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"ISOLATION, SCREENING AND OPTIMIZATION OF NOVEL PECTINASE PRODUCING FUNGAL STRAIN FOR FRUIT JUICE CLARIFICATION AND EXTRACTION"

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ABSTRACT

Microbes are the major source of the enzymes produced industrially. Pectinase share about 10% of the total enzyme production on global scale. Our study is also focussed on pectinase enzyme production by more efficient method and optimising the conditions required for the best activity of the enzyme. In the present study results showed that solid substrate fermentation is best enzyme production method for fungus and orange peel (0.76 IU/ml) proved to be the best substrate with 24 hr (0.75 IU/ml) of incubation with 5ml inoculum (0.80 IU/ml) at 30°C (0.80 IU/ml) and 4 pH(0.76 IU/ml).Activity of pectinase was later calculated for all the parameters. After optimisation, characterisation and calculation of enzyme activity was done and isolated pectinase enzyme was used for clarification of fruit juices and also for extraction of juice from pulp.

KEYWORDS: Pectinase, fermentation, enzyme activity, galacturonic acids, Hydrolysis.

INTRODUCTION

Enzymes are very well established product in biotechnology. These are the bio-active compounds that regulate many chemical changes in living tissues. Among the various enzymes commercialized many are products of fermentation of filamentous fungi [1]. Bulk of the industrial enzymes fall into different groups, out of these the most important group of enzymes used in fruit and vegetable processing industry is pectinases. Pectinases (E.C 3.2.1.15) were some of the first enzymes to be used in homes. Their commercial application was first observed in 1930 for the preparation of wines and fruit juices[2]. Pectinase (E.C

3.2.1.15) is a general term used for a mixture of several enzymes, which break down pectin. Pectin is a high molecular weight polysaccharide, primarily made up of α (1→ 4) linked D-galacturonic acids and are found in the middle lamella and primary cell wall of higher plants[3]. These enzymes are classified based on their preferred substrate (pectin, pectic acid or oligo-D-galacturonate), the degradation mechanism (transelimination or hydrolysis) and the type of cleavage (random [endo] or terminal[4] [exo]). Based on the type of modifications of the backbone chain, pectic substances are classified into protopectin, pectic acid, pectinic acid and pectin[5].Pectinases are the acidic polysaccharides consisting of 3 main classes. They include polymethyl esterase's (E.C 3.1.1.11) (PME), Polygalacturonase's (E.C3.2.1.15) (PG), and Pectate lyase's (E.C 4.2.2.10) (PAL) . Polygalacturonases causes the breakdown of α (1-4) -glycoscidic linkage between the Galacturonic acid residues. Pectate lyase acts on pectin eliminating oligosaccharides of α (1-4) linked galacturonic acid residues. Poly methyl esterases act on pectin methyl esters releasing methanol[6]. Commercial enzyme preparations used in processing of food, traditionally, comprising of the mixtures of polygalacturonase, pectatelyase, and pectin esterase, are almost exclusively derived from fungal species, especially *Mucor and Aspergillus*[7].In this context, the objective of the present study is "**Isolation, screening and optimization of novel pectinase producing microbial strain for fruit juice clarification and extraction.**"to produce pectinolytic enzymes by a fungal species and to study the production pattern in detail.

Table 1. Pectinolytic fungi and substrate used.

Sr no	Fungal strain	Substrate
1	*Aspergillus carbonicus*	Wheat bran
2	*Aspergillus niger*	Wheat bran and Glucose
3	*Fusarium moniliforme*	Wheat bran and orange pulp
4	*Penicillium viridicatum*	Orange bagasse and wheat bran
5	*Thermoascus auriantacus*	Wheat bran or orange bagasse
6	*Aspergillus niger*	Sugarcane bagasse and pectin

MATERIAL AND METHODS

1) Collection of soil sample: Various pectin rich rotten fruits and vegetables have been targeted for isolation of pectinase producing fungus.

2) Isolation and screening of pectinase producing microbes. 1 g of each pectin rich fruits, vegetables and soil sample were dissolved in 9 ml of distilled water and mixed thoroughly.

The serially diluted sample were then plated on potato dextrose agar plates at 30ºº C for 48h for fungal isolates and on Nutrient agar plates at 37ºº C for 24h for bacterial isolates. Enzyme production was identified by clear zone around colonies of pectinase producing microbes. Isolated predominant, morphologically distinct colonies were selected and all isolates were titrated to calculate the enzyme activity.

3) Determination of Enzyme activity

Pectinase activity was assayed by the colori-metric method of (Miller, 1959).Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method. For this, to 0.2 ml of 1% pectin solution, 2.0 ml of sodium citrate buffer of pH 5.0 and 1.0 ml of enzyme extract was added. The reaction mixture was incubated at 35°C±1°C for 25 min After 25 min, 1.0 ml of this reaction mixture was withdrawn and added to test-tubes containing 0.5 ml of 1M sodium carbonate solution. To each test-tube, 3.0 ml of DNS reagent was added and the test-tubes were shaken to mix the contents. The test-tubes were heated to boiling on the boiling water-bath for 10–15 min. Then these were cooled and 20 ml of distilled water was added to the contents of each tube and the absorbance was measured at.570 nm using Spectrophotometer. The enzyme and substrate blanks were run parallel. The standard curve was prepared for reducing sugars with glucose. One enzyme unit of endopolygalacturonase is the number of µM of reducing sugars measured in terms of glucose, produced as a result of the action of 1.0 ml of enzyme extract in 1 minute at 35°C ± 1°C.

4) Protein Estimation: protein was estimated by lowery method.

5) Characterisation: Isolated predominant, morphologically distinct colonies were selected and all isolates were identified on the basis of cultural , morphological ,and biochemical characteristics.

6) Physical characterisation; Hyphal morphology, shape,diameter ,colour ,and appearance were observed in overnight plate culture on media and noted down.

7) Fungal morphology: Prepare a wet mount of each yeast culture in following manner. Suspend a loopful culture of fungal strain in few drops of lactophenol cotton blue solution on a microscope slide and cover with a coverslip.

8) Growth curve of Fungus: A loopful culture of the fungus was inoculated in the sterile 250ml PDB production media and was kept for incubation. Sampling was done from the flask at constant interval of time(After 24 hrs) and the growth pattern of the fungus was observed after plotting the growth curve.

Optimisation of fermentation method for fungal strain for profound enzyme activity: Two types of fermentation methods exists for the microbial strain development either solid substrate fermentation or submerged fermentation. The optimisation of fermentation method for fungal activity was determined using both solid substrate fermentation technique and submerged fermentation method.

Submerged fermentation: For submerged fermentation, different media were employed for fungal growth. P.D.B, S.D.B and N.B were inoculated with 5ml of seed culture and were then incubated for 5 days at 30°C .The reaction mixtures were then used to determine the profound enzyme activity by assay of the mixture.

Solid-substrate fermentation: For solid–substrate fermentation, various solid substrate media were employed which includes Orange peel, Wheat bran and Sugarcane bagasse. The powered substrates (5g each) were added 10ml sterile solution composed of $Cuso_4.5H_20(0.5)$,$MnSO_4.7H_2O(0.01)$,$FeSO_4.7H2O(3.00)$, $NaNO_3(1.0)$, $ZnSO_4(0.50)$ in 1000 ml phosphate buffer (pH6.2) and the prepared media was inoculated for 15 days. Then 30 ml distilled water was added to the flasks and filtered. The collected filtrate was centrifuged at 5000 rpm for 20 min and then cell free supernatant was the crude enzyme for fermentation medium. Solid state fermentation carried out for 15 days using 3 substrates incubated for regular interval of 5,10,15 days respectively and enzyme production & enzyme activity determined.

Enzyme production

The inoculum was prepared by inoculating the loopful culture of strain in P.D.B and it was incubated for 24h. 5ml of this seed culture was transferred aseptically to 250ml production medium (g/l).Enzyme production was carried out using solid substrate fermentation method using Orange Peel as substrate.

9) Optimisation of parameters for crude pectinase enzyme using SSF

Effect of varying substrates: For maximum pectinase activity various substrates were employed. The substrate employed was rich in pectin like orange peel, wheat bran, sugarcane bagasse. The media containers were incubated at desired parameters and then the assay was performed. Substrate showing maximum production of enzyme was selected for further screening.

Effect of varying inoculum size on pectinase production: The media composed of orange peel as substrate was inoculated with varying amount of seed culture(5 ml,10 ml,15 ml and20 ml). After inoculation the flasks were incubated at 30°C for 5 days and then the maximum production will be observed by DNS method of enzyme activity determination.

Thermostability of pectinase: The thermostability of pectinase fraction was studied by incubating the enzyme at different temperatures (25°C-60°C) for 1h. The residual pectinolytic activity was determined using orange peel as substrate.Then assay was carried out under standardized conditions.

pH stability of enzyme: For pH stability enzyme extract was incubated using different pH buffers (4-9) for1h .The reaction mixtures were incubated as per standard assay and residual pectinolytic activity was then determined using orange peel as substrate.Then assay was carried out under standardized conditions.

Effect of time of incubation: For studying effect of time of incubation the enzyme extract was incubated at various time intervals (5,15,30,45,60 min) and as per standard enzyme assay, the residual enzyme activity was determined using orange peel as substrate. Then assay was carried out under standardized conditions.

Effect of Metal ion on enzyme production: For increasing the yield of enzyme by the fungal strain mill molar (1mM, 3mM, 5Mm) solution of various metal ions were prepared. The metal ions include Zn^{2+}, $Ba3^{+}$,Ca^{2+} and $Cu^{2+}$and later their effect was studied on enzyme production. Then assay was carried out under standardized conditions.

RESULTS

Isolation of micro-organism from fruit, vegetable and soil samples: Isolation was done using pectin rich fruits, vegetables and soil sample dumped with kitchen wastes. Total 40 fungal species were isolated Liquid medium containing pectin and inoculated with culture

medium was incubated for 7 days and sampling was done every day and further enzyme assay was performed. The pectinase activity was measured using DNS method of enzyme assay. Out of total 40 isolated fungal strains, Maximum enzyme production recorded in case of strain 11 (fungal strain) i.e. 0.53IU/ml. (Fig.1)

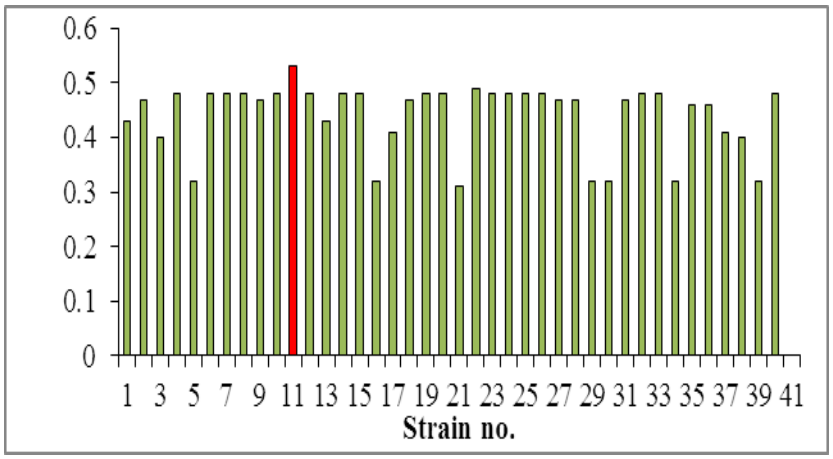


Fig 1: All recovered fungal isolates with their enzyme activity.

Growth pattern of fungus under submerged fermentaion condition: The growth pattern of fungus was observed by plotting the growth curve. This growth curve depicts that maximum fungal growth was observed on 6th day of incubation i.e (2.557 Abs) and later the growth of fungal strain decreases under optimized conditions.The growth curve was plotted after taking the absorbance at 660 nm.**(**Fig 2).

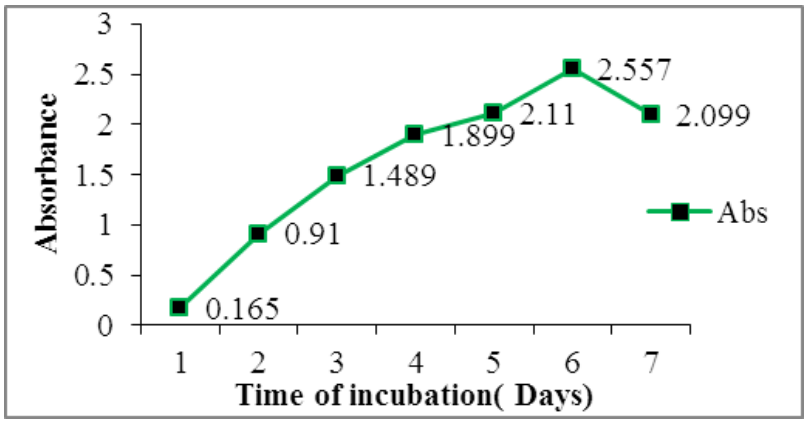


Fig 2. Growth curve of fungus (Hyper-enzyme producer)

Characterisation of hyper-producer pectinase strain (11): Out of these 40 fungal, the best strain that produced highest amount of pectinase and showed highest level of enzymeactivitywas selected. Out of the fungal isolates the fungal Strain 11 produced highest levelofdesiredenzyme and showed the best enzyme activity (0.53 IU/ml) of all the other.

Fig 3 A) Initial growth of fungus

Fig 3 B) Mature fungal hyphae

Morphological characterisation: The recovered fungal isolate (Strain 11) was characterised morphologically on the basis of colour, growth pattern, and microscopic appearance (Table 2.), (Fig 3A and 3B).

Table 2. Morphological characterisation

Sr no.	Colony characterisation	Observation
1	Colour	Creamywhite and turned to hairy cottony growth as it matures.
2	Size	Irregular.
3	Arrangement	Flat over entire plate.

Staining: Staining of culture with Lactophenolcotton blue affirms that the isolate was having non septate mycelium, Oval sporangiophore, Brown spores and the rhizoids were absent. It was then suspected to be *Mucor* species (Table 3.)

Table 3. Microscopic appearance

Mycelium	Sporangiophore	Spores	Rhizoids
Septate	Oval	Brown	Absent

Optimization of fermentation method used for fungal pectinase production: Fungal pectinase production was observed to be more in case of solid substrate fermentation as compared to submerged fermentation. Solid substrate fermentation was carried out using three different substrates. In submerged fermentation, P.D.B medium showed the maximum enzyme activity of 0.08 IU/mg whereas orange peel showed the enzyme activity of .76 IU/mg. Therefore, Solid substrate fermentation is more beneficial for the pectinase production.(Fig 5a and 5b).

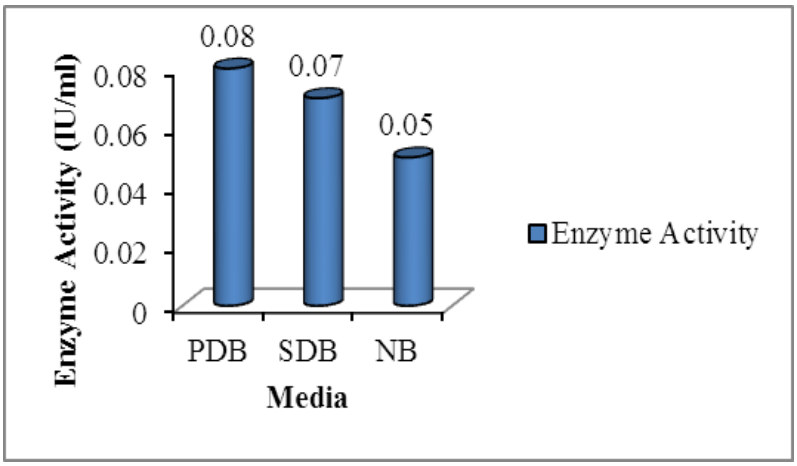


Fig 5a Effect of submerged fermentation on enzyme production

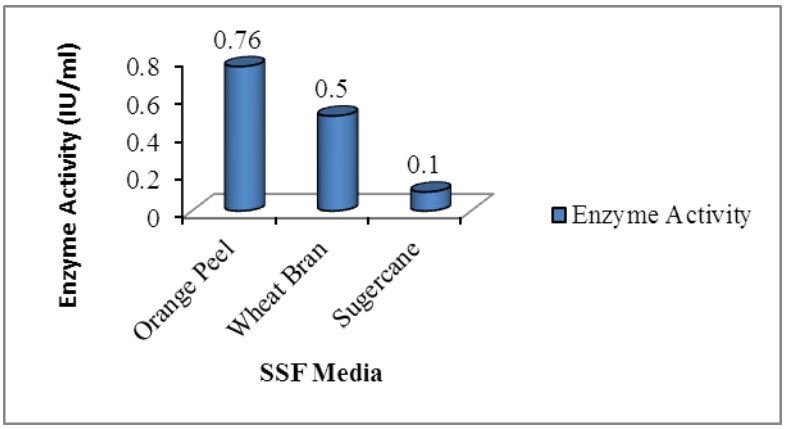


Fig 5 b) Effect of solid substrate fermentation on pectinase production

Effect of varying solid substrate media on pectinase production: The expression of pectinase activity mainly depend upon the substrate employed for the production. The fungal strain showed maximum enzyme with orange peel(0.72 IU/ml) as a solid substrate for enzyme production as compared to wheat bran and sugarcane baggase.

Effect of inoculum size on production of pectinase: The prepared medium was inoculated with varying amount of active culture (5ml-25ml).The maximum enzyme production was observed in the medium inoculated with 5ml medium i.e 0.77 IU/ml using orange peel as substrate. The increasing amount of inoculum has negative effect in production of enzyme (Fig 7).

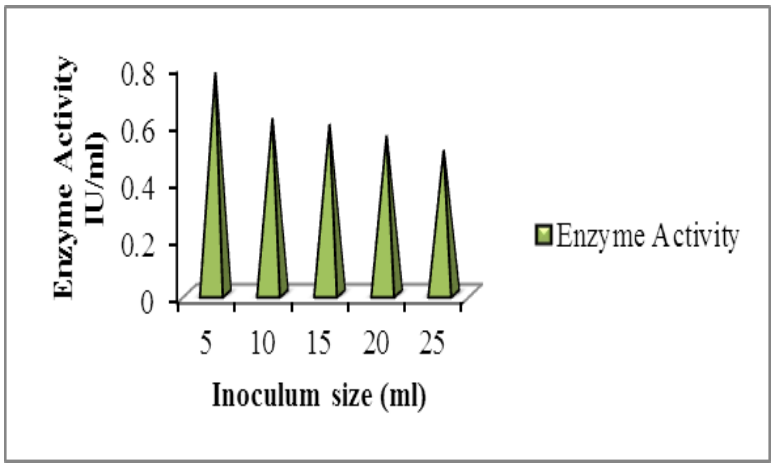


Fig 7: Effect of inoculum size on pectinase production

Optimization of crude pectinase enzyme parameters

Thermo-stability of pectinase: In order to find the optimum condition for the fungal strain to produce high enzyme activity, culture temperature was varied from 30°C to 50°C.The peak value of enzyme activity for pectinase production was recorded 0.77 IU/ml at 30°C under all optimized condition (Fig 8).

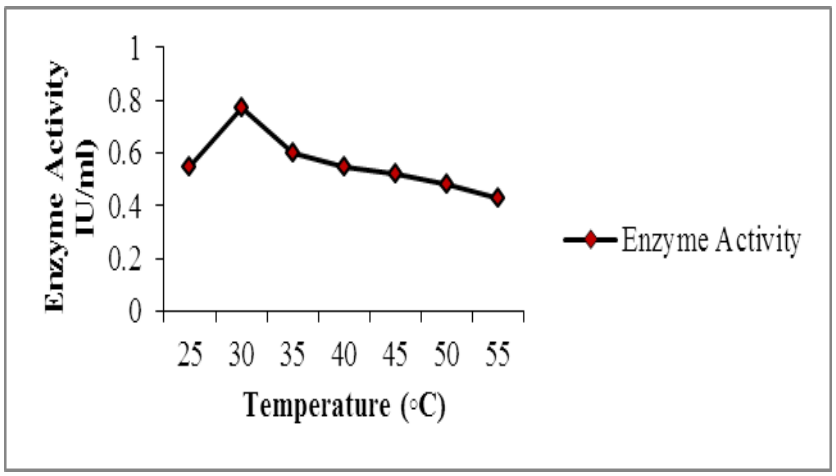


Fig 8 Effect of variable temperature ranges on pectinase production

pH stability of enzyme: The effect of pH on activity of crude pectinase was studied at different pH under standard assay conditions. The maximum amount of enzyme activity bserved was 0.76 IU/ml at pH 4.0(Fig 9).

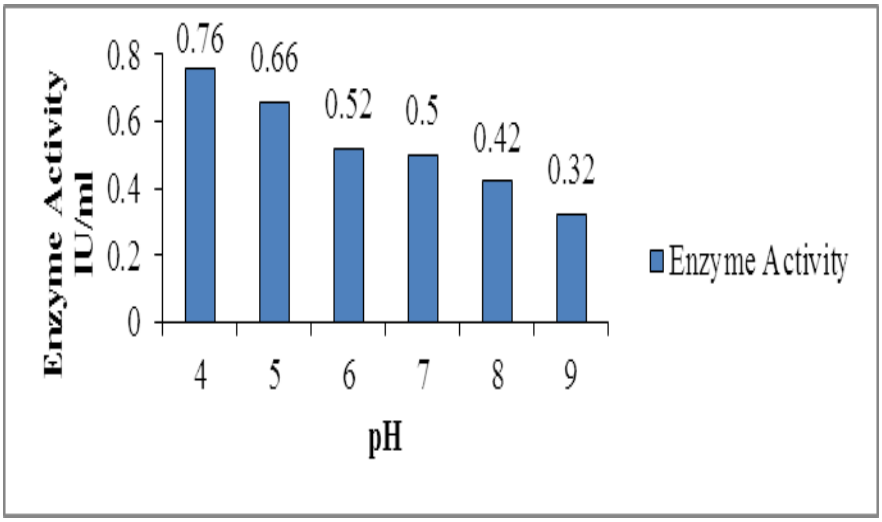


Fig 9: Effect of variable pH on enzyme production

Effect of time of incubation: The crude pectinse was studied under different time of incubation under standard assay conditions. The peak value of enzyme activity was recorded 0.75 IU/mlat 60 minutes (Fig 10).

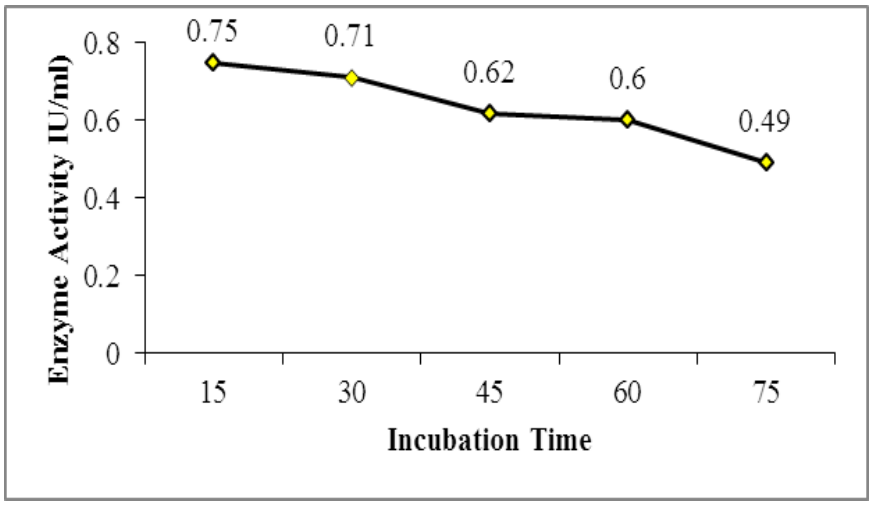


Fig 10: Effect of time of incubation on pectinase production

Effect of Metal ion on enzyme production: The effect of different metal ions on the activity of crude pectinase was studied at 1mM, 3mM,5mM.It was observed that every metal ion had a negative effect on enzyme production i.e metal ions reduced the enzyme production rate.(Fig 11)

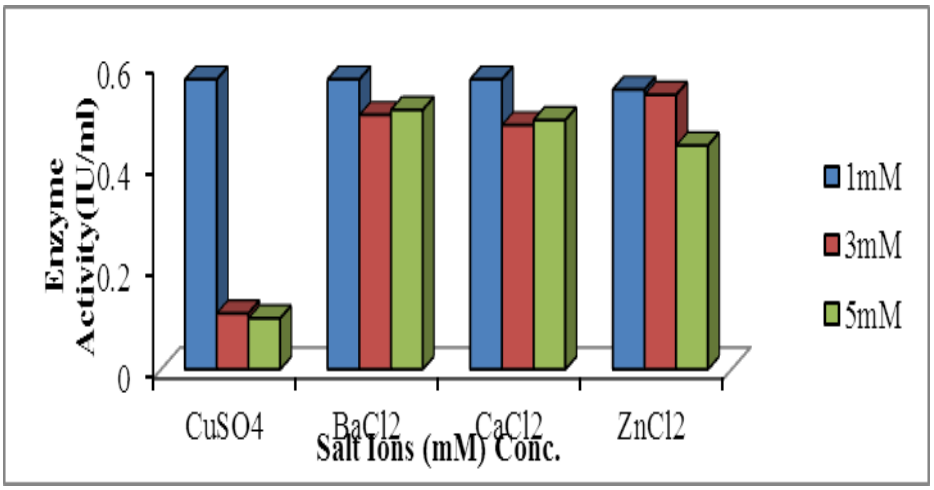
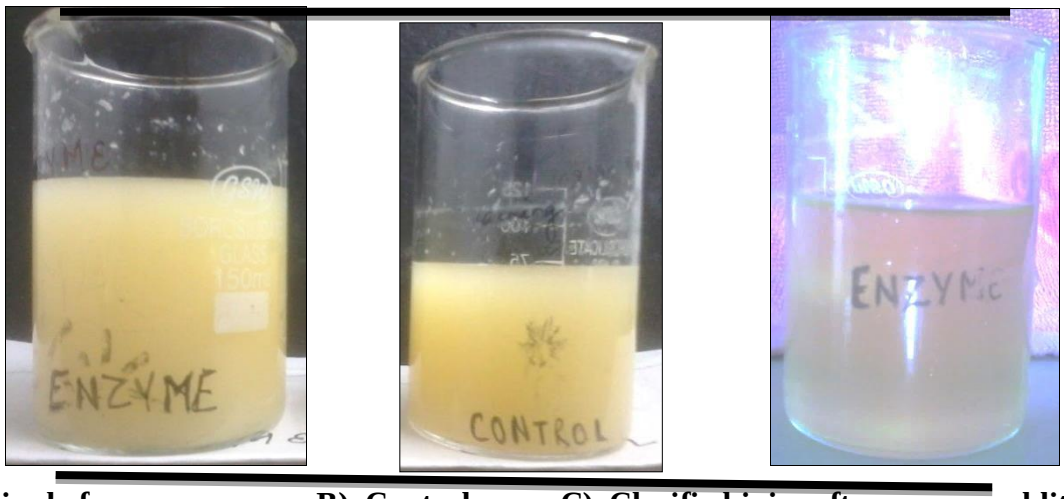


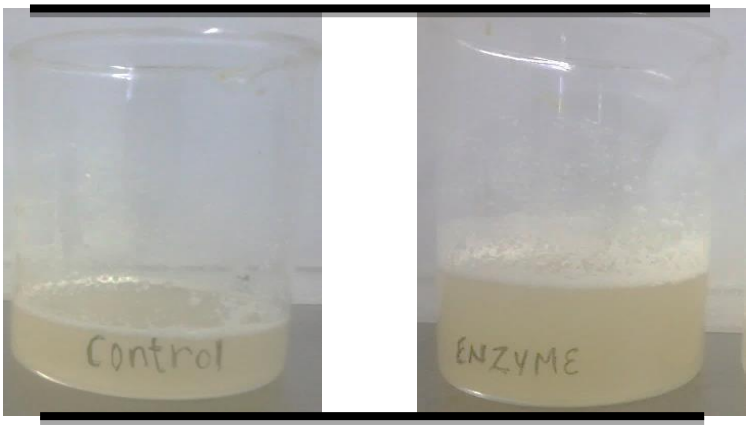
Fig 11: Effect of metal ion concentration on enzyme production.

Application of pectinase in juice extraction and clarification: Fruit juices are turbid and cloudy after their extraction from pulp. This unclarified juice can be clarified using pectinase enzyme under its optimised conditions. Moreover the extraction of juice from the pulp can also be done by using pectinase enzyme. The experiment was carried out for 3 days and the results were recorded. From the figures (Fig 12a and 12b) it is cleared that raw juice is Clarified under all optimized conditions after 3 days of incubation.



A) Juice before enzyme B) Control C) Clarified juice after enzyme addition

B) Fig 12a Clarification of juice by Pectinase



A) Extraction of juice from pulp Without enzyme **B) Extraction of juice from pulp with enzyme**

Fig 12 b) Extraction of juice from pulp by Pectinase

5.6) Protein estimation of enzyme: The estimation of protein was carried out by Lowery method.[8] The protein content of the enzyme was found to be 1.85mg/ml.

CONCLUSION

In the present study total 10 pectinase rich rotten fruits and vegetables and 5 different soil samples were collected from different location of Paonta sahib. Out of the all samples taken 40 fungal isolates were collected. Clear zone on PDA plates gave the indication of pectinase production and then recovered isolates was characterised for production of (mono) galacturonic acid. The isolate which showed maximum activity for the liberation of free galacturonic acid liberation was screened for further investigation. The recovered isolate was characterised and identified on the basis of morphological and microscopic techniques.

The enzyme that is produced from recovered isolates provide optimum activity with orange peel(.76 IU/ml),30°C,pH 4 after an hour of incubation from the parameters of solid substrate(orange peel, wheat bran and sugarcane bagasse),time of incubation,temperature(30°C to 50°C) and pH (4,5,6,7,8,9,10).The recovered isolate was suspected to be *Mucor sp.* In our study maximum production of pectinase by *Mucor* was obtained with orange peelat pH 4.0 and temperature 30°C for an hour in standard assay conditions. In the present study the juice clarification and extraction is observed in 3rd day only. The substrates used are cheap as compared to the chemicals used in submerged fermentation and more appreciable amount enzyme of pectinase enzyme is produced (.76 IU/ml) in SSF as compared to SmF .When orange peel alone was used as the carbon source, a better production of ploygalacturonse was observed. In concern with use of various agricultural wastes and agro industrial wastes

especially fruit processing industries, wine industries generates gallons of wastes during preparation of different juices.[9] It's dumping in nature causes pollution problems. Such problems can be solved by exploiting these agro wastes for pectinase production by using potential microorganisms by fermentation process.

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