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Isolation and Identification of Pectinase Producing Fungus and Enzyme Quantification

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A B S T R A C T

Naturally occurring polysaccharide pectin, the methylated ester of polygalacturonic acid, is very important in both scientific and commercial world due to its biodegradability. A large group of pectinase enzymes causing breakdown of pectin polysaccharides of plants and fruit are used in industrial sector to increase the yield and clarity of fruit juices. In this study, a fungal strain was isolated using PSA Medium from the peel of rotten oranges. Isolated fungal strain was identified based on staining and biochemical tests. The fungus was found to be *Aspergillusniger* due to its spore and head structure. Pectinase enzyme was produced by Liquid State Fermentation. A light grey coloured mat is observed on the liquid broth (production medium) after 8 days of inoculation, this indicates the release of pectinase enzyme. The obtained Pectinase enzyme is purified by salt precipitation and dialysis. Pectinesterase activity can be done by performing the titration of sample using phenolphthalene indicator. One enzymatic unit (U) was defined as the quantity of enzyme which liberates 1 microequivalent of carboxylic group in 1 h of reaction. The enzymatic activity was expressed in U/g. It was calculated and found to be 0.043µmol/min/mg. So the isolated fungal strain is efficient and has potential to be implicated commercially to increase the clarity and quality of fruit juices.

Keywords: Pectinase, liquid state fermentation, Pectinesterase activity

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Biotechnology offers an increasing potential for the production of goods to meet various human needs. In enzyme technology – a sub-field of biotechnology – new processes have been and are being developed to manufacture both bulk and high added-value products utilizing enzymes as biocatalysts, in order to meet needs

such as food (e.g., bread, cheese, beer, vinegar), fine chemicals (e.g., amino acids, vitamins), and pharmaceuticals. Enzymes are also used to provide services, as in washing and environmental processes, or for analytical and diagnostic purposes. Pectinases or pectinolytic enzymes are today one of the upcoming enzymes of the commercial sector. It has been reported that microbial pectinases account for 25% of the global food enzymes sales. Investigation of pectinases is a central issue in enzymology research due to their wide applications in Pharmaceutical, food, Agricultural products and Bioremediation processes. Pectinases account for 10% of the total worldwide production of enzymes. The economic and ecological function of pectinase enzymes in industries is gaining much attention with the need of highly productive strains of microorganisms to reduce production cost

2. Materials and methods

Materials and methods

crystal violet, grams iodine, saffronin, nutrient agar medium, Peptone, Bea for yeast extract, Sodium Chloride, agar-agar, sodium nitrite, potassium hydrogen phosphate, potassium chloride, magnesium sulphate, pectin, methyl red, dextrose, Ethanol and Distilled water all the chemicals were laboratory grade.

Selection and collection of samples:

Spoiled and fungal infected oranges were collected from the local market. Fungal infected orange peels were selected as the source of isolating the pectinase yielding fungi as the skin of the fruits has high content of pectin. The fungal colonies developing on the spoiled fruits would more likely to have pectinase positive fungal samples. Orange peels are separated and made into small pieces. Those fungal infected orange peel pieces are soaked in water overnight and filtered on next day morning. The inoculum is further used for pure culture preparation.

Screening test for Enzyme Production

Preparation of Screening Media:

All the ingredients are weighed accurately and dissolved in required quantity of water as listed below. Pectinase Screening Agar Medium (PSAM) is used for the selective growth of those microbes which release pectin.

Preparation of pure culture:

The petri dishes were taken and sterilized using Hot air oven. The sterilized media were poured into sterile petri dishes and allowed to solidify. The pure culture of the fungi prepared in the previous step was inoculated onto the media plates. All the plates were incubated at 25◦C for 3 to 4 days. For the development of spores the culture must be 7 to 8 days old.

Identification of Fungi by Staining and Microscopy:

Lacto phenol Cotton Blue Staining

Preparation of staining solution:

Dissolve 20 g carbolic acid (solid), 20 ml lactic acid and 40 ml glycerol into 20 ml distilled water (heat as gently as possible). Add 0.05 g cotton blue, shake until well mixed, and filter before storing.

Staining protocol:

Place a drop of lactophenol cotton blue staining solution onto a clean slide, and mix the fungal culture or clinical sample with the staining solution. Place a cover slip on top and heat gently. Press the cover slip gently to remove any bubbles. Observe the slide under an oil immersion lens.

Sub culturing of Pure Cultures:

Pectinolytic Activity:

The secondary screening for pectinase producing bacteria was done by the Well diffusion method. Nutrient broth was prepared and inoculated with the isolate in separate tubes. 1 ml of cell free supernatant was poured on the Vincent's agar well of diameter 5 mm prepared in plates by sterile borer. After pouring the broth cultures, plates were incubated at 37° C for 24 hours. After incubation plates were flooded with the iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330ml H_2O) and the zone of clearance was observed. The cultures which showed highest zones were selected for further study.

Preparation of Production Media:

All the ingredients are weighed accurately and dissolved in required quantity of water as listed below and sterilized using autoclave. Inoculate the media with selected colonies of the master plate and then it is placed in the incubator for 24hrs at 37◦C.

Purification:

Centrifugation method:

Transfer the production media into centrifuge tubes into equal parts of the volume then centrifuge the tubes by maintaining 6000 rpm for 15mins. After centrifugation collect the liquid from the tubes and then measure the volume of liquid.

Salt Precipitation:

Ammonium sulfate precipitation is a method used to purify proteins by altering their solubility. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used, as its solubility is so high that salt solutions with high ionic strength are allowed.

Principle:

Weigh 8.88 grams of Ammonium sulfate for 40ml of suspension add pinch by pinch. Ammonium sulphate in to the enzyme suspension in ice cold conditions, on magnetic stirrer for 1hour.Keep it in ice-cold conditions for overnight for complete saturation.

Dialysis:

Dialysis is the process of separating molecules in solution by the difference in their rates of diffusion through a semipermeable membrane, such as dialysis tubing. Dialysis is a common laboratory technique that operates on the same principle as medical dialysis.

Procedure:

Activation of Dialysis Membrane:

Take 100ml of distilled water and boil it. Add the dialysis membrane into boiling water. Boil it for 10min. Add 2% Sodium bicarbonate, boil it for 10minutes.Take another 100ml of distilled water and boilit. Transfer the dialysis membrane into this boiling water and boil it for 10 minutes.

Take out the membrane with the help of forceps. Tie the one side of the membrane. Add 10ml of enzyme suspension into the dialysis membrane. Tie another side of the dialysis membrane. Place it in distilled water containing beaker. Place the beaker on magnetic stirrer for 2 hours or keep it in refrigerator for overnight

Assay of Pectinase

Pectinesterase activity can be measured by testing the increase in free Carboxyl group at the end of the reaction. This can be done by performing the titration of sample using PH indicator. To analyze pectin esterase activity.20ml of 1% pectin dissolved in 0.15M NaCl (pH - 7) is used to which 4ml of crude enzyme extract must be added. This should be incubated for 1hour.Later the solution is titrated against 0.02 N NaOH to reach PH 7 using Phenolphthalein as an indicator. Heated crude enzyme extract acts as a control. One enzymatic unit (U) was defined as the quantity of enzyme which liberates 1 icroequivalent of carboxylic group in 1 h of reaction under the described conditions. The enzymatic activity was expressed in U/g.

3. Results and Discussion

Selection and collection of samples: Fungal infected orange peels were selected as the source of isolating the pectinase yielding fungi. From the collected orange peels, inoculum for pure culture preparation was collected.

Pure Culture Preparation:

Fungal colonies were isolated by using Sabouraud Dextrose Agar (SDA) medium after inoculating the inoculums collected from the orange peels. Eight days of incubation was done to observe the growth of fungus on the medium.

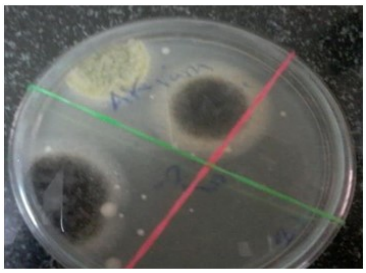


Fig 1: SDA Media Plate showing Light grey and black colonies on 8 day after inoculation of the source

Screening test for Enzyme Production:

Pectinase Screening Agar Medium (PSAM) is used for the selective growth of those microbes which release pectin. Fungal colonies were observed as pure cultures on the screening medium.

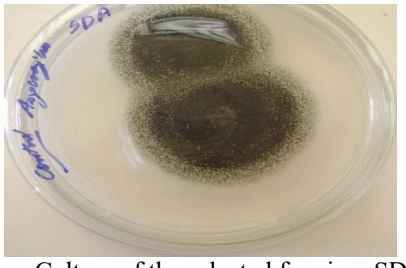


Fig 2: Pure Culture of the selected fungi on SDA Plate

Identification of Fungi by Staining and Microscopy:

By using Lacto phenol Cotton Blue Staining method the fungal was identified as *Aspergillusniger* due to its spore and head structure.

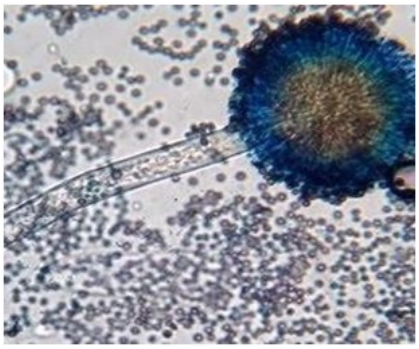


Fig 3: Microscopic Observation of Isolated Fungi

Production of Pectinaseenzyme:

A light grey to black coloured mat is observed on the liquid broth (production medium) after 8 days of inoculation, this indicates the release of pectinase enzyme. Protein excretion by filamentous fungi is mainly restricted to the tips of growing hyphae.

Fig 4: Enzyme Production in Liquid state fermentation

Purification of Produced Enzyme

Salt Precipitation:

By using ammonium sulfate, the produced enzyme is purified as white color precipitate from the spent medium. As the solubility of proteins vary according to the salt concentration, the high concentration of ammonium sulfate precipitates out the enzyme.

Fig 5: Salt precipitation performed on the ice pack

Dialysis: The precipitated enzyme is further purified by a process called Dialysis. In this process the molecules in solution are separated by the difference in their rates of diffusion through a semipermeable membrane. After performing the process for 3 hours at room temperature, it was kept in the refrigerator overnight. On the next day, the enzyme was collected in pure form.

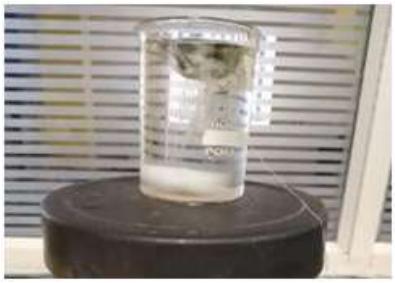
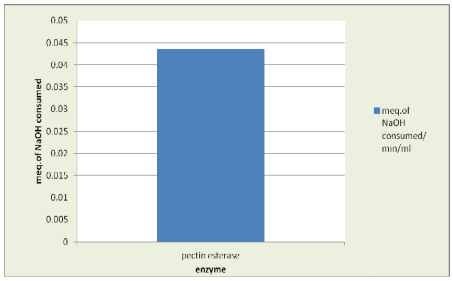


Fig 6: Dialysis performed on a magnetic stirrer



Graph 1: Graph showing NaOH consumed on Y axis and X axis showing the enzyme released.

Therefore pectin esterase activity was calculated to be 0.043µmol/min/mg

4. Conclusion

Naturally occurring polysaccharide pectin, the methylated ester of polygalacturonic acid, is very important in both scientific and commercial world due to its biodegradability. A large group of pectinase enzymes causing breakdown of pectin polysaccharides of plants and fruit are used in industrial sector to increase the yield and clarity of fruit juices. In this study, a fungal strain was isolated using PSA Medium from the peel of rotten oranges. Isolated fungal strain was identified based on staining and biochemical tests. The fungus was found to be *Aspergillusniger* due to its spore and head structure. Pectinase enzyme was produced by Liquid State Fermentation. A light grey coloured mat is observed on the liquid broth (production medium) after 8 days of inoculation, this indicates the release of pectinase enzyme. The obtained Pectinase enzyme is purified by salt precipitation and dialysis. Pectinesterase activity can be done by performing the titration of sample using phenolphthalene indicator. One enzymatic unit (U) was defined as the quantity of enzyme which liberates 1 microequivalent of carboxylic group in 1 h of reaction. The enzymatic activity was expressed in U/g. It was calculated and found to be 0.043µmol/min/mg. So the isolated fungal strain is efficient and has potential to be implicated commercially to increase the clarity and quality of fruit juices.

Table 1: Composition of SDA medium

Ingredients	**Gm/L**
Mycological peptone (enzymatic digest of casein and animal tissues)	**10 gm**
Dextrose	**40 gm**
Agar	**15 gm**
pH adjust to 5.6 at 25^0 C	

Table 2: Composition of Screening Media

Ingredients	Quantity (gm/100ml)
Sodium Nitrite	0.2gm
Potassium hydrogen phosphate	0.1gm
Potassium Chloride	0.05gm
Magnesium sulphate	0.05gm
Yeast Extract	0.1gm
Pectin Pract	1gm
Agar Agar	5gm

Table 3: Composition of Production media

Ingredients	Quantity (gm/100ml)
Sodium Nitrite	0.2gm
Potassium hydrogen phosphate	0.1gm
Potassium Chloride	0.05gm
Magnesium Sulphate	0.05gm
Yeast Extract	0.1gm
Pectin	1gm

Table 4: results for pectin Pectinase assay

Volume of NaOH used to titrate the sample	45ml
Volume of NaOH used to titrate the blank	7ml
Normality of NaOH	0.02N
Volume of incubation mixture	29ml
Time	60min

Pectinesterase activity is calculated using the following formula Pectin esterase activity

$$= V_S - V_b \text{ (Normality of NaOH)} \times 100/Vt$$

Where, Vs- Volume of NaOH used to titrate sample (ml), V b- Volume of NaOH used totitrate blank (ml), V - Volume of incubation mixture (ml), t - Reaction time (min). Pectinesterase activity is expressed as milli equivalents of NaOH consumed $min^{-1}ml^{-1}$of crude enzyme extract under the assay conditions.

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