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OPTIMIZATION OF PECTINASE PRODUCTION KINETICS BY *CANDIDA TROPICALIS* AND ITS APPLICATIONS IN FRUIT JUICE CLARIFICATION

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ABSTRACT

Enzyme pectinase cleaves the polysaccharide pectin into galacturonic acid monomers. Pectinases are produced commercially using bacteria and molds, however the production of pectinase employing yeasts, remains unexplored. The present study was undertaken to isolate pectinase producing microorganism and to optimize the condition for its cost-effective production. In this study, 28 pectinolytic microbes were isolated from soil samples and were qualitatively and quantitatively screened for pectinase production. The isolate giving maximum enzymatic activity was identified as Candida tropicalis by 18S rRNA sequencing. The kinetics of enzyme catalyzed reaction were optimum at temperature 37ºC, pH 5.5 and reaction time of 30 min. The enzyme fraction was partially purified by ammonium sulfate purification and later by dialysis. The Vmax and Km were found to be 13.90 U/ml and 10.19 mg/ml respectively. The specific activity of the dialyzed enzyme fraction was found to be 76.40 U/mg. The extracted enzyme was immobilized in calcium alginate matrix and its efficiency in clarification of orange juice was evaluated. The immobilized pectinase led to an increase in the yield of juice as compared to control and brought about 95% clarification in 2 hours of incubation.

KEY WORDS

Candida tropicalis, fruit juice clarification, immobilization, pectinase, 18S rRNA sequencing

INTRODUCTION

Pectins are heteropolysaccharides present in the cell wall of the plant kingdom which consist of a backbone of α-1, 4-linked D-galacturonic acid residues.[1] Pectinases are a group of enzymes including both depolymerizing and demethoxylating enzymes which are capable of degrading pectin to its galacturonic acid monomers. Depolymerizing enzymes or polygalacturonases (E.C. 3.2.1), cleaves the α-1, 4 glycosidic bonds between two galacturonic acid residues, [2] and pectin-lyase (E.C. 4.2.2), catalyzes the β-elimination reaction between two methylated residues, [3] while De-esterifying enzymes including pectin esterase (E.C. 3.1.1), produces methanol as by-product while demethoxylation of the methylated pectin.[4]

Pectinases have crucial industrial significances in improving the juice yields, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction etc.[5] It has found an important role in the food and wine industry for the processing of fruit juices. The use of pectinases in fruit juice clarification improves the color, turbidity and viscosity of the juice, it also Increases the juice yield and reduces the bitterness of citrus juices.[6] Many of the industrial enzymes are produced using microorganisms in relatively inexpensive and eco-friendly processes. Enzymatic catalysis is specific and generates less toxic by-products, hence preferred over other methods. Industrial production of pectinase has employed many different genera of bacteria, fungal molds and yeast,

including *Aspergillus, Saccharomyces, Bacillus, Penicillium, Erwinia* etc.[7]

Naturally occurring pectic substances like citrus peels are usually acidic, hence fungal molds and yeasts are preferred for pectinase production using naturally occurring cost-effective pectic substances. Commercial preparations of pectinase from fungal molds contain a complex mixture of different enzymes with pectinolytic activity, including polygalacturonases, lyases, the undesirable pectinesterases, and also others enzymes. Yeasts have advantages as compared to filamentous fungi, because they are unicellular, their growth is relatively simple, and usually yeasts do no secrete pectin methylesterases hence they can clarify fruit juices without releasing methanol.[8] The high tolerance against inhibitors present in natural substrates along with the rapid growth rate, makes yeast a potential candidate for industrial production of pectinase enzyme.[9] These significances justify the need for the search of new yeast sp. capable of overproducing the desired enzyme.

Applications of immobilized pectinase for fruit juice clarification has many advantages like increased tolerance to inhibitory action of high concentration of substrate, lower cost of downstream processing and elimination of purification steps, increased stability and also it can be reused.[10]

In our present study, an effort was made to isolate and screen a potential yeast strain which exhibit the ability to produce pectinase enzyme. The partially purified enzyme fraction was characterized, and physico-chemical properties of polygalacturonase was determined. The effect of physical parameters like temperature and pH etc., on its catalytic activity was investigated, in order to evaluate the application of the immobilized enzyme in fruit juice clarification.

MATERIALS AND METHODS

Sample collection and Isolation of microorganism:

Soil samples were collected from vegetable and fruit market dumping site from nearby areas of Badlapur, Ulhasnagar. One gram of the soil sample was inoculated in sterile Hankins mineral Pectin (MP) broth medium[11] and incubated at 25 °C for 72 hours in a rotary shaker. After 72 hours, 1 ml of enriched broth was inoculated into fresh sterile MP broth medium with 1 % pectin and incubated at R.T. for 3-4 days, under shaker conditions. A loopful of the enriched media was streaked on sterile MP agar plates, pH 6. The plates were incubated at RT for 48hrs. After 48hrs, morphologically distinct colonies were isolated and maintained on MP agar slants.

Qualitative screening:

The isolates obtained were spot inoculated on the MP agar plates and incubated at RT for 48 hrs. After 48 hrs, Congo red overlay method was used for observing clearance zones[12]. Isolates showing clearance zone around the colony were further screened using semi-quantitative screening.

Semi-quantitative Screening:

1% of the culture suspension was inoculated in sterile MP broth, pH 6 and incubated at room temperature (RT) for 3-4 days. The broth was centrifuged at 10000 rpm for 10 min at 4ºC. Wells were bored on sterile MP soft agar plates (1.5% agar) with a borer of 6 mm diameter. 0.1 ml of cell-free supernatant was inoculated in the respective wells and the plates were incubated at RT for 48 hrs. After 48 hrs, the plates were stained by Congo red overlay method[12]. The diameter of clearance zone was measured and the relative enzyme activity (REA) was calculated as, REA= $(D^2-d^2)/d^2$, where 'D' is the diameter of zone of clearance &'d' is the diameter of the well. The isolates showing maximum REA were further screened using quantitative assay method.

Quantitative Screening by polygalacturonase assay:

The cell- free supernatant of the isolates was assayed quantitatively using 3, 4-dinitro salicylic acid (DNS) assay method[13]. The tubes showing turbidity were centrifuged before taking absorbance. The isolate giving maximum absorbance was used as potent isolate for the production of pectinase. The amount of reducing sugar released was determined from glucose standard chart. The enzyme activity (E.A) was calculated as µmoles of glucose released per min per ml of enzyme.

E.A

$$(U/ml)=\frac{[\ (\mu g/ml\ of\ glu)\ /\ mol.\ wt\ of\ glu\ ]}{Time\ (min)\ x\ vol.\ of\ enzyme\ (ml)}\ x\ Dil.\ Factor\ of\ enzyme$$

Optimization of fermentation parameters for lab scale enzyme production:

The optimization of fermentation parameters for pectinase production using potent isolate was carried out. For carbon source optimization of pectin & orange bagasse, 1% culture suspension was inoculated in sterile Pectin yeast extract (PYE) broth, pH 6 and orange bagasse media respectively, with different concentrations of carbon source (0.5 - 3.0 %) and 1% yeast extract. For nitrogen source (yeast extract) optimization, culture suspension was inoculated in St.

PYE broth with different concentrations of yeast extract (0.5 - 3.0 %) and 1% pectin solution. For temperature optimization, the inoculated St. PYE media was incubated at different temperatures as 10°C, 28 °C, 37 °C, and 55 °C. For pH optimization, the inoculated St. PYE broth was prepared in buffer with varying pH (4-8). The broth media for each test were incubated for 72 hours and later centrifuged at 10000 rpm for 10 minutes at 4°C, so as to obtain cell free supernatant which was assayed by DNS method[13] to determine the optimum values.

Economic Production of pectinase enzyme using dried orange bagasse:

The orange bagasse (containing peels and remains after juice extraction) was obtained from fruit juice vendors. The bagasse was thoroughly cleaned with water and kept for drying in oven at 55ºC for 2 days. After 2 days, it was powdered and sieved. The orange bagasse powder was stored in air-tight container. 100 ml of the 24-hr. grown culture of the potent isolate in PYE broth was inoculated in 900 ml St. PYE broth medium in which pectin is replaced with orange bagasse powder and incubated at the optimum fermentation conditions. After fermentation, the broth was centrifuged at 10000 rpm for 10 minutes at 4°C, so as to obtain cell free supernatant.

Partial purification of enzyme by ammonium sulphate precipitation and dialysis:

The amount of ammonium sulphate (AS) required was calculated according to the volume of cell free supernatant, using reference table. The entire procedure was carried out in an ice-bath, kept on a magnetic stirrer. Initially 20% saturation was carried out by adding the required amount of AS, as per the table. Since no precipitate was obtained, 40% saturation was carried out. When all the AS has been added, the solution was stirred for half an hour for complete precipitation. Later the whole content was centrifuged at 10000 rpm for 10 minutes for the separation of precipitate and the supernatant was further subjected to 60% and then 80% saturation[14]. The precipitate obtained was dissolved in minimum amount of 0.1 M Acetate buffer, pH 5.5. The enzyme activity of every fraction was determined by DNSA assay method and protein estimation was carried out by Lowry method[15].

For dialysis, the bag was kept in 500 ml of 0.05 M Acetate buffer, pH 5.5 in a beaker. Once open, one end of the bag was tightened with thread and 10 ml of enzyme solution was kept in one tubing and end of the tube was tightened with thread. The whole assembly was kept in a fridge at 10°C. After every four hours buffer was discarded and new buffer was refilled, this step was repeated 3 times, so as to achieve efficient dialysis. After dialysis, the contents in the dialysis bags were taken out in sterile tubes and stored at -20°C until further use.

Kinetic studies of partially purified pectinase enzyme:

For optimization of substrate (pectin) concentration, a reaction mixture was setup containing equal amount of different concentration of pectin (2.5-30 mg/ml in buffer) and partially purified enzyme and incubated at 37ºC for 30 min. For pH optimization, the reaction mixture was prepared containing the substrate (1% pectin) prepared in buffer of pH 4-8. For temperature optimization, the reaction mixture was incubated at varying temperatures (4 - 80^{0}C).

For optimization of reaction time, the reaction mixture was incubated at 37ºC for varying time period 10-60 min. The reducing sugars released was assayed by DNSA method. A Line-weaver Burk's graph was plotted to determine the Vmax and Km values. The protein content of the enzyme fractions were determined by Lowry method.

Immobilization of the enzyme:

Equal volume of dialyzed enzyme sample (1:10 diluted) was mixed with 3% sodium alginate solution and added drop-wise in chilled 0.2M $CaCl_2$ solution to form beads. The beads were kept at 4ºC for hardening then the beads were washed twice with D/W and stored in 0.05M Acetate buffer, pH 5.5 at 10°C[16].

Orange juice clarification:

Orange fruit was peeled, de-seeded and pulped in a grinder. 20 ml of the pulp was incubated with 2ml free dialyzed enzyme/ immobilized dialyzed enzyme/ D/W (in control) for 2 hrs. After incubation, the juice was extracted by filtering through a muslin cloth. The volume of juice extracted was measured and % Transmittance was determined using UV/Vis Spectrophotometer.

Identification of the isolate:

The potent isolate was identified by cultural and biochemical characteristics. Slide culture technique[17] and Inoculation on *Chrom Agar* media[18] was performed for the identification of yeast species. Molecular identification was done by 18S rRNA sequencing using ITS1 (TCCGTAGGTGAACCTGCGG) & ITS4

(TCCTCCGCTTATTGATATGC) universal primers at *Yaazh Xenomics,* Coimbatore, Tamil Nadu.

RESULTS AND DISCUSSION

Isolation and Screening of pectin degrading microorganisms:

About 28 morphologically distinct pectinolytic colonies were isolated from 18 different soil samples by streaking the enriched broth on sterile MP agar plates. Out of the 28 isolates, 16 isolates showed significant clearance zone around the colony by qualitative screening (Fig 1). The orange clearance zone is found around the growth of isolates indicating pectin hydrolysis. There is zone around the isolates, hence they do hydrolyze pectin. The purple color zone developed because of galacturonic acid which is the product of pectin hydrolysis. Further screening was done by semi-quantitative screening. The cell free extract of the 16 isolates showed clearance zone around the inoculated wells (Fig 2). Hence it can be said that the enzyme is extracellular. Relative enzyme activities (REA) calculated for all the isolates is shown graphically (Fig 3). The isolates 10A2, 12B, 13B & 16A showed maximum relative enzyme activity. These isolates were subjected to quantitative screening by DNSA method. The maximum enzyme activity of 9.70 U/ml was given by the isolate 13B (Table 1). The isolate 13B was used for lab-scale production of pectinase.

Qualitative screening

Figure 1: Orange clearance zone found around isolates 10A2, 13B, and 16A, while no zone for isolate 13A, 16B

Semi-quantitative Screening

Figure 2: Orange clearance zone found around the wells inoculated with cell-free supernatant of isolates 10A2, 12B, 13B, and 16A

Relative enzyme activity

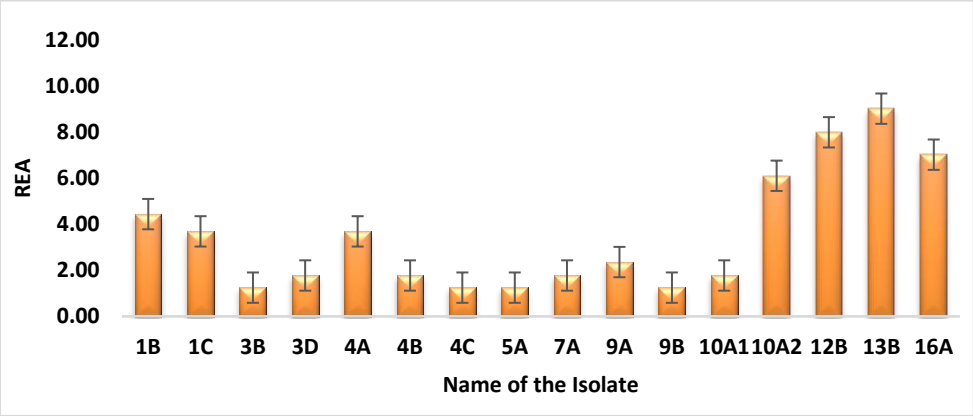

Figure 3: Isolates 10A2, 12B, 13B & 16A showing maximum relative enzyme activity. The standard error bars in the graph represent mean errors from duplicate samples tested

Table 1 - Quantitative Screening

Sr no.	Isolate name	O.D at 540 nm	µg/ml of glucose (from glucose standard graph)	Enzyme activity (U/ml)
1	10A2	0.68	718.47	5.32
2	12B	1.03	1082.55	8.02
3	**13B**	**1.25**	**1309.33**	**9.70**
4	16A	0.79	832.17	6.16

The enzyme activity is calculated as µ moles of glucose released/ min/ml of enzyme.

Table 2 – Enzyme Activity

Sr no.	Enzyme fraction	glucose µg/ml	Enzyme activity (U/ml)	Protein content mg/ml	Specific enzyme activity U/mg	Purity fold
1	Crude enzyme	1280.60	9.49	0.90	10.54	-
2	Ammonium sulphate purified 60% fraction	1444.79	10.70	0.20	53.50	5.07
3	**Dialyzed**	**1547.40**	**11.46**	**0.15**	**76.40**	**7.24**

Specific enzyme activity of the crude, AS purified and dialyzed enzyme is compared.

Table 3 - Result of orange juice clarification experiment

Test	Volume of juice extracted	% yield	% Transmittance
Immobilized Enzyme	**14 ml**	**75**	**0.95**
Free enzyme	12 ml	50	0.68
Control (D/W)	08 ml	-	0.41

Optimization of fermentation parameters and lab scale enzyme production

From the Fig 4, it can be said that enzyme activity increases with increase in the concentration of pure pectin and orange bagasse. The enzymatic yield was found to be decreasing with increase in concentration of orange bagasse to more than 1.5%. This can be because the orange bagasse was not pre-treated and hence some inhibitory components may be present, which at a higher concentration is inhibiting the

enzymatic yield. Hence 1.5% orange bagasse, which was giving maximum enzymatic yield, was used for the fermentation.

Optimization of fermentation parameters.

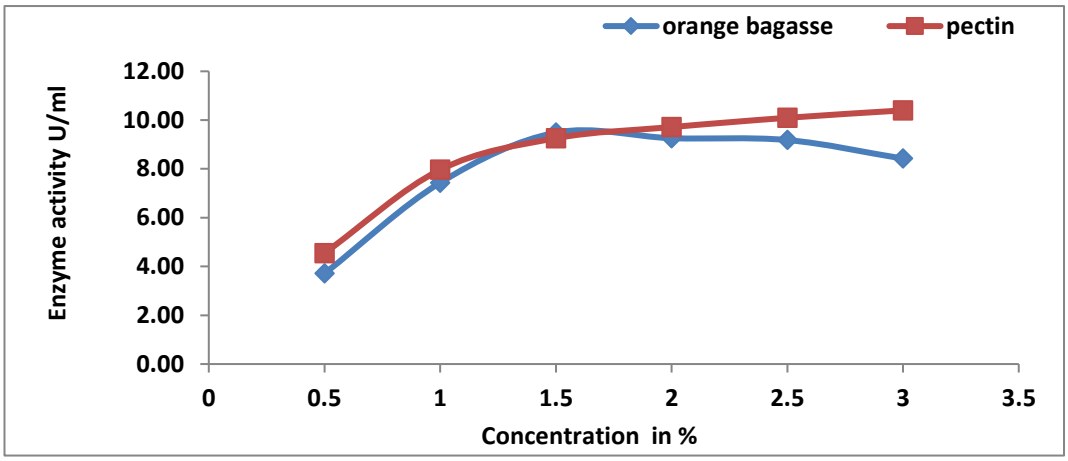

Figure 4: Optimization of carbon source

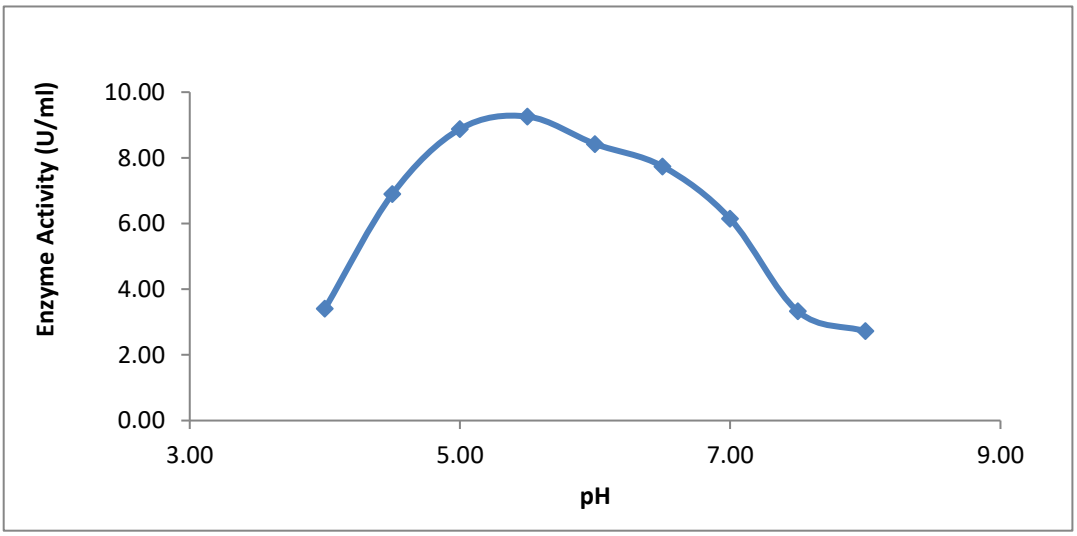

Figure 5: Optimization of nitrogen source (yeast extract)

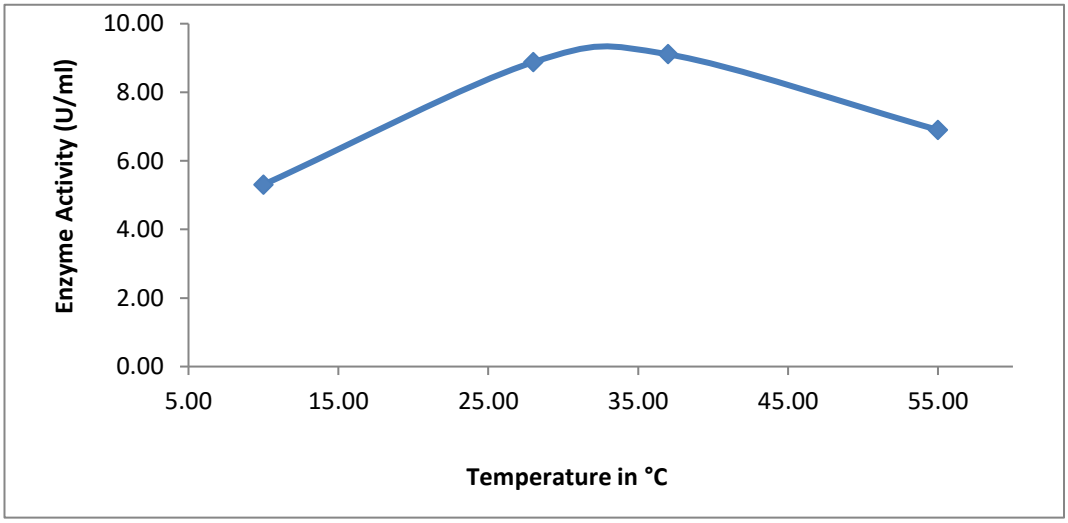

Figure 6: Optimization of pH

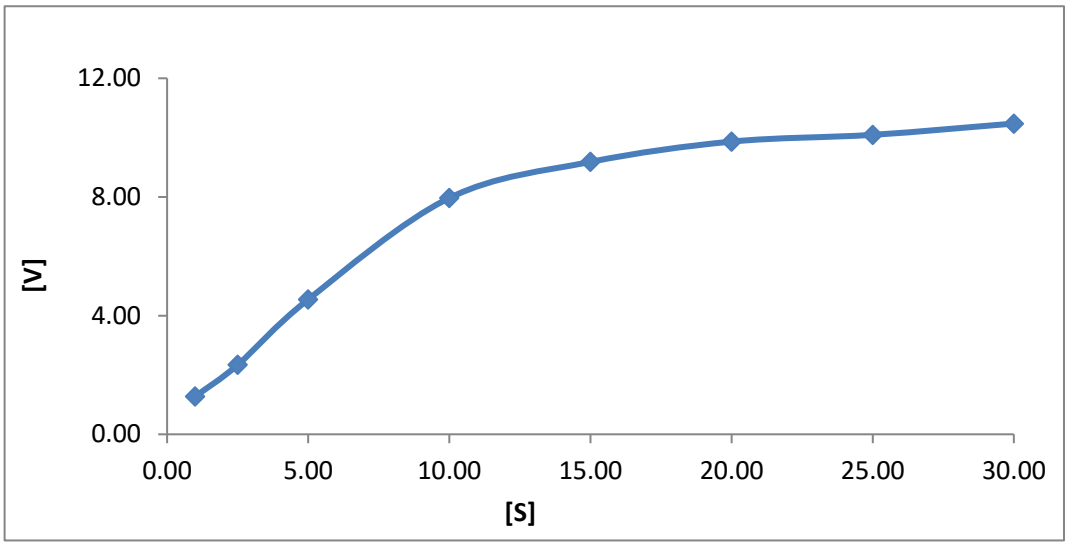

Figure 7: Optimization of Temperature

Kinetic studies of partially purified pectinase enzyme

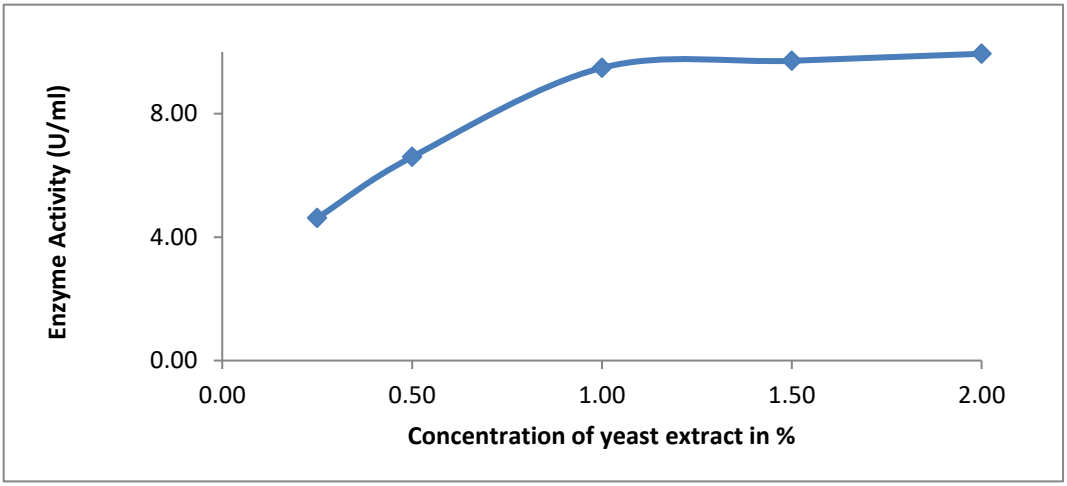

Figure 8: Michelis menton plot

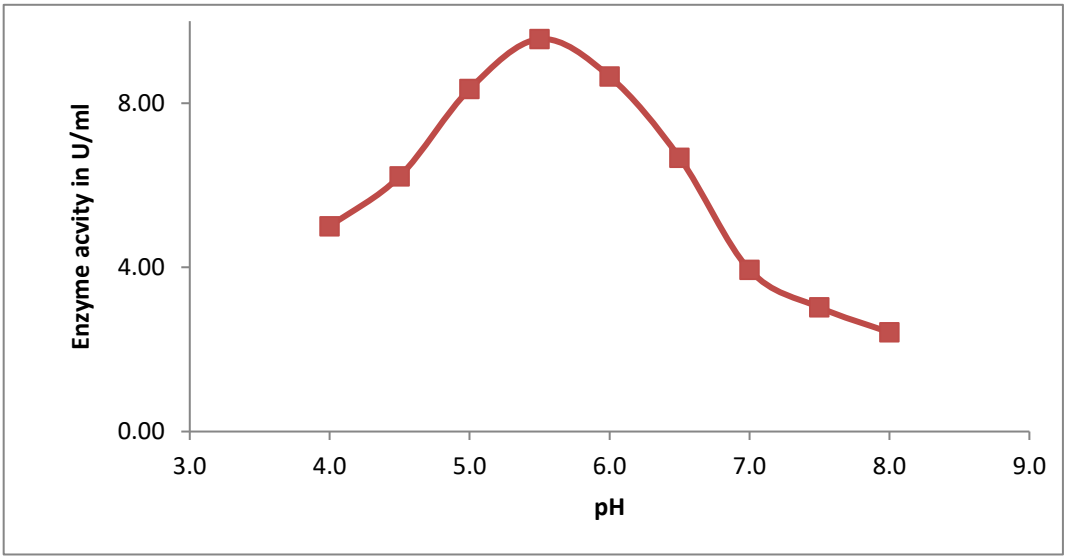

Figure 9: Line-weaver Burk plot

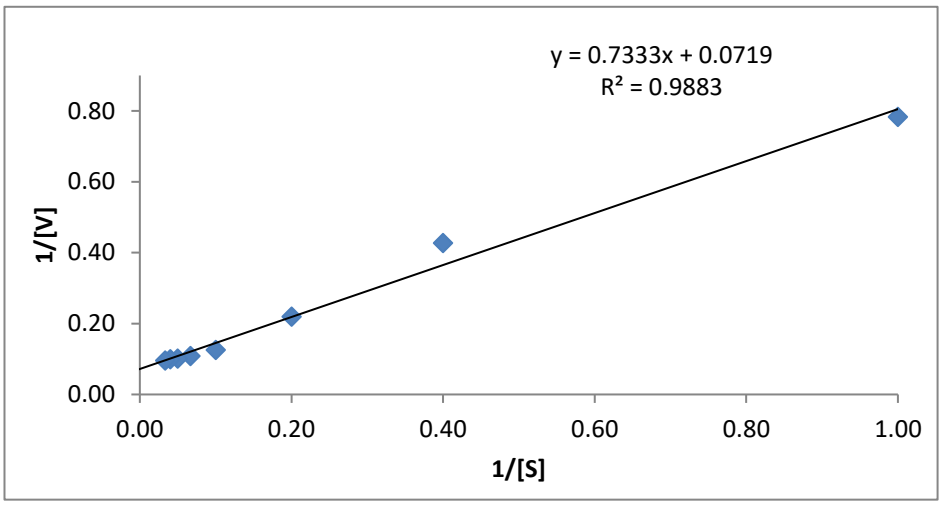

Figure 10: pH optimization

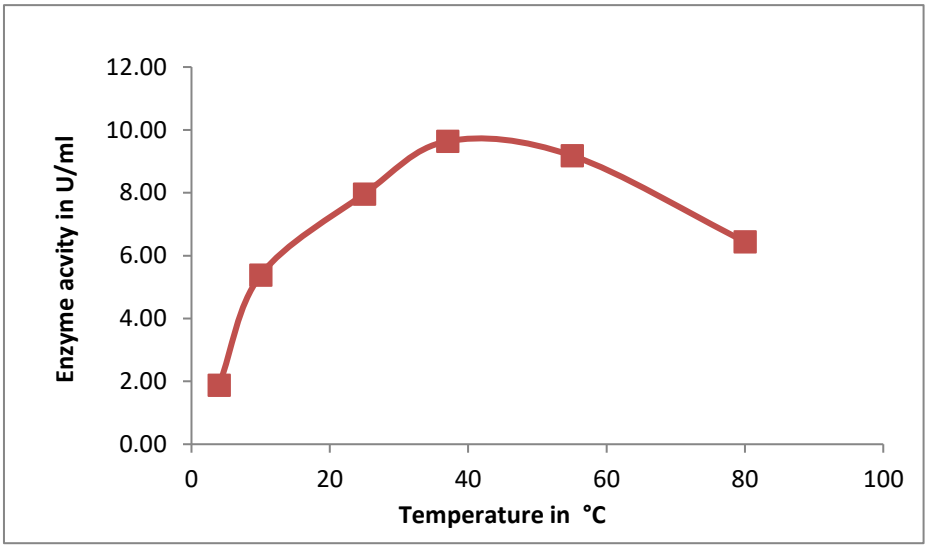

Figure 11: Temperature optimization

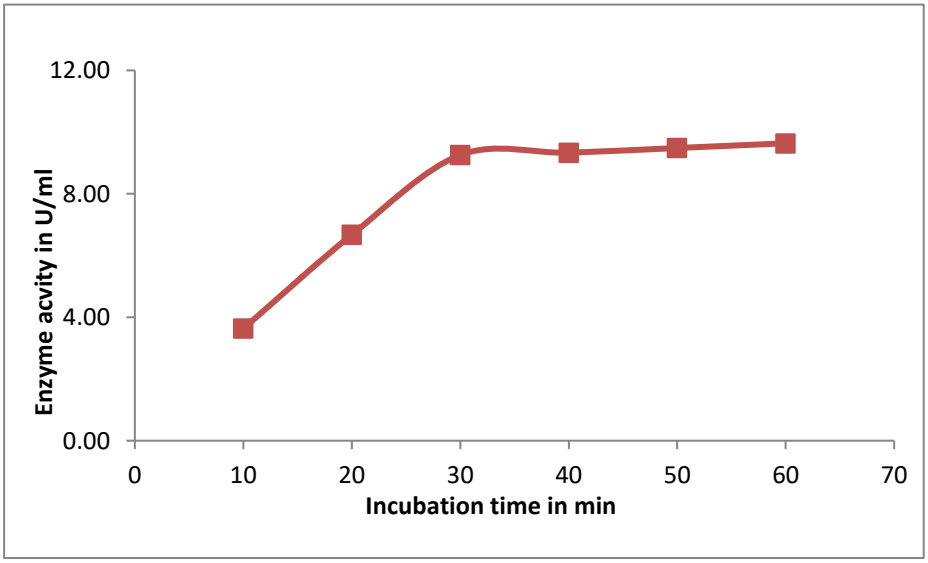

Figure 12: Reaction time optimization

Immobilization of dialyzed enzyme

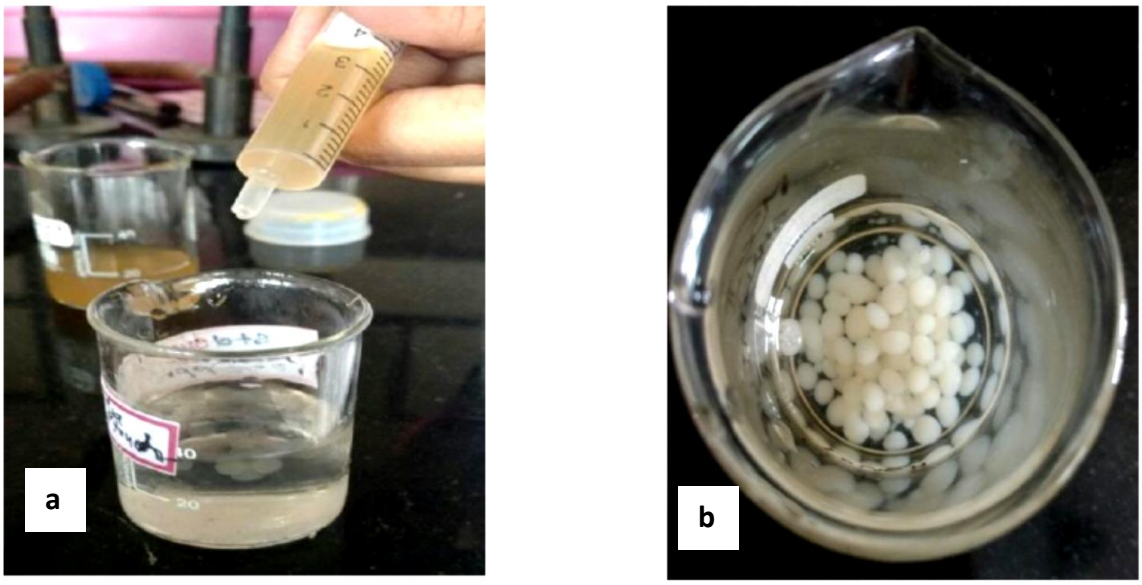

Figure 13: a) Preparation of immobilized enzyme beads; b) Enzyme beads after hardening

Fruit Juice clarification

Figure 14: Orange juice clarification experiment setup
(From left to right: 1- Immobilized enzyme treatment, 2- Free enzyme treatment, 3- Distilled water treatment as control)

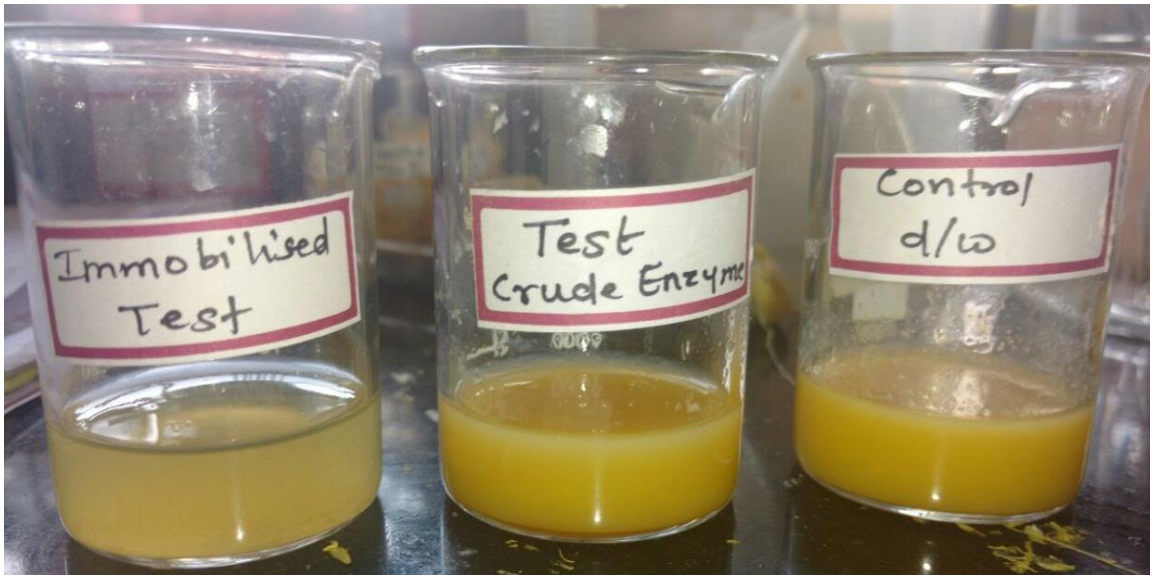

Figure 15: Extracted clarified juice after treatment with immobilized, free enzyme and control (from left to right Identification of the isolate

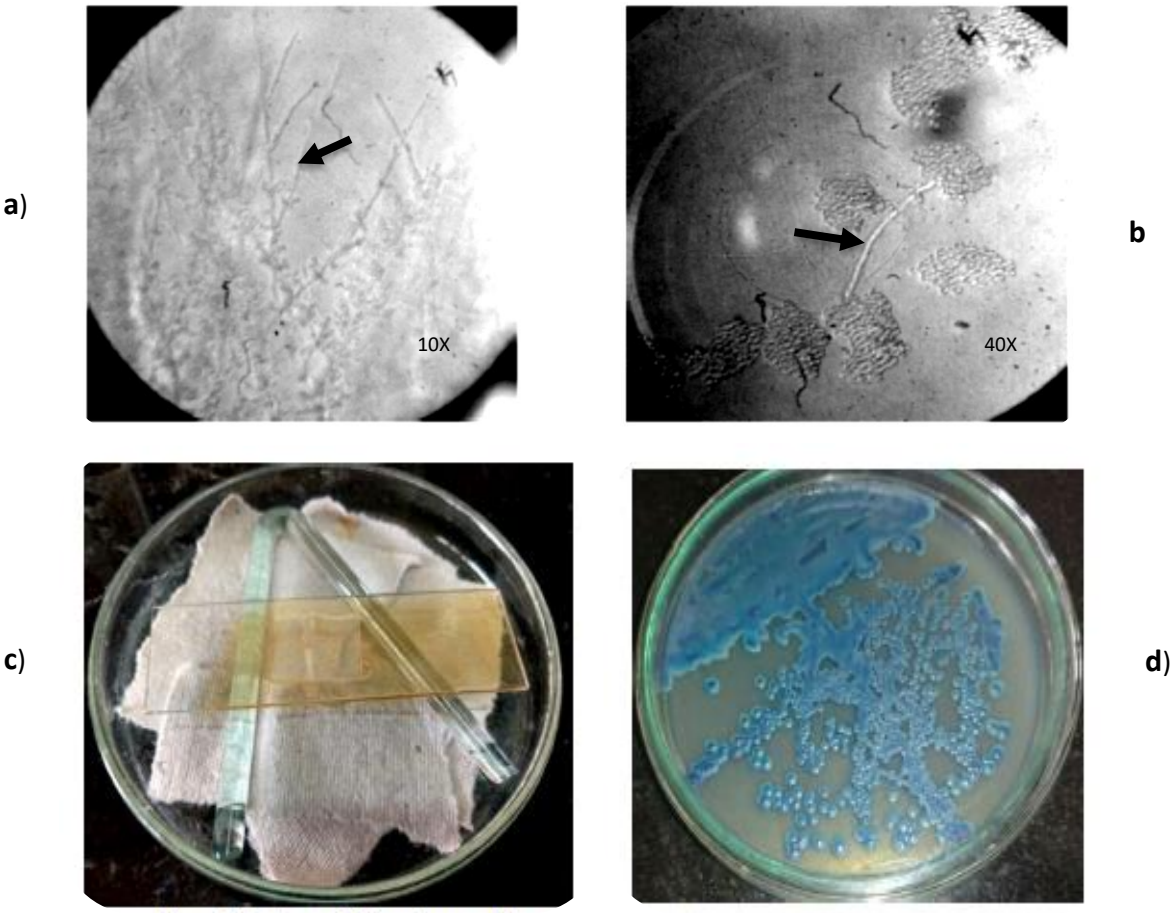

Figure 16: Slide showing long pseudo hyphae and yeast cells as observed under 10X bright field microscope (a) and 40X bright field microscope (b), Setup of Slide culture technique (c), Isolation on Candida specific Chrom Agar (d).

Phylogenic analysis of the isolate

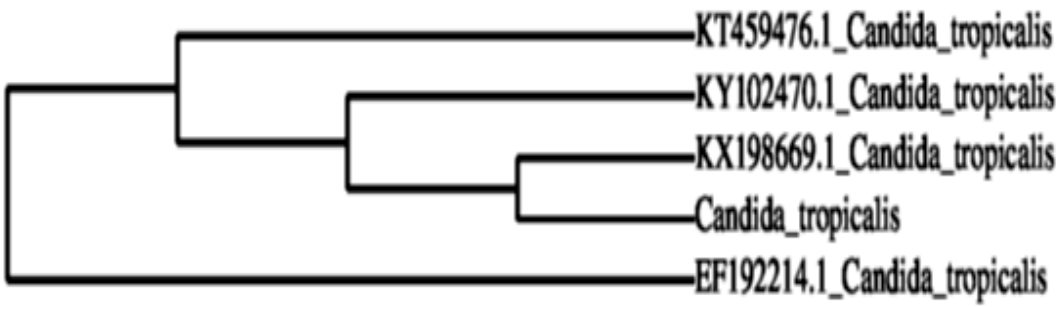

Figure 17: Phylogeny tree of the isolate
GenBank accession no: MH330689 Candida tropicalis

From the Fig 5, the optimum concentration of yeast extract was found to be 1%. Further increase in the yeast extract concentration did not increase the enzymatic yield to much extent. Hence optimum concentration of yeast extract was determined as 1%. The enzymatic activity increased as pH increased from 4 to 5.5. Further increase in pH led to a steep decrease in the activity (Fig 6). Hence optimum pH was 5.5, which was maintained in fermentation media for pectinase production. The temperature optimization graph *(*Fig 7*)* showed that the maximum enzymatic activity was obtained at 37°C. Further increase in temperature decreases the enzyme yield. This could be due to the reduction in growth rate at higher temperatures.

Partial purification of the enzyme

The lab-scale production of pectinase was carried out using orange peel media at the optimized conditions. The broth was centrifuged at 10000 rpm for 15min at 4°C and cell-free supernatant was processed for partial purification.

The Crude extract was purified by salting out method using ammonium sulfate and the 60% fraction known to contain pectinase, showed highest activity of 53.50 U/ml and after dialysis its activity increased to around 76.40 U/ml. The protein content of the enzyme fractions were determined by Lowry method. Dialysis increased the purity fold of the enzyme by 7.24, as compared to crude enzyme. The enzyme activity increased with each step of purification due to purification and concentration of the enzyme.

Kinetic studies of partially purified pectinase enzyme

Pectinase activity was determined by varying the substrate (pectin) concentration keeping other conditions constant. It was found that enzyme activity increases with increase in substrate concentration from 2.5 to 15 mg/ml (Fig 8). However, at higher concentration of pectin, the enzyme becomes saturated with substrate and reaches *V*max, the enzyme's maximum rate. Further increasing the concentration of substrate does not increase the enzyme activity significantly. From the Line-weaver Burk graph, Km and Vmax was found to be 10.19889 mg/ml and 13.90821 U/ml (Fig 9)

pH optimization graph (Fig 10) showed that the maximum enzyme activity was obtained at pH 5.5. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. Hence the enzyme activity is found to be affected by the change in pH, as increase in pH above 5.5, decreases the activity of the enzyme. As the temperature increases, the rate of reaction increases. But at very high temperatures (more than 40 °C) the enzyme get denatured. Here the activity of pectinase decreases with change in temperature below 25°C and above 60°C, optimum temperature was found to be around 40°C (Fig 11). The longer an enzyme is incubated with its substrate, the greater the amount of product will be formed. Enzyme activity increased with increase in incubation time from 5-30 min, after which the increase in its activity was not significant (Fig 12). This shows that the rate of formation of the product is not a simple linear function of the time of reaction. All proteins suffer denaturation, and hence there was no increase in catalytic activity, after incubation for more than 30 min.

Immobilization of extracted pectinase enzyme and its application in fruit juice clarification:

The dialyzed enzyme fractions was immobilized in calcium alginate matrix. (Fig 13) The free and immobilized enzyme was checked for their efficiency in fruit juice clarification. (Fig 14, Fig 15) The volume of juice extracted and % Transmittance obtained is shown in Table 3. The immobilized enzyme showed maximum clarification of juice with % Transmittance of 0.95 and an increase in yield by 75% as compared to control (D/W) (Table 3). The immobilized enzyme was found to be more effective as compared to free enzyme, since the immobilized enzyme is least affected by any inhibitory action of the surrounding.

Identification of the isolate

Gram staining showed Gram positive oval cells similar to yeast cells. Sugar fermentation test was found to be positive with glucose and sucrose only, as shown by many yeast cells. IMViC was negative while catalase and oxidize test were positive. Based on the colony characteristics, a microscopic examination and biochemical test, the isolate was found to be yeast strain. To study the morphology, slide culture technique was carried out *(fig. 6.3)*. The slide culture technique showed presence of pseudo hyphae, which is a characteristic of *Candida sp. (fig. 6.1 & fig 6.2).* Hence the yeast was isolated on Candida specific Chrom Agar media to find the species. The isolate showed dark blue colonies, which is a characteristic of *Candida tropicalis (fig. 6.4).* This was further confirmed by 18S rRNA sequencing.

Sequencing result obtained by 18s rRNA sequencing using ITS1 & ITS4 primers

GTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAATCC
TACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACA
GTCAAACTTGATTTATTATTACAATAGTCAAAACTTTCAAC
AACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCG
AAATGCGATACGTAATATGAATTGCAGATATTCGTGAATC
ATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAA
AGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAACCCCC
GGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGA
ATTTAACGTGGAAACTTATTTTAAGCGACTTAGGTTTATCC
AAAACGCTTATTTTGCTAGTGGCCACCACAATTTATTTCAT
AACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTT
AAGCATA

GenBank accession no: MH330689

CONCLUSION

One of the main reason people prefer soft drinks over fruit juices is that of its high cost compared to soft drinks. Cost increases due to the tedious process of juice clarification especially of pulpy citrus fruits. Pectinase (polygalacturonase) is an enzyme which catalyzes the hydrolysis of α (1-4) linkage in the pectin polymer, a major constituent of plant cell-wall. Fruit juice treated with pectinase, don't have haziness as the pectins are hydrolyzed. This improve clarification and also increases the juice yield. Immobilized pectinase enzyme, produced using *Candida tropicalis* showed 95% clarification of the fruit juice in 2 hrs of incubation and also increased the juice yield by 75%.

Production of pectinase enzyme using yeast has several advantages, as it has higher growth rate and produces only a single type of pectinase, as compared to fungal molds. Use of orange bagasse as a substrate also lead to the economic production of pectinase.

In the future study, the stability of the immobilized enzyme at different temperatures and pH and its efficiency on reuse has to be checked. The orange bagasse used for fermentation was found to be decreasing the enzymatic yield at a higher concentration. Hence the need for the pretreatment of the substrate will be studied.

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