INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
Volume-6, Issue-3, July-Sept-2016 Coden: IJPAJX-CAS-USA, Copyrights@2016 ISSN-2231-4490
Received: 24th May 2016 *Revised: 25th June 2016* *Accepted: 28th June 2016*
DOI: 10.21276/Ijpaes *http://dx.doi.org/10.21276/ijpaes* *Research article*

COMMERCIAL PRODUCTION OF PECTINASE USING FLASK FERMENTATION BY BACILLUS CERUS

K.Mehraj Pasha

Department of Humanities and Sciences, Kuppam Engineering College, Kuppam-517425, Andhra Pradesh, India

ABSTRACT: Pectinases which are generally classified into two types, find applications in a number of biotechnological processes used processing, pharmaceuticals, leather and textile industries. In this work the growth characteristics, individual isolate was inoculated into the nutrient broth tubes and incubated at 37°C for 24hours for growth and the growth pattern was observed for pectinase producing bacteria. From these results it was confirmed that the isolate was Bacillus cereus. The maximum Pectinase production was 24.14 % DCW. The optimum temperature for maximum Pectinase accumulation was found to be between 35^0- 40^0C. The addition of Organic Nitrogen sources increased Pectinase accumulation.
Key words: Pectinase, Bacillus cereus, Flask fermentation, physiological parameters

*Corresponding author: K.Mehraj Pasha, Department of Humanities and Sciences, Kuppam Engineering College, Kuppam-517425, Andhra Pradesh, India Email: k.mehraj@gmail.com Mobile: 91-9703398983

INTRODUCTION

Most of the reactions in living organisms are catalysed by protein molecules called enzymes. The role of enzymes in many processes have been known for a long time. The existence of enzymes was associated with the history of ancient Greece where they were using enzymes from microorganisms in manufacturing beer, wine, vinegar production, in bread making, cheese making and in preserving activities [1]. First commercial enzyme was reported from Germany [2]. In recent years, revolutionary progress has been made in the production of beverages, vitamins, organic acids, antibiotics and enzymes on large scale by fermentation processes. Pectinases which are generally classified into two types, find applications in a number of biotechnological processes, viz., in food processing, pharmaceuticals, leather and textile industries [3-6-]. The applications of pectinases will keep increasing in future as with the need for stable biocatalysts capable of withstanding harsh conditions of operation [7, 8]. Since pectinases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals and micro organisms. Micro organisms represent an attractive source of pectinases as they can be grown in large quantities in a relatively short time by established fermentation methods, and they produce an abundant regular supply of the desired product [9]. Furthermore, microbial proteins have a longer shelf life and can be stored under less than ideal conditions for weeks without significant loss of activity. In general microbial pectinases are extra cellular in nature and are directly secreted into the fermentation broth by the organism, thus simplifying downstream processing of the enzyme as compared to pectinases obtained from plants and animals [10]. A large number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline pectinases of the serine type [11]. Relative ease of isolation of Bacilli from diverse sources has made these organisms the focus of attention in biotechnology [12].

The earliest isolate is the *Bacillus stearothermophilus* which is stable at 60^0C. Most commercial pectinases mainly neutral and alkaline pectinases are produced by a variety of micro organisms such as *Bacillus subtilis, Lactococcus, Serratia, Pseudomonas, Aeromonas, Vibrio, E.coli* etc. Bacterial neutral pectinases are active in a narrow pH (pH 5 to 8) environment and have relatively low thermotolerance. Due to their intermediate rate of reaction neutral pectinases generate less bitterness in hydrolysed food proteins than do animal proteinases and hence are valuable for use in the food industry [13-15]. Neutrase, a neutral pectinase, is insensitive to the natural plant proteinase inhibitors and is therefore useful in the brewing industry. The bacterial neutral pectinases are characterized by their high affinity for hydrophobic amino acid pairs.

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 pectinase producing organisms

Sample collection

Soil samples were collected near industries from Hyderabad, for screening of micro flora exhibiting pectinase 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 micro organisms diametere of zone was measured and used for further study.

Purification of the isolates

The selected 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.

Pectinase 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.)

Pectinase 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.

Effect of pH

Effect of various pH levels on Pectinase production was observed by incubating the culture broth at pH levels ranging from 5 to 8 with a difference of 0.5. The different pH levels were adjusted using 2N NaOH(Sodium Hydroxide) to the 50 ml production medium taken in 250 ml conical flask, inoculated with active culture of *Bacillus cereus* and incubated at 37^0 C for 24 hours. The Pectinase production was estimated at an interval of 24^{th} and 48^{th} hour using the procedure mentioned earlier.

Effect of temperature:

Effect of various temperatures on Pectinase production was observed by incubating the active culture broth at various temperatures 25^0 C, 30^0 C, 35^0 C, 40^0 C, 45^0 C and 50^0 C for 48 hours. The cell suspension was collected at an interval of 24^{th} and 48^{th} hour and the Pectinase production was estimated with the procedure mentioned earlier.

Effect of various carbon sources:

The effect of various carbon sources on Pectinase production was observed by incubating the culture in 50 ml production medium-1 containing various carbon sources like Sucrose, Glucose, Galactose, Fructose, Lactose, Xylose etc. in 250 ml conical flasks, incubated with active culture of *Bacillus cereus* strain. The incubation was carried out for 48 hour and the Pectinase production was estimated at an interval of 24^{th} and 48^{th} hour using the procedure mentioned earlier.

Effect of Nitrogen sources

The influence of various nitrogen sources on Pectinase production was investigated by using different nitrogen sources at a concentration of 2gm /l in the medium. The various nitrogen sources tested are Casein, Peptone, Yeast extract, Potassium nitrite, Malt extract and Urea. The production medium with these nitrogen sources was inoculated with active culture of *Bacillus cereus* and incubated at 37^0 C for 24 hrs on shaker at 250 rpm / min. The Pectinase production was estimated at an interval of 24^{th} and 48^{th} hour by using the procedure mentioned earlier.

Effect of various Phosphorous sources

The Influence of various phosphorous sources on Pectinase production was investigated by using various phosphorous sources at a concentration of 1gm/l. The various phosphorous sources used are Na_2HPO_4, K_2HPO_4, NaH_2PO_4 and KH_2PO_4. The production medium with these phosphorous sources was inoculated with active culture of *Bacillus cereus* incubated at 37^0C for 24hrs. the Pectinase production was estimated at interval of 24^{th} and 48th hour using the procedure mentioned earlier.

Effect of Inoculum size

The Pectinase production was studied with various volumes of inoculum. The production medium in different flasks was inoculated with 1%, 2%, 3%, 4% and 5% of active culture and incubated at 37^0C for 24hrs. After incubation, Pectinase production was estimated following the procedure mentioned earlier.

RESULTS

streak plate technique

Color less colonies were observed over the medium. This isolated organism was grown on Nutrient Agar medium by Streak plate technique.

Gram staining

On Gram staining blue colored rods were observed. Hence it is a Gram positive Bacterium.

Catalase activity

After the addition of hydrogen peroxide gas bubbles were observed which is the indication of positive test. Hence *isolated organism* is positive for catalase.

Hydrogen sulphide production test

Black coloration along the line of stab inoculation was not observed. Hence the organism may be H_2S negative

Indole production test

As development of cherry red color is not observed in the top layer of the tube so isolated organism is an indole-positive bacterium.

Methyl-red and voges-proskauer tests

As in the methyl red test red color is not observed hence, it is negative test. In the VP test, red color is observed hence, it is positive test.

Citrate utilization test

From the above observation it is said that *isolated organism* is positive to this test.

Urease test

From the above observation it is said that *isolated organism* shows positive test. Its shows deep pink coloration of the medium thus showing positive reaction for the degradation of urea by means of the production of an enzyme urease.

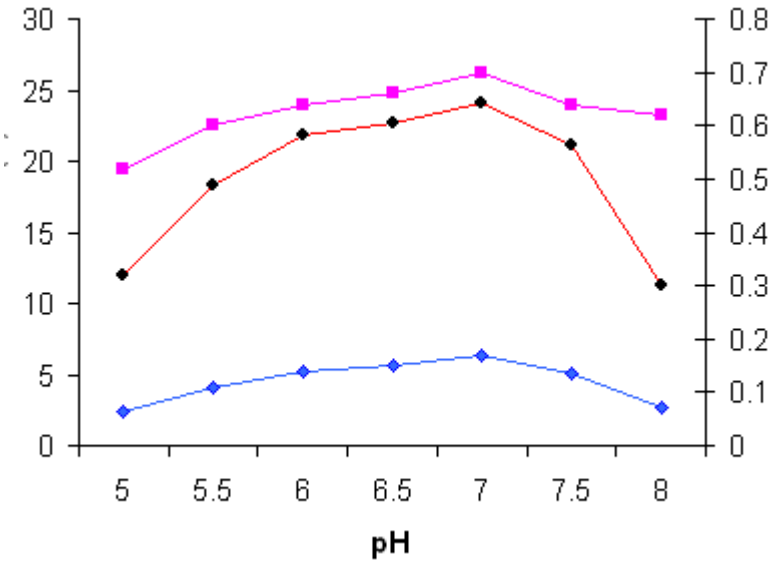
From these results it was confirmed that the isolate was Bacillus cereus.

Optimization of physiological parameters of Bacillus cereus for enhanced production of Pectinase

The various parameter carried out were temperature, pH, carbon, nitrogen, phosphorus, head space and inoculum size.

Influence of pH on production of Pectinase by Bacillus cereus:

The influence of various pH levels on Pectinase accumulation was determined by incubating Bacillus cereus at different pH levels for 24 h at 37^0 C. The pH levels used are 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Among these, the highest Pectinase accumulation was found to be at pH 7.0. pH 6.0 and 6.5 were also found to be suitable for Pectinase accumulation. PH 5.0, 5.5, 7.5 and 8.0 were found to be unsuitable for Pectinase accumulation. The maximum Pectinase production was 24.14 % DCW. The influence of pH on growth and accumulation is shown in (figure 1).



Here

Blue color shows concentration of pectinases. Pink color dry weight of cell.

Orange color shows yield of pectinase.

Then

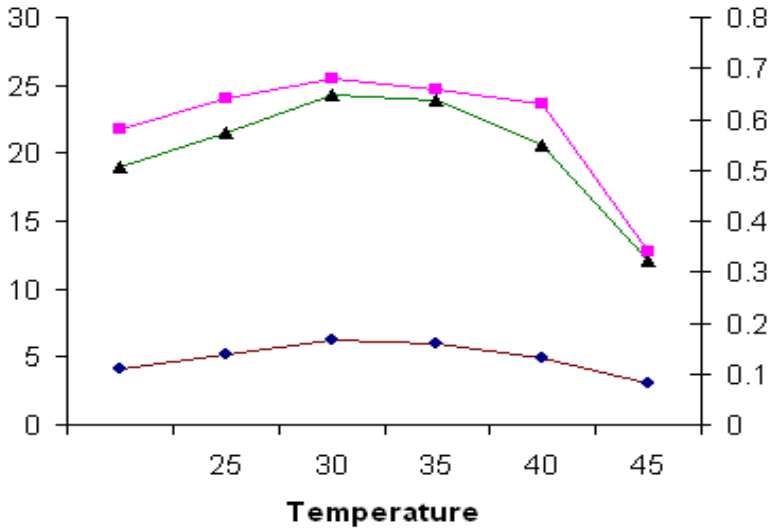
Left side values shows yield of pectinase. Right side values show dry weight of cells

Fig 1: Production of Pectinase by Bacillus cereus at different pH

Influence of Temperature on production of Pectinase by Bacillus cereus:

The influence of temperature on Pectinase accumulation was determined by incubating the culture, Bacillus cereus at different temperatures for 24 h and Pectinase accumulated in the cells was assayed by Law and Slepecky method mentioned earlier. The various temperatures for which the culture is exposed are 25^0 C, 30^0 C, 35^0 C, 40^0C and 45^0C.

The optimum temperature for maximum Pectinase accumulation was found to be between 35^0- 40^0. The temperatures like 25 and 45 suppressed the Pectinase accumulation. The Pectinase yield was approximately 24.26 % DCW at 35^0C (figure 2).



Here

Orange color shows concentration of pectinases. Blue color dry weight of cell.

Green color shows yield of pectinase.

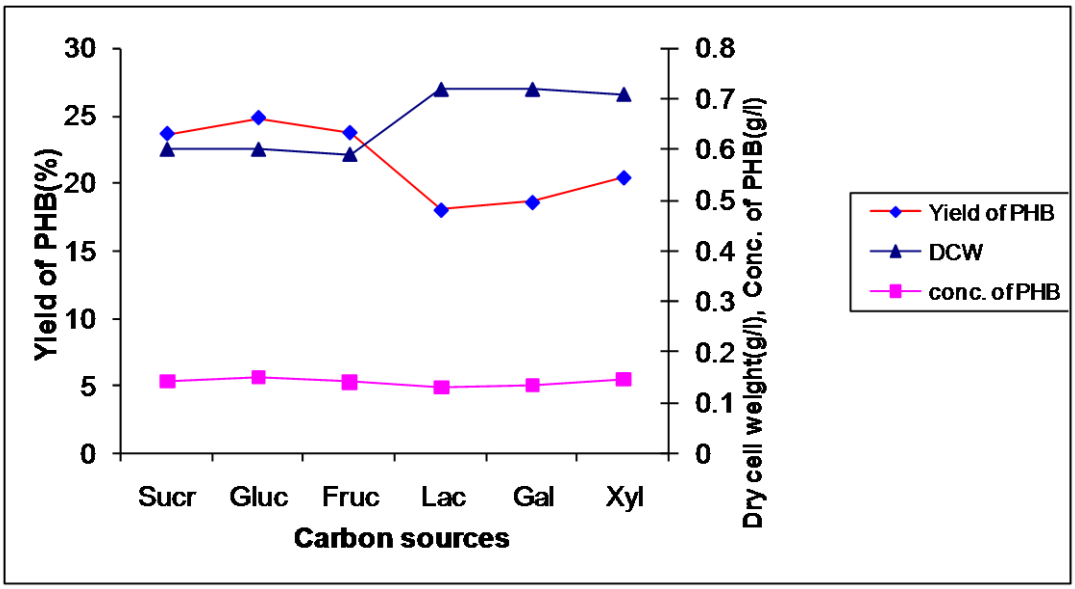
Then

Left side values shows yield of pectinase. Right side values show dry weight of cells

Fig 2: Production of Pectinase by *Bacillus cereus* at different temperatures

Influence of carbon sources on production of PHB by *Bacillus cereus*

The ability of the organism to grow and accumulate PHB appears to the carbon source specific. The effect of carbon sources on PHB production was tested by incubating the culture with different carbon sources at 37^0 C for 24 hours and assayed by Law and Slepecky method mentioned in the materials and methods. The various carbon sources used are Glucose, Fructose, Sucrose, Lactose, Xylose and Galactose at 20 g/l .Among the carbon sources used, Glucose was found to induce the PHB accumulation. Fructose and Sucrose were also suitable for PHB accumulation. Lactose, Xylose and Galactose were found to be less supporting for PHB production (fig 3). The maximum PHB accumulation was approximately 24.83 % with Glucose as carbon source.

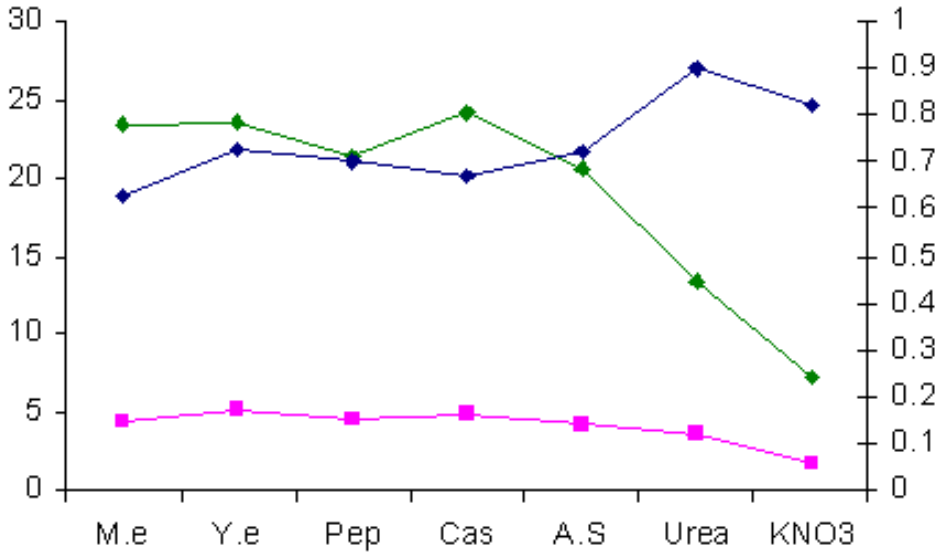


(Where Sucr = Sucrose; Gluc = Glucose; Fruc = Fructose; Lac = Lactose; Gal = Galactose; Xyl = Xylose)
(DCW=Dry Cell weight)

Fig 3: Production of PHB by *Bacillus cereus* with different carbon sources

Influence of Nitrogen sources on production of Pectinase by *Bacillus cereus*

The addition of Organic Nitrogen sources increased Pectinase accumulation. The effect of Nitrogen sources on Pectinase production was investigated by incubating the active culture with different Nitrogen sources at 37^0C for 24 hours and Pectinase accumulated in the cells was assayed by Law and Slepecky method mentioned earlier. The various Nitrogen sources used were Yeast extract, Malt extract, Peptone, Casein, NH_4SO_4, KNO_3 and Urea. Among these sources, Casein was found to induce more Pectinase accumulation when compared to other nitrogen sources (Fig 3.10). Yeast extract, Malt extract, Peptone and NH_4SO_4 were also induced Pectinase accumulation after casein. Inorganic sources like KNO_3 did not support the accumulation of Pectinase. The maximum Pectinase accumulation was found to be 24.18% with Casein as Nitrogen source (fig 4).



Here

Pink color shows concentration of pectinases. Blue color dry weight of cell.

Green color shows yield of pectinase.

Then

Left side values shows yield of pectinase. Right side values show dry weight of cells.

(where M.e = Malt extract; Y.e = Yeast extract; Pep = Peptone; Cas = Casein; A.S = Ammonium Sulphate; KNO_3 = Potassium Nitrite) **(**DCW=Dry Cell weight)

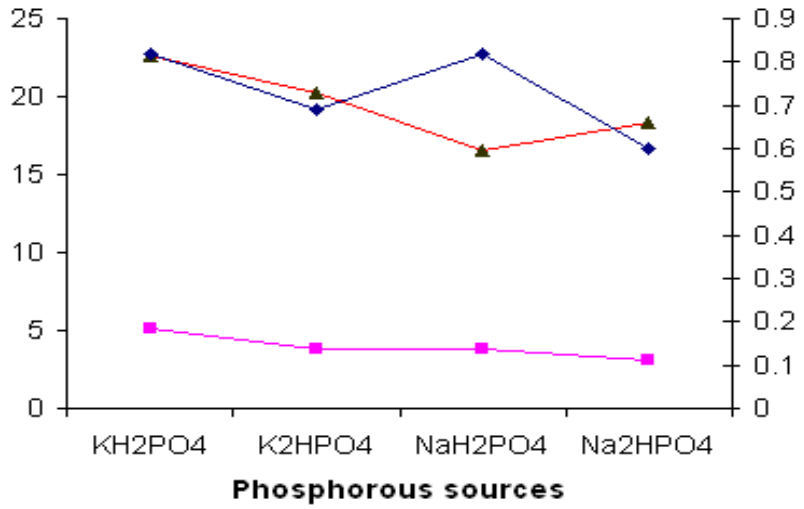
Fig. 4: Pectinase production by *Bacillus cereus* with different Nitrogen sources

Influence of Phosphorous sources on Pectinase accumulation

The influence o various phosphorous sources on accumulation of Pectinase was determine by incubating the culture, Bacillus cereus with different phosphorous sources at 37^0 C for 24 h. The various phosphorous sources used are KH_2PO_4, K_2HPO_4, Na_2HPO_4 and $NaHPO_4$, at a concentration of 1 g/l. Among these sources KH_2PO_4 found to induce more Pectinase accumulation of about 22 %. K_2HPO_4 also supported the Pectinase accumulation. Na_2HPO_4 and NaH_2PO_4 were found to be less inducing Pectinase accumulation. The maximum Pectinase production was approximately 22.56 % (figure 5).

Influence of Inoculum size on production of Pectinase by *Bacillus cereus*

The influence of Inoculum size on Pectinase accumulation was determined by incubating the culture; Bacillus cereus with different Inoculum sizes for 24 h and Pectinase accumulated in the cells was assayed by Law and Slepecky method mentioned earlier. The different inoculum size investigated is 1%, 2%, 3%, 4% and 5% v/v. The maximum Pectinase yield was obtained when 4% and 5% of inoculum v/v was added. The other volumes like 1%, 2% and 3% were found to be less suitable for Pectinase accumulation. The Pectinase accumulation was approximately 24.49 % and 24.10 % DCW at 5% and 4% respectively (figure 6).



Here

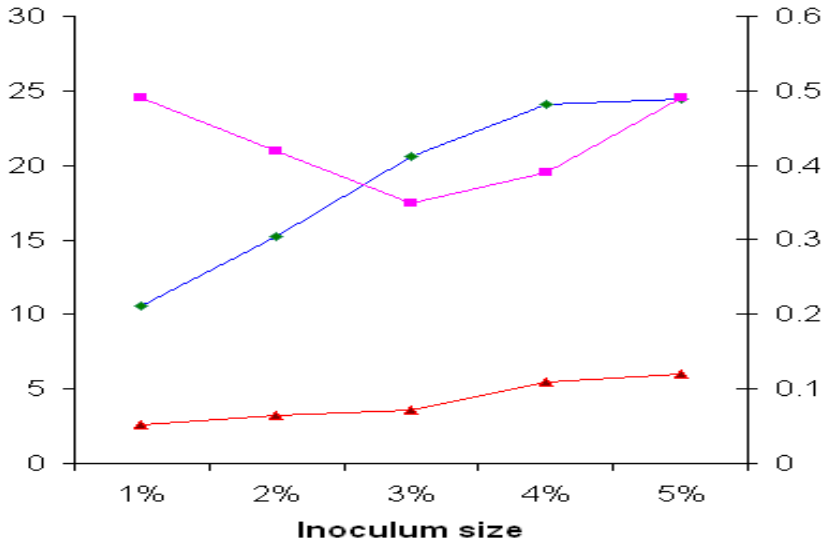
Pink color shows concentration of pectinases. Blue color dry weight of cell.

Orange color shows yield of pectinase.

Then

Left side values shows yield of pectinase. Right side values show dry weight of cells.

Fig.5: Production of pectinase by *Bacillus cereus* with different Phosphorous sources



Here

Red color shows concentration of pectinases. Blue color dry weight of cell.

Pink color shows yield of pectinase.

Then

Left side values shows yield of pectinase. A right side value shows dry weight of cells.

Fig.6: Production of *Pectinase* by *Bacillus cereus* with different Inoculum size

CONCLUSION

Soil is the potential source for isolation of microorganism strains producing pectinases. To accomplish this, a brief study was carried out to know the presence of different bacteria in soil that produce pectinase. An efficient isolate was selected, identified and studied further for pectinase 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 pectinase by the bacillus cereus was studied.

REFERENCES

[1] Riley.R.G., T.G.Garland, K.Shiosaski, D.C.Mann and R.E.Wildung.1981. Alkylpyridines in surface water, Groundwater and subsoil's of a drainage located adjacent to an oil shale facility.Environ.Sci.Technol.15:697-701.

[2] Rogers.J.E., R.G.Riley, S.W.Li., and M.L.O'Malley and B.L.Thomas.1985. Microbial transformation of alkyl pyridines in groundwater Air Soil Pollut.24:443-454

[3] Kuhn.E.P., and J.M.Suffita.1984. Microbial degradation of nitrogen, oxygen and sulfate heterocyclic compounds under anaerobic conditions, Studies with aquifer samples.Environ.Toxicol.Chem.8:1149-1158.

[4] Stuermer.D.H, D.J.Ng, and C.J.Morris.1982. Organic contaminants in groundwater near underground coal gasification site in northwestern Wyoming. Environ. Sci. Technol. 16:582-587.

[5] Maya.J.A.1981. Pyridines in food, J.Agric. Food chem.29:895-898.

[6] Sims.G.K., and E.J.O'loughlin.1989. Degradation of pyridine in the environment .Crit.Rev. Environ. Control.19:309-340.

[7] Kaiser.J.P., and J.M.Bollag.1992. Influence of soil inoculums and redox potential on the degradation of several pyridine derivatives. Soil Biol.Biochem.24:351-357.

[8] Shukla, O.P.1973. Microbial decomposition of pyridine. Indian J.Exp.Biol 11:463-465

[9] Houghton.C., and R.B.Cain.1972. Microbial metabolism of the pyridine ring. Biochem.J. 130:879-893.

[10] Shukla,O.P., and S.M.Kaul.1974. A constitutive pyridine degrading system in Corynebacterium sps.Indian J.Biochem.Biophys. 11:201-207.

[11] Shukla,O.P., and S.M.Kaul.1975. Succinate semi aldehyde,an intermediate in the degradation of pyridine by Brevibacterium sps.Indian J. Biochem.Biophys. 12:326-330.

[12] Sims,G. K.,L. E. Sommers, and A. Konopka.1986. Degradation of pyridine by micrococcus luteus isolated from soil Appl.Environ. Microbiol.51:963-968.

[13] Watson, G.K., and R.B.Cain.1975. Microbial metabolism of the pyridine ring. Biochem. J. 146:157-172.

[14] Shukla, O.P., and S.M.Kaul.1986. Microbiological transformation of pyridine N-oxide and pyridine by Nocardia sps. Can.J. Microbiol.32:330-341.

[15] Rogers, Z., and J.M.Bollag.1991. Pyridine metabolism by a denitrifying bacterium.Can.J. Microbiol. 37:725-729.

INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES

ISSN 2231-4490

International Journal of Plant, Animal and Environmental Sciences