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# Bioconversion of orange peel residue for the production of pectinase using *Calocybe indica* and its application in biomedical research

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## ABSTRACT

Conversion of organic material to valuable products like pectinase by biological agents like fungi and bacteria has been a leading approach in recent times. Production of pectinases for various commercial uses by a cheaper method is a big challenge. An edible mushroom *Calocybe indica* cultivated under solid state fermentation using orange peel residue as substrate can produce significant amount of pectinase. Since, orange peel is highly rich in pectin could be used for the growth of many fungi. *Calocybe indica* seed was inoculated on PDA agar medium and allowed to grow on the plate for seed inoculum production, then; it was inoculated onto the substrate as orange peel residue. Five different substrate concentrations were prepared using orange peel residue with wheat bran and rice bran as subsidiary with moisture content of 1:2.The growth was stopped on 25$^{th}$ day of fermentation by mixing distilled water and enzyme was extracted. Among substrates, substrate type-1 (only residue) and 4 (residue + wheat bran + rice bran) showed higher enzyme activity 0.3435 and 0.335µmol/min respectively this was determined by DNS method. Molecular weight of crude pectinase was determined by SDS-PAGE which showed band of 30kDa which indicated the presence of pectinase in substrate. In this study, we found out that orange peel residue could be cheaper and better substrate for production of pectinase using *Calocybe indica* an edible fungus and make the pectinase enzymes commercially viable.

**Keywords:** Pectinase, Hydro-distillation, Mushroom, Ammonium salt, Dialysis, SDS-PAGE

## 1. INTRODUCTION

The term bioconversion refers to the process of conversion of organic wastes from plants and animals into valuable products utilising biological agents like microbes. Numerous species, including bacteria,

fungus and algae have been utilised as biocatalysts in the bioconversion process. Among all microorganisms employed for the bioconversion process fungi are the best due to their high yield and resistance to the environmental changes like pH and temperature. The bioconversion process is usually carried out in small to large vessels depending on the requirement. Different bioreactor configurations may be suited for the bioconversion process, which is typically carried out in batch or continuous process (Kennes, 2018; Jadaun and Jyoti, 2018).

Enzymes are biocatalysts which accelerate the rate of metabolic reaction. Enzymes are catalysts that bring about specific biochemical reactions in cellular metabolism. More than 3000 enzymes have been identified, but only about 5% are exploited industrially (Patel et al., 2017). Pectinase, an enzyme that converts pectin into a simple polysaccharide, is produced by many fungi. Pectinase production accounts for about 10% of the total production of enzyme preparations. Since orange peel is highly rich in pectin could be used for growth of many fungi which can degrade it by producing pectinase (Castilho et al., 2000). Production of pectinase can be accomplished either through submerged fermentation or solid-state fermentation, with solid state fermentation being preferred due to its higher yield (Irshad et al., 2014). In industrial field, when pectin needs to be broken down pectic enzymes are used, many microorganisms are used in the production of pectic enzymes (Haile et al., 2022).

Pectinase is of industrial importance, especially in the juice industry, to improve the quality of juice, in the food industry to produce vegetable oils (Mahmoodi et al., 2019). Pectinases are a group of enzymes that catalyse the breakdown of pectic substances by depolymerization and de-esterification reactions (Danielle et al., 2009). In 1930s, the commercial use of pectinases was first observed to produce wine and fruit juice. The main applications of pectinases are in juice industry, textile industry, paper industry, coffee and tea fermentation, wastewater treatment, retting and degumming of fibre crops and so on (Kashyap et al., 2001). These enzymes are used to increase juice extraction by reducing concentrate viscosity in cellulosic fibre production, coffee/tea fermentation, and juice and wine clarification (Phutela et al., 2005).

Pectinase is used in the maceration of fruit tissue in the wine industry. It is utilised in numerous food goods to create unique flavours. To reduce viscosity and mucilage, they are employed in the manufacturing of instant coffee and tea. In the paper and pulp business, alkaline pectinase is used to enhance paper quality without producing harmful effluent and to cleanse wastewater from industries that produce pectinaceous wastes (Jadaun and Jyoti, 2018). The pectinase complex enzymes, which are of the utmost importance, are polygalacturonase, pectin lyase, pectin esterase and pectate lyase. The pectin hydrolases as polygalacturonases catalysis α-1,4-glycosidic linkage hydrolysis in pectic acid (Palagiri et al., 2019).

Fruit waste is one of the most grown and consumed fruits in the world, orange are produced at a rate of over 80 million tonnes annually (Alexandrino et al., 2007). The Nagpur orange, one of several orange varieties, is of mandarin origin (*Citrus reticulate*) and it is typically grown in the Maharashtra districts of Amaravati, and Wardha. It is well-known for its rind that is simple to peel. The geographical indication for Nagpur orange was filed with the Indian G I registrar and became official in April 2014. Citrus fruit is one of the most valued crops because it contains numerous active phytochemicals that are used to treat a variety of medical conditions. The key nutrients in this fruit include vitamin C, folic acid, potassium and pectin (IIFPT, 2020). Citrus fruit peels are already available in the market as a by-product of the orange processing industry (Hachemi et al., 2015). The orange peel contains a significant amount of pectin, which is broken down by microorganisms to produce pectinase (Haile et al., 2022). They are major waste that are used in cattle feed because they are high in molasses, pectin, and limonene (Rafiq et al., 2016). Wheat bran mostly consists of non-starch carbohydrates such as

arabinoxylans or cellulose, starch and crude proteins is used as substrate to produce pectinolytic enzymes.

In this study, an attempt is made for the first time to produce pectinase in a solid-state fermentation using *Calocybe indica*, an edible mushroom strain and orange peel residue as a substrate. *Calocybe indica*, commonly known as milky white mushroom, grows during the summer in the gangetic plain of West Bengal of India (Chakravarty et al., *1981*). This mushroom is a relatively new introduction from India (Purkayastha et al., *1976;* Sandeep et al., 2018). Its robust size, sustainable yield, attractive colour, delicacy, long shelf-life, and lucrative market value have attracted the attention of both mushroom consumers and prospective growers (Chakraborty et al., 2010). The *C. indica* is rich in protein, lipids, fiber, carbohydrates, and vitamins and contains an abundant amount of essential amino acids (Alam et al., 2008). Various studies have used *Aspergillus, Penicillium, Rhizopus, Trichoderma* and other fungi in the synthesis of pectinase enzyme and majority of them produce toxins. At the time of harvesting, this can be expensive. The primary safety concern with the production of commercial enzymes is not the enzyme, but the inclusion of known mycotoxins in the product. An edible fungus, *Calocybe indica* could be utilised to produce enzymes to overcome this limitation (Heerd et al., 2012; Karittapattawan and Benchawattanon, 2021).

## 2. METHODS AND MATERIALS

### 2.1 Preparation of orange peel residue

Orange peels were collected from local fruit shop from Bengaluru and dried in a hot air oven for three days at 40°C. The peel was ground into powder using a mixer, after complete drying it was stored in a polythene bag. Hydro-distillation is the procedure used to extract the essential oil from orange peel power. Each time the distillation process was started, 30g of powder was placed in a round-bottom glass flask with 200ml of distilled water. The temperature is initially kept at 80°C for 30 minutes to induce fumes, and then it is reduced to 55-60°C for 2.5h, after which oil is separated. The whole content is filtered to remove the water, the filtered residue is dried in a hot air oven at 40°C until complete dry. The fully dried residue was made into powder by using mixer and stored carefully in a polythene bag. The powder was sieved using medium size mesh, wheat bran and rice bran were also sieved with medium size mesh.

### 2.2 Organism and culture condition

An edible mushroom, *Calocybe indica* is used in the production process. The seed was inoculated on PDA agar medium and allowed to grow completely on the plates. Mycelial plugs of agar plates containing 15 day mycelium were used as inoculum A total of 10g of substrate was made using orange peel powder, wheat bran, and rice bran in five 250 ml Erlenmeyer's flasks labelled 1, 2, 3,4 and 5 respectively. The substrate in the first flask had only orange-peel powder residue which is used as control. Second flask contained 7.5g of wheat bran and 2.5g of residue. The same amount of wheat bran and orange peel powder (5g each) were used in the third set, there were 7.5g of residue and 2.5g of wheat bran added in fourth flask (**Table 1)**. The final fermentation substrate was made up of 2.5 g of residue, 3.75 g of wheat bran, 3.75 g of rice bran and 3.75 g of residue in the fifth flask. Distilled water in the ratio 1:2 is added to each of the flasks and the flasks were then sterilised at 121°C for 15 min. Mycelial plugs were inoculated into the flasks and then incubated at room temperature to allow the fungus to fully develop on the substrate that breaks down pectin to pectinase. After 25 days, the growth was stopped by adding distilled water and enzyme was extracted.

**Table 1** Different composition of substrates

| Substrate type | Substrate Composition |
|---|---|

| | |
|---|---|
| 1 | Control (only residue) 10g |
| 2 | 2.5g residue + 7.5g wheat bran |
| 3 | 5.0g residue + 5.0g wheat bran |
| 4 | 7.5g residue + 2.5g wheat bran |
| 5 | 2.5g residue + 3.75g wheat bran + 3.75g rice bran |

**2.3 Enzyme extraction**

Enzymes were extracted from the fermented mycelial substrate in solid state fermentation by homogenising the entire substrate with 45ml of distilled water. It is agitated at 100 rpm in 4°C using a rotary shaker. After centrifuging the biomass at 4000 rpm for 20 min, the supernatant was collected and used as crude enzyme (Mrudula and Anitharaj, 2011).

**2.4 Determination of enzyme activity**

The filtrate was assayed for pectinase activity; determined at 50 °C using 0.1% (w/v) pectin as substrate at pH 5.0. Reducing substances were measured using DNS method (Miller, 1959; Carmona et al., 1998). Enzyme activity was expressed as U/mL (Alexandrino et al., 2007). The reducing sugar released were estimated by the 3, 5-dinitrosalicylic acid method (DNS) (Rafiq et al., 2016)

**2.5 Preparation of standard curve**

The 3,5-dinitrosalicylic acid (DNS) reagent was used to measure the amount of reducing sugars generated because of pectinase's action on orange pectin to determine the activity of the pectinase enzyme (**Figure 1)**. Polygalacturonase hydrolyses the polymer of pectin into the galacturonic acid monomers. The free galacturonic acid units produced as a result of polygalacturonase activity reacts with 3-5 dinitrosalicylic acid (DNS) reagent and form a colored complex. The degree of change of color was measured by spectrophotometer at wavelength of 550 nm. Greater the amount of galacturonic acid produced, darker the color of the enzyme- galacturonic acid complex formed and more the light absorbed. With the help of DNS reagent, several dilutions of known glucose concentrations were made, and the absorbance of those dilutions was then evaluated in a spectrometer at 540 nm. 1ml of the 14µmol galacturonic acid solution was serially diluted into 1ml of distilled water taken in 5 test tubes. About 1ml of DNSA reagent was added into each test tube and incubated in boiling water bath for 10 min. 1ml of Sodium Potassium tartrate to each tube then measure the optical density. Determined the reducing sugars released by enzymatic hydrolysis. To correct the non-enzymatic release of sugars, a distinct blank was prepared. The quantity of enzyme needed to release 1µl mole of reducing groups per minute using glucose as the standard under the assay conditions is referred to as one unit of enzyme activity. The of pectinase activity concentration will be shown on a glucose standard curve. By enzymatic action to determined amount of reducing sugars released in five categories of samples by same method and absorbance was read at 540nm. In each tube take sample of 20µl of crude enzyme then addition of 1ml of distilled water, 1ml of DNS reagent then incubated in boiling water bath for 10min after adding 50µl of sodium potassium tartrate, measure optical density at 540nm.

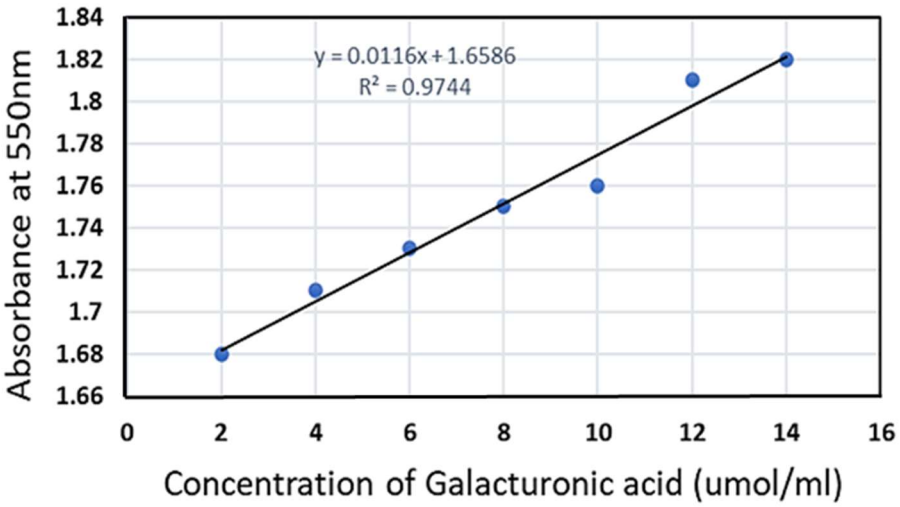


**Figure 1** Standard curve of Galacturonic acid.

**2.6 Purification of enzyme**

To determine the proportion of ammonium sulphate concentration that will precipitate most of the protein from the crude enzyme sample, ammonium salt precipitation is done. Each flask is filled with 30ml of crude enzyme, and then 60% (w/v) solid ammonium sulphate is added while stirring continuously in a cold condition and the flasks are then stored at 4°C overnight. The precipitate that resulted was separated by centrifugation at 4000 rpm for 30 min in cold condition. Discard the supernatant and use distilled water to clean the pellets. For additional purification, settled precipitate is dissolved in sodium acetate buffer (Ubani et al., 2015).

**2.7 Dialysis**

The dialysis bag (Sigma Aldrich) of 10000KDa molecular cut off was thoroughly rinsed with distilled water. The precipitate was combined with 5ml of 0.1M sodium acetate buffer. The dissolved solution was put into dialysis bags and both ends were tied, then, submerged in a 100ml solution of sodium acetate buffer. It was stored at 4°C for 24 hours (Leelamani et al., 2018).

**2.8 Molecular weight determination by SDS-PAGE**

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular mass of the crude and half purified pectinase. Electrophoresis was performed at room temperature for 2.5h with a 100V and gel was kept in fixing solution and then washed with wash buffers. Coomassie blue stain was used to visualize the protein bands.

**3. RESULTS**

**3.1 Culture Condition**

*Calocybe indica* was used in the present study since it is an edible mushroom and has the ability to grow on orange peel residue to breakdown pectin. After incubation, type-5 substrate yielded highest mycelial mass followed by 4, 3, 1 and 2.

**3.2 Determination of enzyme activity**

Enzymes were extracted from the fermented mycelial substrate in solid state fermentation by homogenising the entire substrate with distilled water. After centrifuging, supernatant was collected and used as crude enzyme (**Figure 3)**. Results of determination of enzyme activity were showed in Table 2.

**Table 2** Determination of enzyme activity by DNSA method

| Substrate type | O D (540nm) | PA (µmol/ml) |
|---|---|---|

| | | |
|---|---|---|
| 1 | 2.222 | 1.407914764 |
| 2 | 1.444 | -0.565702689 |
| 3 | 1.789 | 0.309 |
| 4 | 2.196 | 1.341 |
| 5 | 2.151 | 1.227 |

**Note:** OD- optical density: nm- nanometer: PA- Polygalacturonic acid

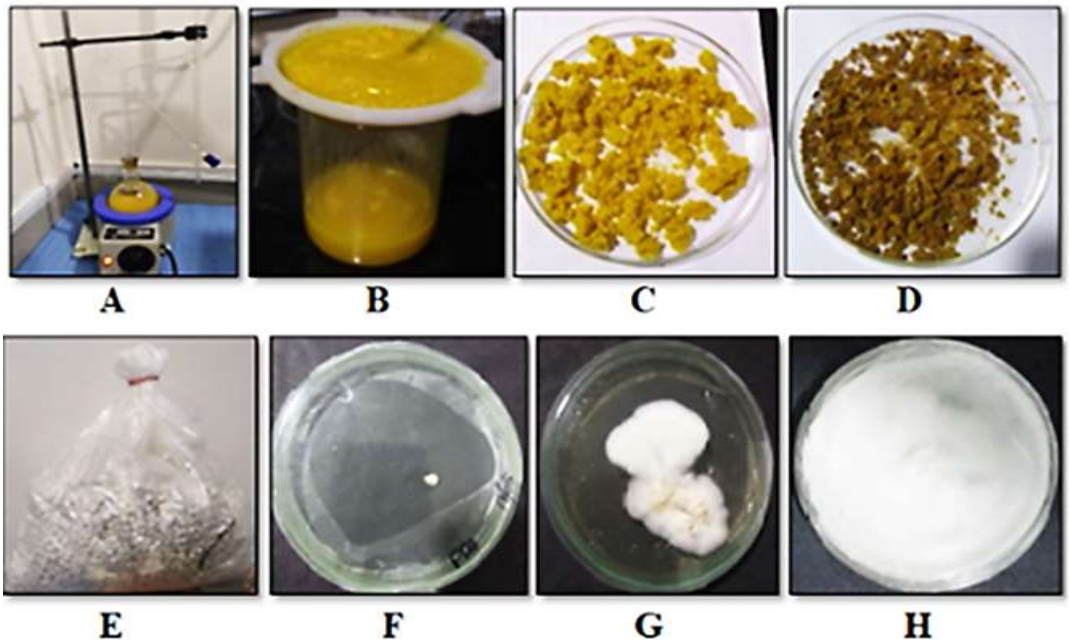


**Figure 2.** Preparation of substrate: A. Hydro-distillation of orange peel powder: B. Filtration after hydro-distillation, C. residue collected, D. dried residue. *Calocybe indica* seed culture at different stages, E. Seed inoculum, F. Seed inoculation on PDA medium on day 1, G. mycelial growth on day-7 and H. mycelial growth covering whole petriplate on day 14.

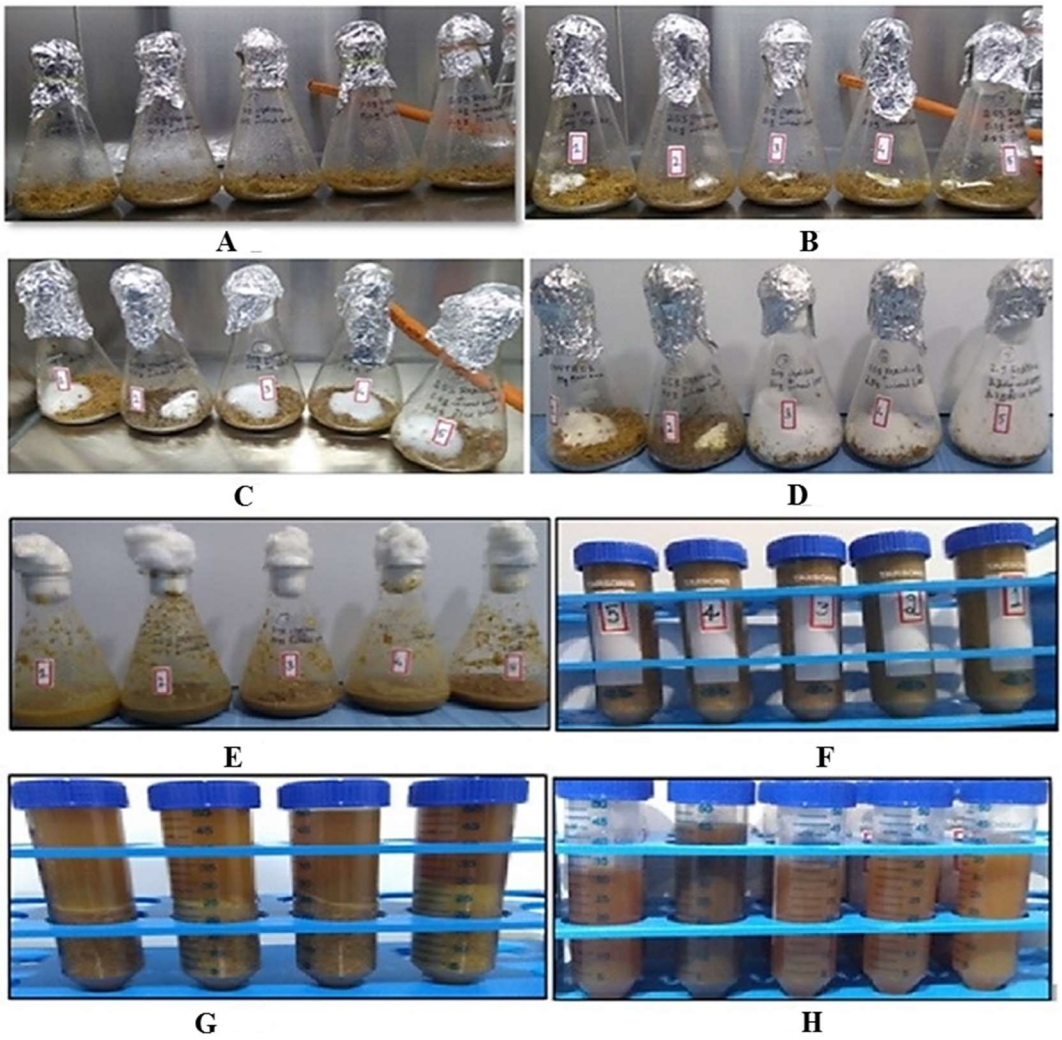


**Figure 3.** Growth of *Calocybe indica* on orange peel residue (solid-state fermentation): A. Substrate before inoculation, B. *Calocybe indica* culture on day 1, C. Culture growth on substrate (15$^{th}$ Day), D. Culture growth on substrate (25$^{th}$ Day). Culture harvest and enzyme extraction: E. Culture inhibition, F. Crude extracts after harvest, G. Crude extracts centrifugation, H. enzyme extract.

**3.3 Molecular weight determination by SDS-PAGE**

The molecular weight of crude pectinase was analysed by SDS-PAGE separations and all the five substrate types showed protein band of 30 kDa (Figure 4E) clearly indicating again that orange peel residue is one of the best substrates added with wheat bran and rice bran. Crude enzyme was concentrated by subjecting it for ammonium salt precipitation and dialysis to further confirm the presence of pectinase by SDS-PAGE (Figure 4F). It is very evident by all these results that the orange peel residue is a very good and economical substrate to produce pectinase in a cheaper simple process.

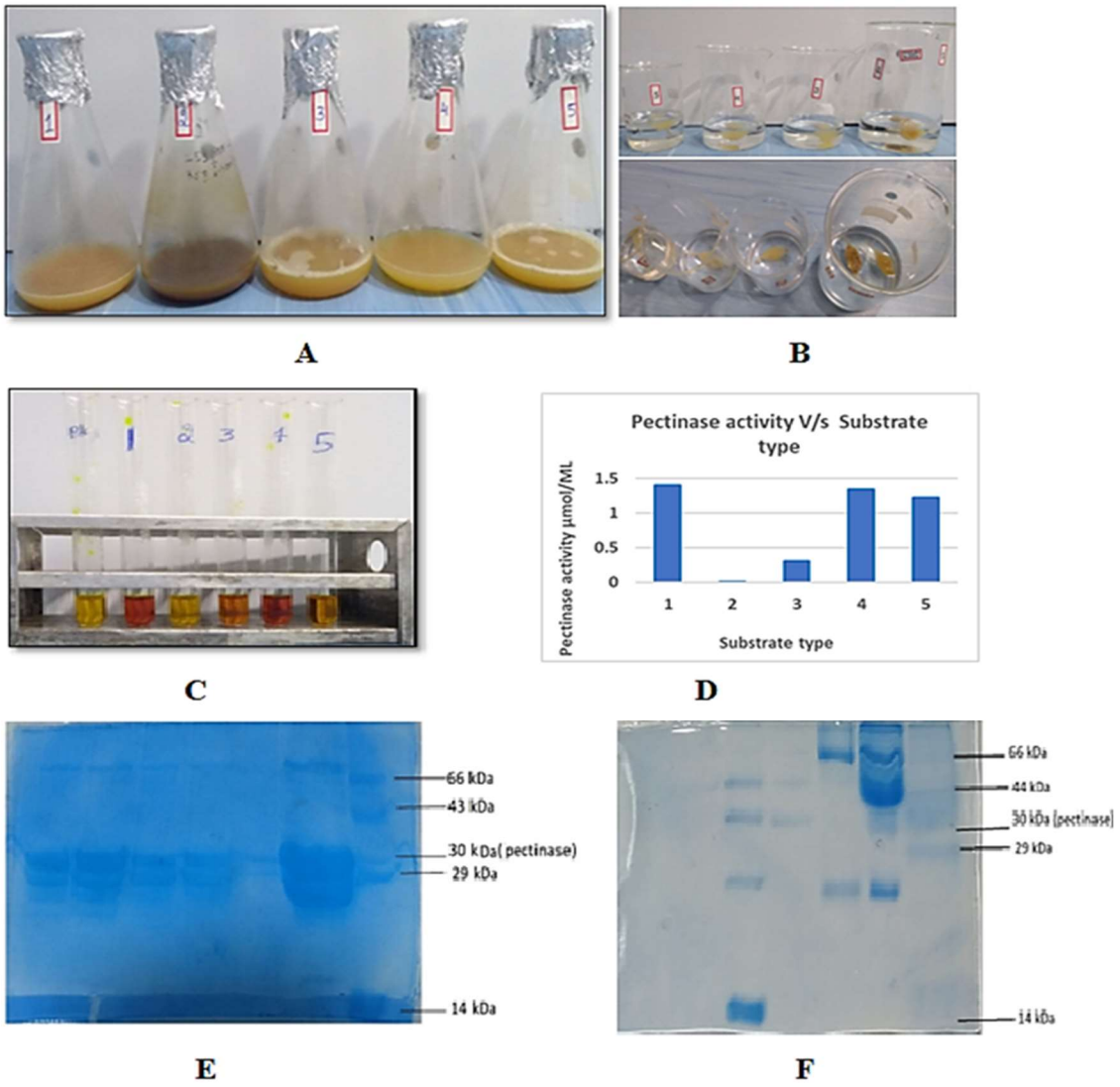


**Figure 4.** Enzyme purification and activity determination: A. Ammonium salt precipitation, B. dialyzed pectinase, C. Determination of enzyme activity by DNS method: D. histogram: enzyme activity v/s substrate type, SDS-PAGE separation: E. crude pectinase, F. half purified pectinase.

## 4. DISCUSSION

The substrate used in this study can be considered practical and economically feasible due to availability throughout the year at little or no cost in large quantity. Utilization of these agrocellulosic wastes for the production of pectinase from *Calocybe indica* could be more economically and ecologically friendly. In the present investigation orange peel residue used as substrate for production of pectinase from *Calocybe indica* strain. Increase in the amount of orange peel residue enhanced the growth of fungus indicates that pectin is the main substrate and wheat bran, rice bran are the subsidiary substrates (Figure 2). The substrate Type-1 showed maximum pectinase activity (1.407914764µmol/ML) followed by Type-4.3,5 and 2 indicating that presence of pectin in the substrate induce production of more and more pectinase as fungus utilize the pectin as main source of carbon (Figure 3 A-H). Orange peel residue is the best substrate to produce pectinase. Among five categories, substrate type 1 and 4 showed more activity 1.40µmol/ml and 1.341 µmol/ml respectively, as these contained more orange-peel residue and which is highly rich in pectin. Similar to these

findings, Anuradha et al. (2014) reported that the pectin rich raw substrates like orange peel, Jack fruit rind, carrot peel and beet-root peel, the maximum pectinase production was found in Jack fruit rind (38 U/ml) followed by carrot peel (36 U/ml) bee-root peel (24 U/ml) and orange peel (16.8 U/ml) by *Aspergillus awamori*. Okafor et al. (2010) during their investigation on two pectinase producing fungal isolates, *A. niger* and *P. chrysogenum* using the different agro-wastes, including pineapple peel, orange peels, sawdust, sugarcane pulps and wheat bran, as the sole carbon source reported the highest pectinase activity of 350.28 and 478.25 $Uml^{-1}$ protein using *A. niger* and *P. chrysogenum,* respectively. From various agricultural waste and agro-industrial by-products such as banana peel, wheat bran, sugar cane bagasse, and orange bagasse, the best substrate for pectinase production by *Penicillium* species was found to be in orange bagasse with enhanced enzyme production of 64.5 U/mg followed by wheat bran 53.6 U/mg (Okafor et al., 2010; Vijaykumar et al., 2014). The Similar reports were also approved out by Silva et al. (2002) where orange bagasse and wheat bran gave higher yields of pectinase by the culture *P. viridicatum* RFC3. Orange bagasse is very cheap, abundantly available and could be easily stored after sun drying. This waste is generated after the extraction of juice and available in high quantity from fruit processing industries. Hence its eco-friendly utilization is essential which interested to use agro waste for pectinase production by solid state fermentation (Afifi and Foaad et al., 2002). The previous research reported that natural substrate, orange and xylan supported the pectinase production with 363.0 ± 2.1 U/g and 697.23 ± 11.7 U/g pectinase activity attained, respectively (Oumer and Abate 2017). Though, several substrates for pectinase's substrate specificity have been used in the earlier studies. Siddiqui *et al.,* (2012) stated pectin has been used as a substrate in the substrate specificity study with 8.34 U/ml of pectinase activity (100% relative activity) was obtained.

The results of this study are reliable with the work of Darah et al., (2013), who reported that *Aspergillus niger* HFM-8 produced the highest pectinase activity of 3.0156 IU. it is also reliable with the work of Ezugwu et al. (2014), who examined pectinase production by *Aspergillus niger* using two types of substrates, where higher pectinase activity was observed when using orange peels as the substrate. The molecular weight of crude pectinase was analysed by SDS-PAGE separations and all the five substrate types showed protein band of 30 kDa. It clearly representative again that orange peel residue is one of the best substrates added with wheat bran and rice bran. Various studies discovered different molecular mass of pectinase enzyme produced from different microbial species (Oyede, 1998). Different microbial sources showed wide-ranging divergence in molecular mass of pectinase i.e. *Aspergillus japonicum* (38.0 and 65.0 kDa) (Semenova et al., 2003), *Aspergillus kawachii* (60.0 kDa) (Esquivel and Voget, 2004). The difference has been associated with the substrate employed, type and nature of microorganism, host cell wall and analytical approaches (Oyede, 1998).

## 5. Conclusion

These studies illustrated that when *Calocybe indica* cultivated under solid-state fermentation in orange peel residue is able to produce pectinase. Thus, Orange peel residue which is an agro-biowaste could be used properly instead of disposing it a merely waste. It is found to be good substrate for the production of pectinase by growing *Calocybe indica*. It is very evident that the flask containing high proportion of orange peel residue showed maximum yield indicating that the pectin is the main substrate for the growth of fungus. These studies suggested that *Calocybe indica*, an edible mushroom can be applied to produce good quality pectinase using orange peel residue as substrate very economically. Further works on scale up fermentation with optimization and industrial application are in progress in our laboratory.

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**Authors' contributions**
All authors contributed toward data analysis, drafting and revising the paper and agreed to be responsible for all the aspects of this work.

**Declaration of Conflicts of Interests**
Authors declare that they have no conflict of interest.

**Availability of data and materials**
The datasets used in the current study available from the corresponding author on reasonable request.

**Use of Artificial Intelligence**
Not applicable

**Declarations**
Authors declare that all works are original and this manuscript has not been published in any other journal

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