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PRODUCTION OF PECTINASE BY USING *BACILLUS CIRCULANS* ISOLATED FROM DUMP YARDS OF VEGETABLE WASTES

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ABSTRACT: *Bacillus circulans* strain isolated from dump yards of vegetable wastes was tested for its abilities to hydrolyze the structural polysaccharides. The effect of different production parameters such as pH, temperature, carbon source, nitrogen source (Organic and inorganic), incubation time, inoculum sizes and surfactants on pectinase production by the isolated bacterial strain was studied. The enzyme production was assayed in submerged fermentation (SmF) condition. The maximum pectinase production was observed with Galactose (256 U/ml), Yeast extract (130 U/ml), Ammonium sulphate (72 U/ml), pH 7.0 (236 U/ml), Temperature 40°C (126 U/ml), Tween-80 (144 U/ml), Incubation time 48 hours (166 U/ml) and Inoculum size level 5% (136 U/ml) in the production medium.

INTRODUCTION: Enzymes are delicate protein molecules necessary for life. Pectin is a polymeric material having carbohydrate group esterifies with methanol. It is an important component of plant cell wall. It is present in highest concentration in the middle lamella, where it acts as a cementing substance between adjacent cells. Plant pathogens attack target cells by producing number of cell degrading enzyme which facilitates the entry and expansion of pathogen in the host tissue. The history of pectinases began with an understanding the structure of pectin substances and the mechanism by which pectolytic enzymes degrade pectic substances.

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Later the microbial production of pectinases became prominent for many decades. Many microorganisms viz., bacteria, yeast, fungi could produce pectinases [1].

Evidence showed that pectinases are inducible and they can produce from different carbon sources. In the course of time, numerous reports have appeared on the optimization of fermentation and microbiological parameters and different fermentation strategies for the production of pectinases.

With the advent of molecular biology, vigorous research has been carried out on cloning and expression of pectinase genes in various hosts. Among the various pectinase, bacterial extracellular pectinase are the most significant, compared with animal, plants, viruses and fungal extracellular pectinase. Extracellular pectinase produced by *Bacillus* and *Cocci* species are of main interest from a biotechnological perspective, and are not only in scientific fields of protein chemistry and protein

engineering but also in applied fields such as foods, pharmaceutical and paper industries. These pectinases account for 10% of the total worldwide production of enzymes [2].

The genus *Bacillus* and *Cocci* contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus* and *Cocci*. Pectinases are the single class of enzymes which play an important part in the metabolism of almost all organisms (plants, animals, fungi, bacteria and viruses). Investigation of Pectinases is a central issue in enzymology due to their wide applications in Pharmaceutical, Food, Agricultural products and Bioremediation process.

Pectinase (EC 3.2.1.15) belongs to the class hydrolase which are able to hydrolyse pectin more efficient than other Pectinases and their action are very specific, i.e., they acts only on pectin substrates. Pectin is a major constituent of cereals, vegetables, Pectin fibers are complex, high molecular weight heterogeneous and acidic structural polysaccharide. D-galacturonic acid is one of the major components of pectin [3].

The present study was mainly focused on the production of pectinase form *Bacillus circulans* by optimizing various parameters such as carbon sources, inorganic nitrogen sources, organic nitrogen sources, pH, temperature, substrate concentration, inoculums concentration, incubation time and surfactants.

MATERIALS AND METHODS:

Collection and isolation of sample: Samples were collected from dump yards of vegetable wastes at Solddevanahalli, Chikkabanavara, Devasandra, K.R. Puram, Tannary road and Yashwanthpur in and around Bangalore, Karnataka, India. The samples were labelled after collected. These were spread onto isolation media pectinase screening agar medium and incubated at 37^{o}C for 24 hours after serial dilution of 10^{-1} to 10^{-6}.

TABLE 1: TABULATION FOR SAMPLES DESCRIPTION

S. NO.	Designation of sample	Sample collected area	Sample collected land mark	Sample nature	Sample pH
1	AP-1	Shivaji Nagar	Opposite to Masjid vegetable center	Semisolid sticky Seems to Brown in color	7.64
2	AP -2	Tannery Road	Near to Bus stop vegetable center	Semisolid Seems to Black in color	7.60
3	AP -3	Tannery Road	Opposite to Bus stop vegetable Center	Semisolid Seems to Brown in color	7.72
4	AP -4	Tannery Road	Slaughter house opposite vegetable center	Hard consist clay seems to Brown in color	7.65
5	AP -5	Solddevanahalli	Near to Bus stop vegetable Center	Semisolid Seems to Brown in color	7.62
6	AP -6	Chikka Banavara	Near to Bus stop vegetable Center	Sticky consist clay seems to Brick red in color	7.44
7	AP -7	K.R.Puram	Devasandra vegetable dump	Semisolid Seems to red in color	7.71
8	AP -8	Tin Factory	Opposite to Maszid vegetable center	Semisolid Seems to red in color	7.60
9	AP -9	Tin Factory	Near to Bus stop vegetable Center	Hard consist clay seems to Black in color	7.26
10	AP -10	Yashwanth Pura	Opposite to Fish market vegetable center Near to Railway station	Sticky consist of sand and clay seems to Black	7.34

Isolation of Pectinase Producing Microorganisms: The isolates were screened for pectinase activity. This was done by inoculating the organisms on the pectinase screening agar medium (PSAM) plates containing 1gm pectin, 0.3gm Diammonium orthophosphate, 0.2gm KH_2PO_4, 0.3gm K_2HPO_4, 0.01 gm $MgSO_4$ and 2.5 gm agar in 100 ml, the initial pH of medium was adjusted to 7 and incubated at 37^{o}C for 24 hrs. The plates were flooded with 50 mM iodine solution and incubated for 15 min at 37^{o}C. A clear zone around the growth of the bacteria was indicated to pectinase activity [4].

TABLE 2: TABULATION FOR RESULTS OF COLONY CHARACTERISTICS WHICH SHOWS PECTINASE ACTIVITY

Strain no.	Colony surface	Colony color	Visual characteristics	Shape of the colony	Height of the colony	Pectinase Activity
G-1	Smooth	Brown	Opaque	Irregular	Raised	Positive
G-2	Smooth	Off white	Translucent	Circular	Raised	Positive
G-3	Smooth	Brown	Translucent	Irregular	Flat	Positive
G-4	Smooth	Off white	Opaque	Irregular	Raised	Positive
G-5	Smooth	Brown	Opaque	Irregular	Raised	Positive
G-6	Smooth	Brown	Translucent	Irregular	Flat	Positive

Morphological and Biochemical Characteristics: Gram staining, motility, indole production, methyl red, Voges Proskauer's, citrate utilization, triple sugar iron, nitrate reduction, catalase, oxidase, gelatin liquefaction, urease, hydrolysis of casein, hydrolysis of starch were carried out [5] .

TABLE 3: TABULATION FOR RESULTS OF STAINING TECHNIQUES

Strain no.	Gram staining	Morphology (*Bacillus/Cocci*)	Endospore staining	Capsule staining
G-1	Positive	Rods	Positive	Positive
G-2	Positive	Rods	Positive	Positive
G-3	Positive	Cocci	Positive	Positive
G-4	Positive	Rods	Positive	Positive
G-5	Positive	Rods	Positive	Positive
G-6	Positive	Cocci	Positive	Positive

TABLE 4: TABULATION FOR RESULTS OF VARIOUS BIOCHEMICAL TESTS

S. No.	Samples	Indole	Mr	Vp	Amylase	Nitrate	Oxidase	Catalase	Urease	Gelatinase	Pectinase
1	G-1	-Ve	-Ve	-Ve	+Ve	-Ve	+Ve	+Ve	+Ve	+Ve	+Ve

Pectinase Enzyme Assay: Pectinase was assayed according to Pitt (1988). The reaction mixture consisted of 5.0 mL of 1.0 % pectin solution in 0.05 M Tris-HCl buffer, pH 8.5; 1.0 mL of 0.01 M $CaCl_2$; 1.0 mL of crude enzyme and 3.0 mL of distilled water. After incubation for 2 hours at 30ºC in water bath, 0.6 mL of 9 % $ZnSO_4.7H_2O$ and 0.6 mL of 0.5 M NaOH were added. The precipitated proteins were removed by centrifugation at 3,000 g, for 10 min. A volume of 1.0 mL of supernatant was removed and added into a mixture composed by 3.0 mL of 0.04 M thiobarbituric acid; 1.5 mL of 0.1 M HCl and 0.5 mL of distilled water. After boiling for 30 min. and posterior cooling, the absorbance was measured at 550 nm. A unit was defined as the amount of enzyme that changes 0.1 absorbance unit, under the assay conditions [6].

Protein Estimation of Crude Enzyme: The protein content of the crude enzyme was assayed by using bovine serum albumin as the standard. Total activity and total protein are represented by U/mL and mg/mL, respectively, multiplied by the total volume of enzyme extract [7].

Medium Optimization for Pectinase Production: Carbon Source: To identify the suitable carbon sources for pectinase production by the *Bacillus circulans*. The following different carbon sources were tested such as glucose, sucrose, maltose, lactose, galactose, fructose and dextrose with sample concentration of 0.5% in the optimized carbon sources in production medium at 37°C [8].

Organic and Inorganic Nitrogen Sources: The pectinase production by the selected bacterium was also optimized by supplementing different organic 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 [9].

Effect of pH: The effect of pH for pectinase 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, 9 and 10. The enzyme assay was carried out after 72 hours of incubation at 37°C [10].

Effect of Temperature: Temperature is an important role for the production of pectinase. The effect of temperature on pectinase production was studied by the incubating the culture media at various temperatures 10, 20, 30, 40, 50, 60,70 and 80°C along with arbitrary control at 37°C [10].

Effect of Surfactants: To identify the surfactants facilitating pectinase production, four different surfactants were used for experimentation. They were Tween-20, Tween-80, SDS (Sodium dodecyl sulphate) and PEG (Poly Ethylene Glycol). The surfactants were tested individually at the concentration of 0.2% in the optimized production medium [11].

Effect of Various Incubation Times on Pectinase Production: The pectinase 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 times such as 24, 48, 72, 96 and 120 hours. The enzyme assay was carried out individually after 72 hours of incubation [12].

Effect of Various Inoculum Concentrations on Pectinase Production: The pectinase production by the selected experimental microorganisms was determined by adding bacterium at different inoculum's concentrations such as 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 % to test its ability to induce pectinase production in the production medium [13].

RESULTS:

Screening of Pectinase Producing Bacteria from Dump yards of Vegetable wastes: The bacteria isolated from dump yards of vegetable wastes were screened for pectinase production on pectinase screening agar medium (PSAM). From the soil samples 10 bacterial strains were isolated. But later during screening it was found that only 6 strains showed pectinase activity. Later only one potential isolate was identified by standard morphological and biochemical characterization. After careful biochemical tests, it was confirmed that the isolate was *Bacillus circulans.*

Effect of Carbon Sources on Pectinase Production: Chart 1 shows the effect of carbon sources on pectinase production after 48 hours of incubation period at 37°C. The maximum pectinase production was recorded in galactose (256U/ml) supplemented medium and minimum pectinase production was recorded in glucose (40U/ml).

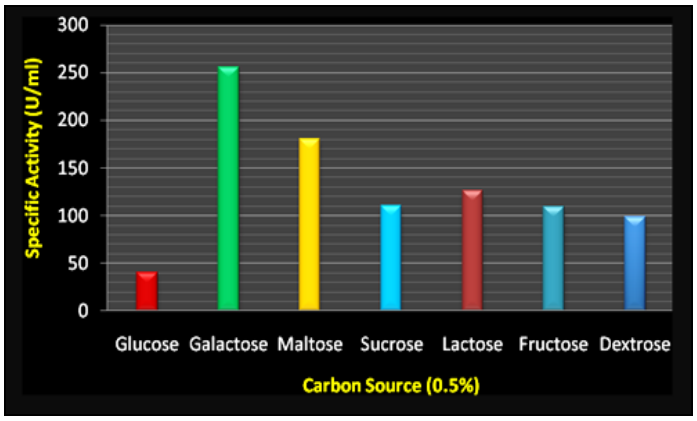

CHART 1: EFFECT OF VARIOUS SUBSTRATES ON PECTINASE PRODUCTION BY *BACILLUS CIRCULANS* UNDER SMF

Effect of Inorganic Nitrogen Sources on Pectinase Production: Chart 2 shows the effect of different kinds of inorganic nitrogen sources on pectinase production after 48 hours of incubation period at 37°C. The maximum amount of enzyme production was observed in ammonium sulphate (72U/ml) supplemented medium and minimum amount of pectinase production was observed in sodium nitrate (46U/ml) supplemented medium.

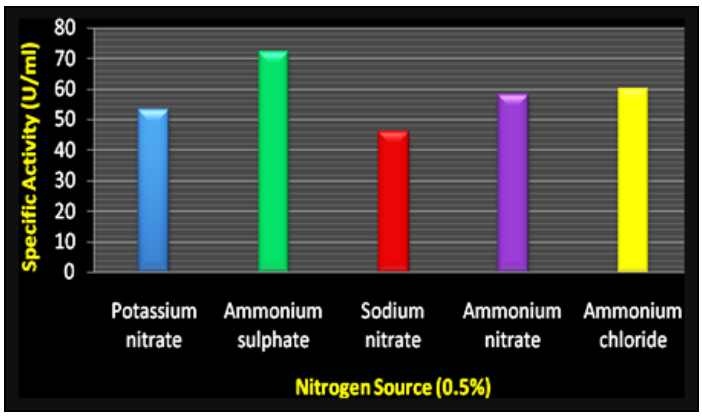

CHART 2: EFFECT OF VARIOUS INORGANIC NITROGEN SOURCES ON PECTINASE PRODUCTION BY *BACILLUS CIRCULANS* UNDER SMF

Effect of Organic Nitrogen Sources on Pectinase Production: Chart 3 shows the effect of different kinds of organic nitrogen sources on pectinase production after 48 hours of incubation period at 37°C. The maximum amount of pectinase production was observed in yeast extract (130U/ml) with supplemented medium and minimum enzyme activity was observed in gelatin (61U/ml).

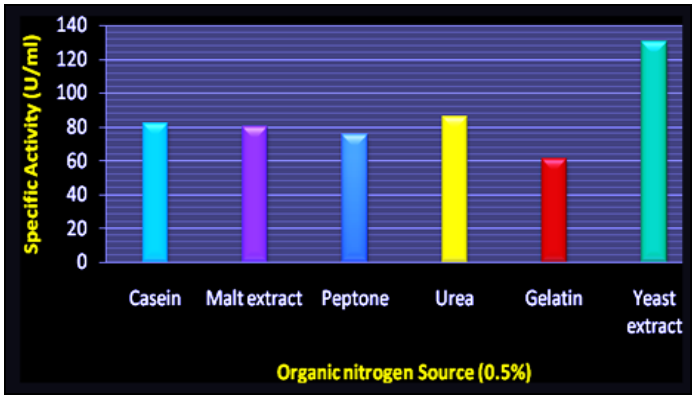

CHART 3: EFFECT OF VARIOUS ORGANIC NITROGEN SOURCES ON PECTINASE PRODUCTION BY *BACILLUS CIRCULANS* UNDER SMF

Effect of pH on Pectinase Production: Chart 4 shows the effect of various pH on pectinase production after 48 hours of incubation period at 37°C. The maximum pectinase production was observed at pH 7.0 (236U/ml) and minimum amount of pectinase production was recorded at pH 10 (72 U/ml).

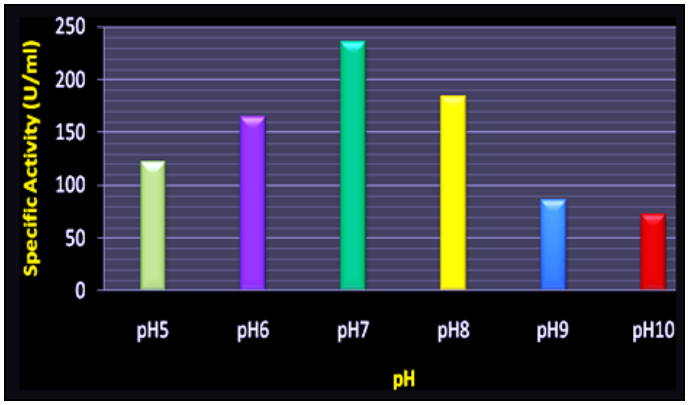

CHART 4: EFFECT OF VARIOUS pHS ON PECTINASE PRODUCTION BY *BACILLUS CIRCULANS* UNDER SMF

Effect of Temperature on Pectinase Production: Chart 5 shows the effect of various temperatures on pectinase production. The maximum pectinase production was obtained at 40°C (126U/ml). Followed by this, 50°C temperature (107U/ml) was the second best temperature on pectinase production. On the other hand, the minimum amount of pectinase production was observed at temperature 80°C (27U/ml).

Effect of Surfactants on Pectinase Production: Chart 6 shows the effect of various surfactants on pectinase production after 48 hours of incubation at 50°C. The maximum amount of enzyme was recorded in Tween-80 (144U/ml) and minimum amount of pectinase was observed in PEG (90U/ml).

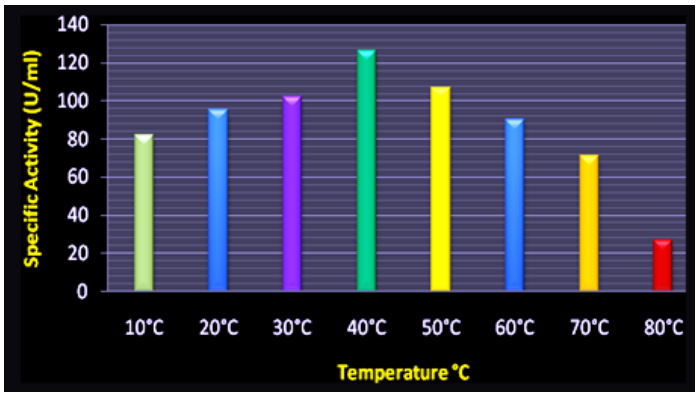

CHART 5: EFFECT OF VARIOUS TEMPERATURES ON PECTINASE PRODUCTION BY *BACILLUS CIRCULANS* UNDER SMF

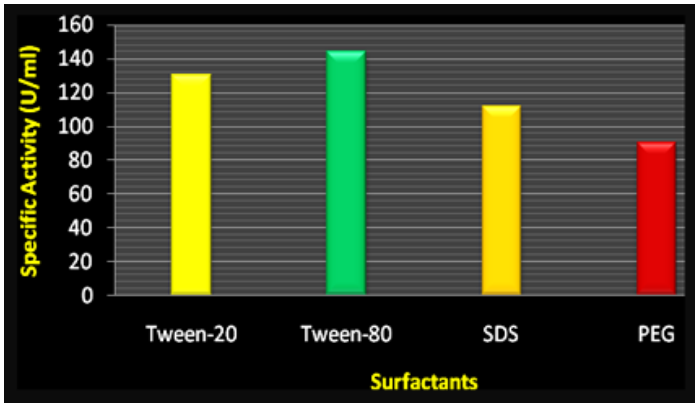

CHART 6: EFFECT OF VARIOUS SURFACTANTS ON PECTINASE PRODUCTION BY *BACILLUS CIRCULANS* UNDER SMF

Effect of Incubation Time on Pectinase Production: Chart 7 illustrates the effect of different incubation times on pectinase production. The maximum amount of pectinase production was observed with 48 hours incubation time (166U/ml). The minimum amount of pectinase production was obtained with 120 hours incubation (98U/ml).

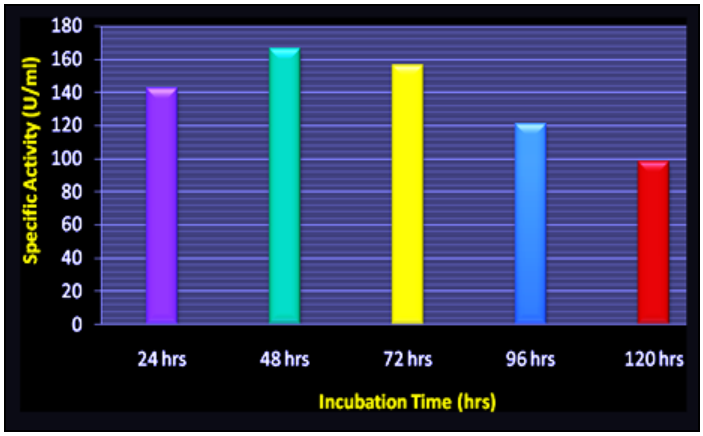

CHART 7: EFFECT OF VARIOUS INCUBATION TIMES ON PECTINASE PRODUCTION BY *BACILLUS CIRCULANS* UNDER SMF

Effect of Various Inoculum Sizes on Pectinase Production: In the present study, the initial inoculum level has played an important role in pectinase production by *Bacillus circulans*. The maximum pectinase specific activity was registered at the 5% (136U/ml) of inoculum level. On the other hand, the minimum amount of pectinase production was observed at 2% of (51U/ml) inoculums level (**chart 8**).

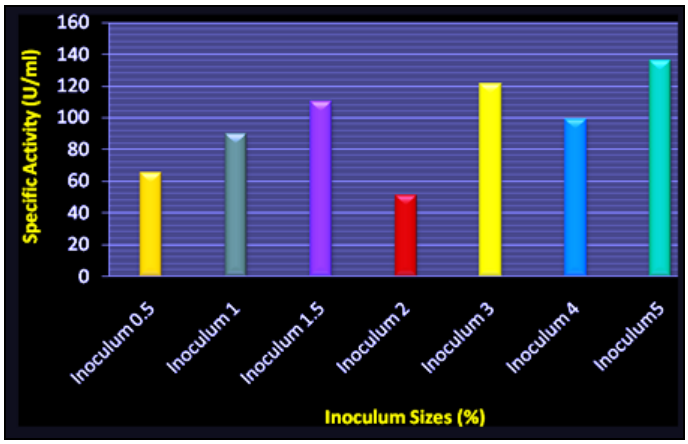

CHART.8: EFFECT OF VARIOUS INOCULUM SIZES ON PECTINASE PRODUCTION BY *BACILLUS CIRCULANS* UNDER SMF

DISCUSSION: The addition of carbon source in the form of either monosaccharide or polysaccharides may influence the production of pectinase enzyme. In our present study, the influence of galactose was more (256 U/ml) than the other carbon sources tested. Maltose was the second best supplementary carbon source (180 U/ml). Glucose gave the lowest pectinase enzyme activity (40U/ml). Kilara *et al.,* [14] reported that the different carbon sources have varied influence on the production of extracellular enzymes especially pectinase strains. These results are similar to the findings of Ward *et al.,* [15] who observed that glucose represses the production of pectinase in the *Aspergillus niger C28B25*. According to them glucose prevented pectinase gene expression and not merely secretion of performed enzyme.

In the present study, ammonium sulphate was found to be the most suitable inorganic nitrogen source for *Bacillus circulans* and the enzyme activity observed was 72 U/ml. The lowest pectinase production was observed in sodium nitrate (46 U/ml) supplied medium. Lonsane *et al.* [16] reported that ammonium salts enhanced the enzyme activity. Sodium nitrate showed a negative influence, showing a steep decrease in "pectinase activity.

McMillan [17] also reported that nitrate was inferior to ammonia in pectinase production. Ammonium sulfate, sodium nitrate and ammonium nitrate (inorganic nitrogen sources) inhibited the enzyme production by *P. chrysogenum* under SSF.

The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The nature of the compound and the concentration that we used might stimulate or down modulate the production of enzymes. In the present study experiment on the effect of supplementary nitrogen sources on pectinase production under SSF, showed that yeast extract was found to be a better nitrogen source for this isolate (130U/ml).

Yeast extract is the best nitrogen source for pectinase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components [18] where it was found that the pectinase production by *Aspergillus oryzae* under SSF of sugar cane bagasse was greatly influenced by organic nitrogen sources especially yeast extract. The pectinase production by *A. oryzae* was also reported as high in yeast extract and casein [19]. Pandey [20] reported that peptone gave an increase in enzyme yield in SSF using coconut oil cake as substrate.

The effect of initial pH on SSF of pectinase showed that the pH range of 5-7 produced more amount of pectinase and it was relatively high in pH 7.0 (236U/ml) and pH 8 (184U/ml).Above this level, the pectinase production decreased, because the metabolic activities of microbes are very much responding to pH change.

Mrudula *et al* stated that at high pH, the metabolic action of bacterium may be suppressed and thus it inhibits the enzyme production. Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the bioprocess are pH, temperature, aeration and agitation [21]. In the present study, the effect of temperature on pectinase enzyme activity by SmF revealed that 40°C was optimum (126U/ml) and at the tested higher temperatures, the enzyme production decreased which might be due to growth reduction and enzyme inactivation or suppression of cell viability [22].

A similar result was reported by Smith [22]. In contrast, low temperature values may reduce the metabolism of the microorganism and consequently, the enzyme synthesis. Surfactants in the fermentation medium are known to increase secretion of proteins by increasing the cell membrane permeability. In the present study, the addition of Tween-80 increases the pectinase production for *Bacillus circulans* (144U/ml).

The effect of incubation time on pectinase production showed that 48 hours was the optimum duration for maximum pectinase enzyme activity (166U/ml). Above this period the pectinase enzyme activity started to decrease. This is because, the cells may reach the decline phase and displayed low pectinase synthesis.

Since the carbon source represents the energetic source that is available for the growth of the microorganism, it could be that the enzyme production is associated and the presence of galactose in the medium stimulated the increased production of the enzyme. An inoculums concentration higher than the optimum value may produce a high amount of biomass which rapidly depletes the nutrients necessary for growth and product synthesis.

On the other hand, lower inoculums levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium. This increases the necessary time to grow to an optimum number to consume the substrate and synthesize the desired product.

In the present study, the highest enzyme activity (136U/ml) was obtained at an inoculums level of 5% by *Bacillus circulans* under SmF. Sharma *et al.*, [23] reported that *Bacillus pumilus* showed increased enzyme production with the increase in inoculums size from the lowest value of 0.5ml and showed maximum enzyme activity at 2ml inoculums.

CONCLUSION: The above report stated the evidence for the production of pectinase with substrate interactions of bacterial strains with simple and effective manner. More over this study gives us values as well as the microbial wealth of pectinase producing bacteria which can be boon for the development of biotechnological processes.

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