B. myloliquefaciens, B. coagulans, B. polymyxa,, B. caldolyticus*

B. stereothermophilus, Escherichia coli, B.licheniformis, B.amyloliquefaciens, B. acdiocaldarius, Lactobacillus sp, Micrococcus sp, Pseudomonas sp, Arthrobacter sp, Proteus sp, and *Serratia sp*, etc., and fungi producing amylase enzymes are *Aspergillus oryzae, A. niger, Penicillium sp*, etc., (Sivaramakrishnan et al., 2006; Vihinen et al., 1989; Goto et al., 1998; Sanchez and Cardona 2008; Gigras et al., 2002).

Optimum of cultural conditions is very important for optimum production of microorganism strains. Because, the expansion conditions conjointly influence their accelerator activity (Bezbaruah et al., 1994).The first amylase enzyme created industrially from a fungal source and supply in 1994, which was used for the treatment of biological disorders.

The production of α-amylase by submerged fermentation (SMF) and solid state fermentation (SSF) has been investigated and rely on a range of chemistry factors. SMF has been historically used for the assembly of industrially necessary enzymes as a result of the benefit of management of various parameters like pH, temperature, aeration and chemical element transfer and wet (Couto and Sanroman 2006; Gangadharan et al., 2008).

The commercial source of industrial supply of starch is corn from that it's extracted by a wet edge method (Aiyer PV., 2005)

Amylase is the second variety of enzymes employed in the formulation of accelerator detergent and 90% of all liquid detergents contain amylase enzymes (Gupta et al., 2003,Hmidet et al., 2009 and Mitidieri et al., 2006,). These enzymes are employed in detergents for laundry and automatic lavation to degrade the residues of starchy foods like potatoes, gravies, custard, chocolate, etc. to dextrins and different smaller oligosaccharides (Mukherjee et al., 2009; Olsen and Falholt 1998).

Amylases enzyme employed in textile trade for desizing method (Ahlawat et al., 2009; Gupta et al., 2003). The utilization of α-amylases within the pulp and paper trade is for the modification of starch of coated paper. The scale enhances the stiffness and strength in paper (Gupta et al., 2003; Van Der Maarel et al., 2002; Bruinenberg et al., 1996).

In this research work Amylases producing bacteria were isolated and tested for its production of Amylases and its enzyme activity. The isolates were tested in various environmental and nutritional factors. Finally the enzymes were purified, characterized and the molecular mass determined by SDS-PAGE. The partially purified amylase enzyme was immobilised. The immobilized enzymes are used dye decolorization studies.

MATERIALS AND METHODS

Isolation of amylolytic organism and screening for amylolytic activity

The soil samples were collected and screened for amylolytic activity by starch hydrolysis test on starch agar plates. The microbial isolates were streaked on the starch agar plates and incubated at 37^{o}C for 48 hrs. The isolates produced clear zones of hydrolysis were considered as amylase producers and were further investigated.

Identification of selected strain

The isolated bacterium was subjected to various tests including colony morphology, staining and standard biochemical tests. The organism used in the present study has been identified based on the test performed as species of *Bacillus megaterium.*

Effect on the p^{H} on enzyme production

The production medium was prepared and the p^{H} of the broth was adjusted 5,6,7,8 and 9 using 0.1 N HCL or 0.1N NaOH. 0.1 ml of 24 hrs old *Bacillus megaterium* culture was inoculated and incubated at 37^{o}C for 48 hrs after incubation, it was observed for amylase production.

Effect on the temperature on enzyme production

The production medium was prepared and 0.1 ml of 24 hrs *Bacillus megaterium* culture was inoculated and incubated at 27^{o}C, 37^{o}C, 47^{o}C and 57^{o}C. After incubation, it was observed for amylase production.

Effect on the incubation time on enzyme production

The production medium was prepared. 0.1 ml of 24 hrs old *Bacillus megaterium* was inoculated and incubated at 24hrs, 48 hrs and 72 hrs. After incubation, it was observed for amylase production.

Effect on the carbon sources on enzyme production

Influence of different five carbon sources such as sucrose, dextrose, lactose, maltose and fructose was analysed at 1% level. The production medium was prepared with 1% selected carbon sources separately and 1 ml of 24 hrs *Bacillus megaterium* was inoculated and observed for amylase production.

Effect on the nitrogen sources on enzyme production

Influence of different organic (Peptone, Beef extract, Meat extract, Yeast extract, tryptone) and inorganic nitrogen (Ammonium sulphate, Sodium chloride, Potassium nitrate, Sodium nitrate, Potassium nitrite) sources were analyzed at 1% level. Production medium was prepared and 0.1ml of 24 hrs *Bacillus megaterium* inoculated and incubated at 24 hrs. After incubation, it was observed for amylase production at 540nm optical density (OD) value.

Estimation of amylase enzyme assay

Amylase was determined by colorimetric method as slight modifications of Fisher and Stein (Arvinder kaur et al., 2012). According to the procedure 1.0ml of culture extract enzyme into test tube and 1ml of 1%soluble starch in citrate- phosphate buffer (pH 6.5) was added in test tube. The test tubes were covered and incubate at 35^{o}C for 5 minute. Then 2.0ml of DNS reagent was added into each tube to stop the reaction and kept in boiling water bath for 15 minutes. After cooling at room temperature, the absorbance was read at 540nm by colorimeter.

Partial Purification of enzymes

Ammonium sulphate precipitation method

The crude enzyme was partially precipitated by ammonium sulphate salt. The solubility of protein is markedly affected by the ionic strength of the medium. To the crude enzyme, different concentration of ammonium sulphate was added to precipitate obtained at each concentration of ammonium sulphate was dissolved in water and assayed for amylolytic activity. The total enzyme activity and the total protein of the precipitate were also determined. The enzyme activity was found to maximum at 60-80% ammonium sulphate saturation and above 90% saturation there was decrease in the enzyme activity.

Dialysis

The partially purified amylase enzyme (using ammonium sulphate precipitation) was subjected to further purification by dialysis. The dialysis tube was cut according to the required size and boiled in distilled water containing a pinch of EDTA at 60^{o}C to 80^{o}C for 10mins rinsed with distilled water. Then the precipitate was mixed with Tris HCL, calcium chloride. The solution was transferred to the dialysis tube. This dialysis tube containing the sample (hypertonic) was placed in a beaker containing 500ml of Tris HCL (hypotonic) for 24 hrs. As a result osmosis occurs and the impurities were discharged into the beaker. These impurities were discarded and same procedure was repeated for 48 hrs.

Purification of lipase enzyme

The partially purified enzyme was subjected to DEAE-cellulose column using ion exchange chromatography to obtain pure and homogenous enzyme. The purified was subjected to determine molecular weight by SDS-PAGE (Laemmli et al., 1970).

Immobilization of crude enzyme

The partially purified amylase enzyme was immobilised. 6gm of sodium alginate and 85 ml of 0.1% NACL solution was taken. Stirrer continuously and kept it for 6-8 hrs at room temperature. Add 0.4 O.D of 30ml of 24 hrs bacterial culture was centrifuged at 5000 rpm for 20 min at 4^{o}C.Take the 15ml supernatant and add it to the slurry. Mix well and add it to 4% $CaCl_2$ solution in drop wise. Beads were maintained in $CaCl_2$ solution for 30 minutes. Beads were washed with in sterile distilled water. Beads were stored in nutrient broth or sterile distilled water and stored in refrigerator and use in further research work.

Decolorization of dyes by the immobilized beads in column

The prepared immobilized beads were taken in column packed with cotton as a supporting material. The dye solution had an initial concentration of 100 ppm was poured and allowed to get adsorbed on the beads by changing the flow rate. The eluent was collected and analyze for percentage was used for this purpose.

Two types of column (narrow and wide) were used to pack the immobilized beads and understand their effect in the decolourization process. The culture volume of 15 ml and 30 ml was added to slurry to delineate the effect of the types of bacterial volumes. The bead numbers were changed as 250 and 500 to study the decolourization ability of the selected microorganisms. Flow rate of the dye was maintained as 25 ml/hr and 25ml / 2hr and the decolourization was observed. The slurry pH was maintained as pH 6.0, 7.0 and 8.0.

Assay of dye decolourization by the immobilized bacterial beads:

Decolorization was expressed in terms of percen tage of decolourization. Dye decolorization by the bacterial isolates was analyzed by adopting colorimetric method. Prior, to this, the absorbance maximum of the dye was determined. The adsorption maxima for Azo dye 420 nm, then the percentage of dye decolourization was calculated by applying the absorbance value using the formula described by (Mane et al., 2008)

$$\text{Percentage of Decolorization} = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} \times 100$$

RESULTS AND DISCUSSION

Enzymes are biological catalyst and have longed been employed in food processing. The amylases are one of the most important families of enzymes in the field of biotechnology especially in employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar. Some bacteria can produce extracellular amylase enzymes catalyzing the degradation of starch substances (Nanik Rahmani et al., 2011).

The amylase producing organism isolated from soil sample, identified and confirmed as *Bacillus megaterium*, based on their morphology, gram staining, motility and standard biochemical test. The amylase enzyme production was checked in various pH with isolated *Bacillus megaterium* and the optimum pH of enzyme production was at pH 7 and 0.155units /ml. **Fig: 1.**

When the time and the temperature taken for maximum amylase production of *Bacillus megaterium*. The higher amount of amylase production at 37°C and the optimum incubation period is 48 hrs and the enzyme production rate is 0.175 units/ml **Fig: 2 & 3**. The production of extracellular amylase by *Bacillus sp* was optimized

in a submerged fermentation. The production of the enzyme was maximum at 10 hrs after inoculation (Vidyalakshmi et al., 2009). But the present study revealed that, *Bacillus megaterium* produced high amount of amylase enzyme for 24hrs. The maltose is the best carbon source for production of amylase enzyme from *Bacillus megaterium* is 0.280units/ml. **Fig: 4.**

Meat extract is the best organic nitrogen source for higher amylase production of *Bacillus megaterium* is 0.299 units/ml. **Fig: 5.**Potassium nitrate is serves as the best inorganic nitrogen source for maximum production of amylase by *Bacillus megaterium*, 0.253 units/ml. **Fig: 6.** Nitrogen sources peptone and sodium nitrate were the best organic and inorganic sources respectively for producing amylase enzyme. They also screened the efficacy of varied carbon source and its role enzyme production and reported that the amylase production is higher in the presence of carbon source maltose producing the highest amount of amylase. Optimization and enzyme estimation in *B. megaterium* through various factors and found the enzyme production was maximum at 35°C, pH 7.5. (Sajitha et al., 2011; Jogezai et al., 2011)

The molecular weight of the partially purified enzymes are found that is approximately 25 kDa determined by SDS-PAGE.

The immobilized enzymes are used for removal of azo dyes by using different parameters. Dye concentration played important role on degradation .It showed negative effect on degradation. When the concentration of dye was increased, the percentage of degradation decreased.

The flow rate greatly imparts the amount of decolourization of the azo dye. It was observed that while we increased the time of flow rate, the decolourization rate increased. Thus, 2 hour flow rate showed a high decolourization than 1 hour flow rate of the azo dye through the beads, both in the column. The maximum dye decolourization was found at an alkaline pH 8.0. Both the 5ml and 10ml enzyme containing 250 and 500 beads showed good adsorption rates. **Table No: 1&2**. The crude enzyme was immobilized and used in the removal of Azo dye. The maximum dye decolorization was observed at 10ml enzyme containing 500 beads in broad column is 89%.

Fig-1: Effect of pH on enyme production by *Bacillus megaterium*.

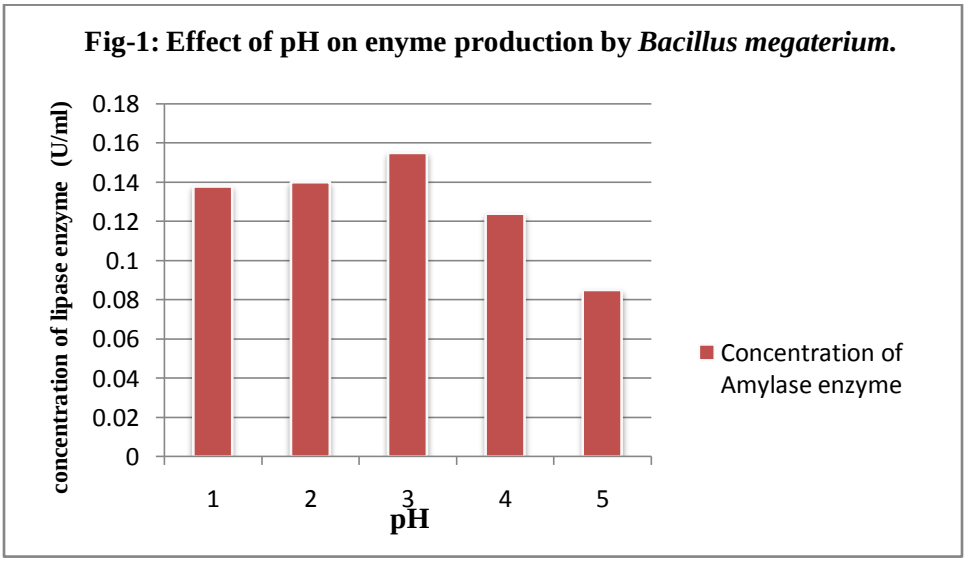

Fig-2: Effect of temperature on enyme production by *Bacillus megaterium.*

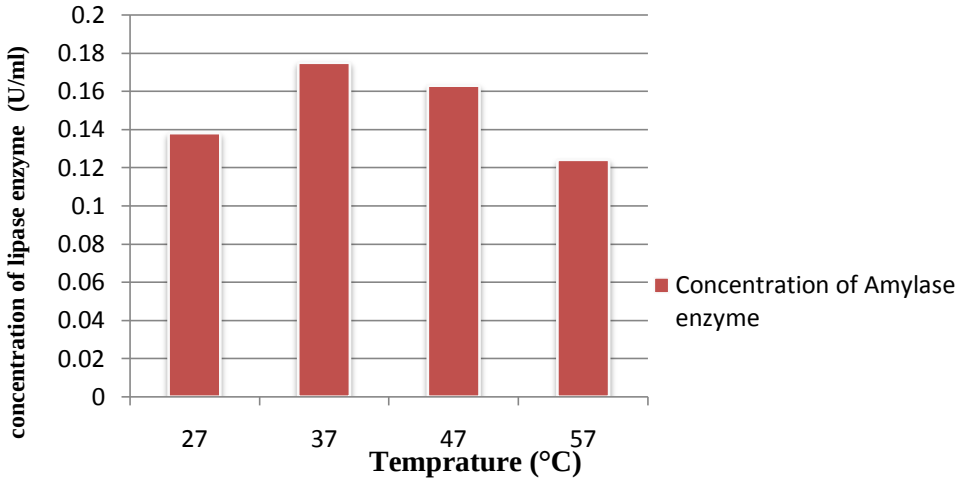

Fig-3: Effect of incubation time on enyme production by *Bacillus megaterium.*

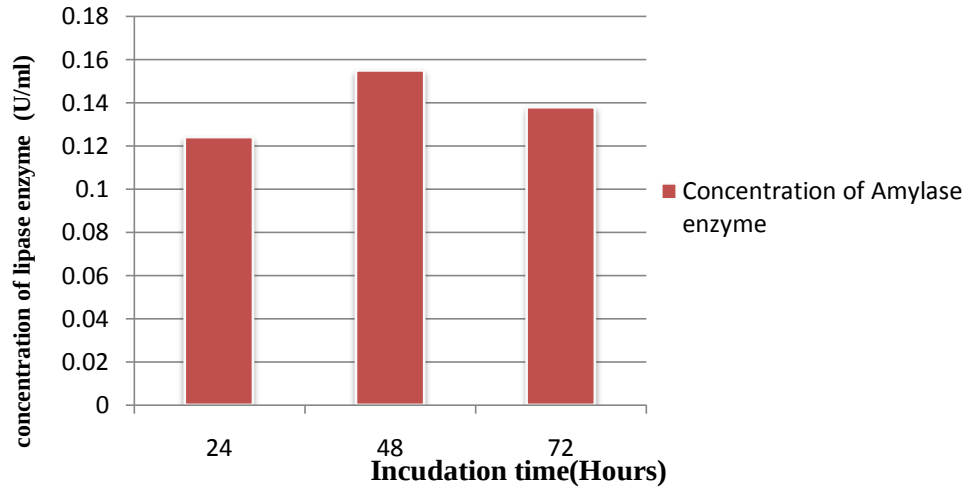

Fig-4: Effect of carbon source on enyme production by *Bacillus megaterium*.

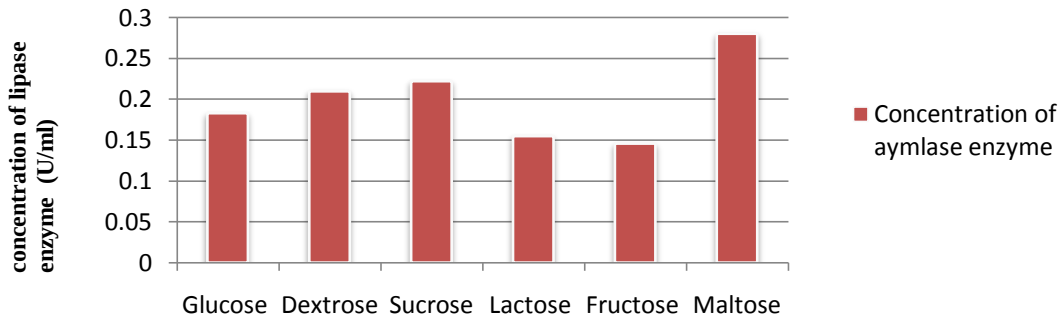

Fig-5: Effect of organic nitrogen source on enyme production by *Bacillus megaterium*.

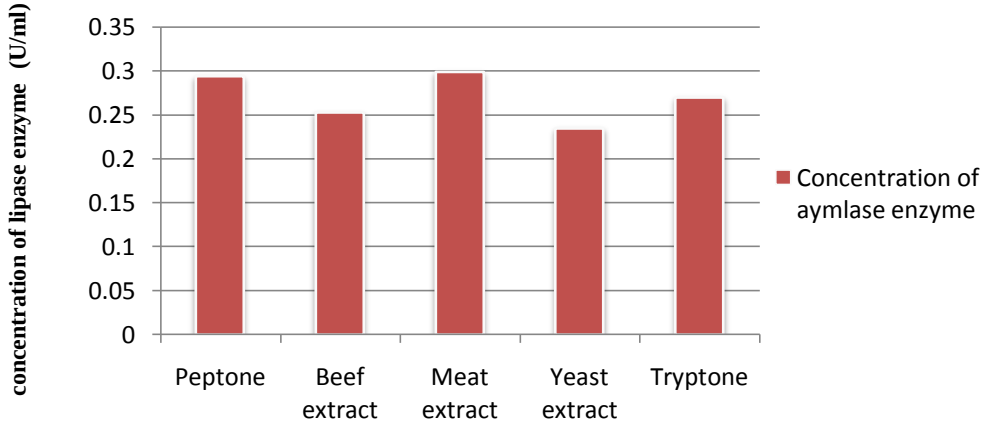

Fig-6: Effect of inorganic nitrogen source on enyme production by *Bacillus megaterium.*

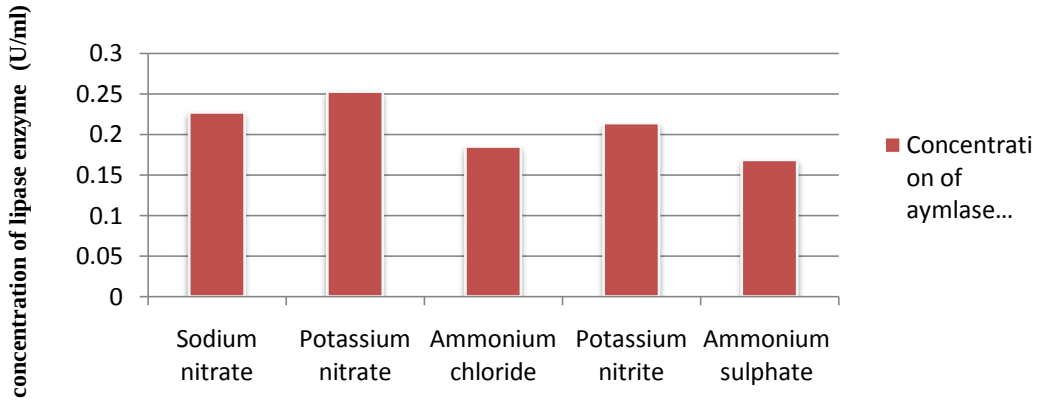

Table: 1
Decolorization of Azo dye by Immobilized Enzymes of *Bacillus Megaterium* in the broad column at 25ml/hour flow rate.

S.No	Dye Concentration (ppm)	Volume of the culture	Number of beads	Percentage of dye decolorized		
				pH 6.0	pH 7.0	pH 8.0
1	100	5	250	66.1	64.2	67.1
2			500	67.8	66.5	70.1
3		10	250	66.5	66.9	69.2
4			500	66.9	67.1	72.8
5	300	5	250	65.2	64.5	68.1
6			500	66.5	67.9	69.2
7		10	250	66.8	65.1	70.3
8			500	68.2	67.2	74.2

Table: 2
Decolorization of Azo dye by Immobilized Enzyme *Bacillus megaterium* in the narrow column at 25ml/hour flow rate

S.No	Dye Concentration (ppm)	Volume of the culture	Number of beads	Percentage of dye decolorized		
				pH 6.0	pH 7.0	pH 8.0
1	100	5	250	78.4	79.2	81.3
2			500	78.9	80.8	83.5
3		10	250	77.1	82.4	84.2
4			500	79.6	82.1	86.1
5	300	5	250	75.6	77.3	80.4
6			500	77.1	78.5	81.1
7		10	250	76.2	79.1	80.9
8			500	78.2	80.5	82.7

CONCLUSION

In conclusion, the selected isolate of *Bacillus megaterium* is good candidate for production of amylase enzyme. The isolated organism is capable of producing amylase enzyme which is a demand of many industries. It was more applicable for many industry especially paper and leather industries, etc. The immobilised enzyme is employed to removal of azo dyes, thus this immobilised enzymes is employed Decolorization of Textile Dyes.

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