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Production of fungal pectinase using mango peel as substrate by submerged fermentation

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Abstract: Mango peels have been found to have a considerable amount of pectin. The objective of the current research was to explore the feasibility of utilizing mango peels as fermentation medium to produce pectinase using fungi isolated from natural resources. Fungal strains were isolated from decaying fruits and grown in Czapek agar supplemented with pectin. Among these isolates, Isolate 2 gave the highest pectinase production rate which was later identified to be *Penicillum* sp. strains through microscopic examinations. This isolate was further used with three different fermentation media for submerged fermentation. The fermentation media are: T1−a defined medium, T2−mango peel powder, T3−6% mango peel powder plus 1% commercial pectin. Highest pectinase activity was achieved with T3 fermentation medium. Nevertheless, such difference does not exist between T1 and T2 (P<0.05). This suggests that mango peel can be used as an alternative base material in submerged fermentation media for pectinase production. To enhance pectinase production, it is further recommended that the mango peel powder be supplemented with small amount of pectin when used as base material in the medium.

Key words: mango peel powder; pectinase; fungi; submerged fermentation

以芒果果皮为基质进行真菌深层液态发酵生产果胶酶

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摘　要：为探寻果胶酶的生产途径，从腐烂的水果中分离得到真菌菌株，在添加有果胶的察氏琼脂中进行培养，在分离的菌株中发现菌株 2 果胶酶产率较高，通过镜检，确认该菌株为青霉菌(*Penicillum* sp.)。采用 3 种培养基(T1 为已知配方的对照培养基，T2 为芒果皮粉，T3 为 6%芒果皮粉+1%商业果胶)液态深层发酵该菌株，比较果胶酶活性。结果表明：T3 培养基中的果胶酶活性显著高于 T1 和 T2(P<0.05)，而 T1 和 T2 培养基果胶酶的活性差异不显著(P＞0.05)。芒果皮可以作为一种替代基质在液态深层发酵中生产果胶酶，在芒果皮粉中加入少量果胶作为培养基质可提高果胶酶产量。

关　键　词：芒果果皮粉；果胶酶；真菌；深层液态发酵

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Mango (*Mangifera indica* L.) is one of the most important tropical fruits in the world and currently ranked 5th in total world production among the major fruit crops[1]. Asia accounts for approximately 77% of

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global mango production, followed by the Americas and Africa at approximately 13% and 9%, respectively. In the Philippines, mango ranks third among the country's agricultural exportable crops. However, the utilization of this fruit for processing receives less attention internationally. It is estimated that only 0.22% of mangoes produced in the world is utilized for processing [2].

One of the major problems in fruit processing is that they produce a large amount of waste, which poses considerable disposal problems and ultimately can lead to pollution problems. Mango processing is no exception.

Mango consists of between 33%–85% edible pulp, with 9%–40% inedible kernel and 7%–24% inedible peel[3]. During the processing of mango, byproducts such as peel and kernel are disposed as waste, which is approximately 40%–50% of total fruit weight.

It has been found out that mango peels are rich sources of pectin, with a high degree of esterification and phenolic compounds. According to researchers at the Central Food Technological Research Institute in Mysore, India, mango peel provides high quality pectin. Since the peel makes up 20% to 25% of the total fruit weight, the average yield from the fruit makes it a sufficient and cost effective pectin source[4].

Pectin is an important component of middle lamella and primary cell wall of higher plants. These high molecular weight acid polysaccharides, which are primarily made up of α(1,4)–linked D–galacturonic acid residues[5], finds wide application in the manufacture of many fruit products like jams, jellies, and preservatives and thus are indispensable to the fruit juice industry[6].

The degradation of pectic substances is catalyzed by the enzyme pectinase in most food processing techniques. Pectinases are a complex and diverse group of enzymes that depolymerize pectin by hydrolysis, trans-elimination and de-esterification reactions, which hydrolyses the ester bond between carboxyl and methyl groups of pectin[7]. These reactions degrade pectic substances to saturated and unsaturated galacturonans, which are further catabolized to 5–keto–4–deoxy–uronate and finally to pyruvate and 3–phosphoglyceraldehyde[8]. This reaction is used in most food industries to prevent the undesirable spoilage and decay of processed foods.

Pectinases are often produced by plants, insects and saprophytic microorganisms such as bacteria and fungi. Microbial production of pectinolytic enzymes is mainly from filamentous fungi, yeasts and filamentous and non-filamentous bacteria[9]. Generally, fungal enzymes are acidic in nature, while alkaline enzymes are produced by bacterial strains. *Aspergillus* and *Penicillium* sp. are the predominant fungal sources of the pectinase[10–11]. Commonly, *Aspergillus* sp. is made to produce pectinase using two different techniques-submerged fermentation (SmF) and solid-state fermentation (SSF). As compared to solid state fermentation, submerged fermentation has been extensively employed for the production of enzymes and to understand physiological aspects of the synthesis of enzymes [12–13].

Pectinases are of great importance in commercial production of high quality fruit juices. However, these enzymes are often costly and must be imported from other countries. Therefore, a need to find an alternative and cheaper source of these enzymes exists.

In view of the above-mentioned points, the present investigation was undertaken and attempts were made to produce pectinase from isolated fungi from natural sources and to explore the feasibility of using mango peel powder as the sole carbon source to grow fungi under submerged fermentation.

1 Materials and methods

1.1 Screening of strain and culture maintenance

The fungal strain used in this study was isolated from decaying fruits procured from a local market. Ten grams of decaying fruits were peeled and poured in 0.8% NaCl solution. Serial dilutions of the sample were made and were spread afterwards in prepared pectinase screening agar medium (Czapek agar). The

medium was prepared by combining 2.0 g/L pectin, 15.0 g/L agar, 3.0 g/L $NaNO_3$, 1.0 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.01 g/L $FeSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to (6.0±0.20) and then sterilized. The sterilized medium was subsequently transferred aseptically into petri dishes.

Three replicates per dilution were made. The plates were incubated at 25 ℃ for 5 d. The colonies thus isolated were then subcultured 3–4 times in Czapek agar until active growth of isolates was obtained. Cultures grown on medium without pectin were used as control.

To test for the presence of pectinase among the isolates, 1% cetyl trimethyl ammonium bromide (CTAB) was added to the cultures to observe for zones of clearance, an indicator of the presence of pectinase[14]. The isolate which gave the highest pectinase production ratio was used in submerged fermentation experiments. Pectinase production ratio was calculated using the formula[15]: $R = Dh/Dc$. where Dh = pectin hydrolyze diameter (mm) and Dc = colony diameter (mm)

Thereafter, colonies were maintained on the same media in a slant and were stored at 4 ℃ for further experimental work.

1.2 Substrate

Dried mature mango peels were used as substrate for pectinase production. These were obtained locally and were cleaned and washed several times with water to remove all adhering substances. The peels were sliced into small pieces using knife and then dried in tray drier at 45 ℃ for 24 h[16]. The dried mango peels were powdered using a mechanical grinder and were homogenized by sieving.

1.3 Culture media and fermentation conditions

Three different fermentation media were prepared. A defined medium consisting of 2% pectin as carbon source, 1% peptone, 1% $(NH_4)_2SO_4$, 0.4% KH_2PO_4, and 0.05% $MgSO_4$ was used as control (T1). To check if microorganisms will be able to grow and produce pectinases on mango peel, a medium containing nothing but green mango peel powder (7%, *w*/*v*) as carbon source were prepared (T2). A second medium was prepared by combining 6.0% (*w*/*v*) green mango peel powder and 1.0% (*w*/*v*) pectin to assess the effect of combining different carbon sources on the production of pectinases (T3). The pH's of the media were adjusted to 6.0 and sterilized at 121 ℃ for 15 min and then used in submerged fermentation experiments.

Submerged cultures were prepared in 250 mL Erlenmeyer flasks with a working volume of 60 mL. The flasks were incubated at 25 ℃ on a rotary shaker for 3 d at 180 r/min.

1.4 Pectinase assay

After fermentation, the samples were centrifuged at a speed of 10 000 g for 10 min at 4 ℃ and then filtered using pre-dried and pre-weighed filter papers Whatman No.1 filter paper. The pH and pectinase activity of the filtrate were analyzed while the filter papers containing the residues were kept for the measurement of cell dry weight.

Pectinase activity was determined using the procedure described by Miller[17]. This method is based on the measurement of the amount of reducing substances liberated from pectin. A reaction mixture, consisting of 0.3 mL of suitably diluted sample, was added to a solution containing 1 mL of 1% of pectin substrate and 0.7 mL of 0.1 mol/L acetate buffer with a pH of 4.5. The mixture was then incubated at 45 ℃ for 30 min. The reaction was stopped by the addition of 2.5 mL 3,5–dinitrosalicylic acid (DNS) and the content was boiled for 5 min. After cooling, the color developed was read at 570 nm. The amount of reducing sugar liberated was quantified using galacturonic acid as standard. One exo-pectinase unit (U/mL) was defined as the quantity of enzyme that liberates one micromole of galacturonic acid per minute under the conditions mentioned above. All measurements were made in triplicate.

1.5 Dry matter

Biomass production was measured as the dry weight(DW) of the solids filtered from the centrifuged

samples. The filter papers containing the solids were dried at 105 ℃ for 18 h, until a constant weight was obtained. DW measurements of the solids from the samples allow the direct estimation of the amount of microbial biomass for each medium and its increase with time, i.e. growth rate[18].

1.6 Statistical analysis

Experiments were carried out in triplicate. The study was laid-out using Complete Randomized Design (CRD). Analysis of Variance (ANOVA) was used in analyzing and interpreting the data. Duncan's Multiple Range Tests (DMRT) was used to determine if there are significant differences existing among means.

2 Results and discussion

The present investigation was undertaken in order to explore the feasibility of using mango peel powder as substrate for the production of pectinase using isolated fungal strain from decaying fruits. Only extracellular pectinases were obtained because intracellular pectinases, in comparison with extracellular, are easier to harvest.

2.1 Isolation of pectinolytic fungi

The ability of each fungal isolate to produce pectinase was evaluated using the clear zone test. Based on visual observation, three isolated strains, gave noticeably large clear zones with diameters ranging from 2.00 mm to 3.75 mm, in contrast with the control which gave no evidence of pectinase production. Among the three isolates, Isolate 2 gave the highest clear zone diameter of 3.75 mm (Table 1). Hence, this isolate was used throughout the study.

Table 1 Selected isolates during the screening process

表 1 菌种筛选分离得到的菌株特征

strains	colony diameter/mm	hydrolyze halo diameter/mm	pectinase production ratio
control	9	0	0.00
isolate 1	9	21	2.33
isolate 2	8	30	3.75
isolate 3	10	20	2.00

Morphological examination of screened isolates was performed using Image Analyser with compound microscope. Most of the isolates, especially the three potential cultures, were identified as *Penicillium* sp. (Figure 1). Mycelium, a highly branched network of multinucleate, septate, colorless hyphae, is evident in the microscope image. Many branched conidiospores sprouting on the mycelia are also observed.

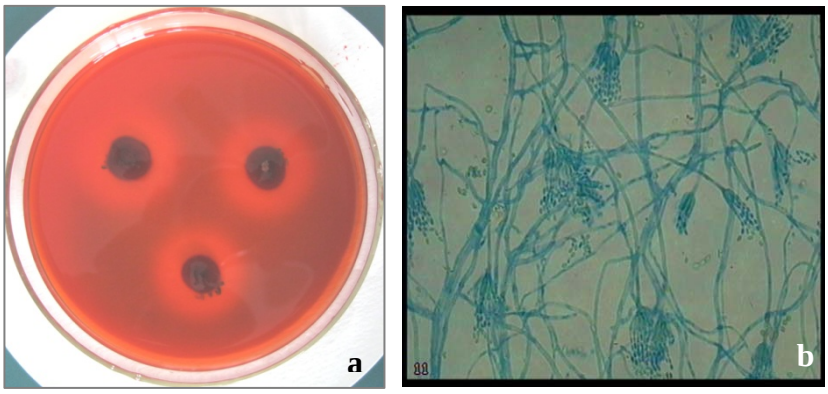

Fig.1 Macroscopic (a) and microscopic images(b) of the fungal isolate *Penicillium* sp. after 5 d

图 1 分离青霉菌株培养 5 d 后的平皿生长形态(a)和显微镜下形态(b)

2.2 Effect of using mango peel powder as sole carbon source versus defined medium in submerged fermentation

To investigate the feasibility and efficiency of using mango peel powder as substrate in pectinase production. All prepared fermentation media (T1, T2, T3) have an initial pH of 6.0. The fermentation media and control were then used in submerged fermentation

using Isolate 2 strain.

Table 2 shows the estimated enzyme activities for each fermentation medium formulations. For each medium, Isolate 2 was able to produced significant amounts of extracellular pectinase under submerged fermentation process. The use of mango powder supplemented with pectin (T3) in the fermentation medium gave the highest enzyme activity with a value of (0.899±0.089) U/mL. The same kind of behavior has been reported by Talboys[18] who stated that PG activity production in *Verticillium* sp. was constitutive but it was increased by incorporation of pectin to the medium. In contrast, the defined medium (T1), which was regarded as the basis of good production of pectinases, gave a pectinolytic enzyme activity of (0.643±0.017) U/mL. Statistical comparison of the means and standard deviation of the enzyme activities of the two treatments shows that they are significantly different at $P<0.05$.

Table 2　Pectinase activity and dry matter of the fungal isolates after a 72 h submerged fermentation

表 2　分离真菌菌株深层液体发酵 72 h 后的 pH、果胶酶活性及干物质量含量

treatments	pH	pectinase activity/(U·mL^{-1})	dry matter /%
T1	3.8	(0.643±0.017)b	(15.779±0.374)b
T2	3.4	(0.582±0.022)b	(15.554±0.330)b
T3	4.2	(0.899±0.089)a	(16.665±0.092)a

Comparison of the pectinase activities of media T2 (0.582±0.022) U/mL and T3 (0.643±0.017) U/mL indicates that substituting commercial pectin with mango peel powder as substrate in fermentation medium formulations is also viable. Statistical comparison of their means and standard deviation denotes that no significant difference exists between the two treatments ($P<0.05$). This may be due to the fact that mango peel powder has been proven to be a good source of pectin, containing about 10%–15% pectin[19–23].

Other parameters such as pH and dry matter of the fungal isolate were also measured. Based from the results obtained, there is an observed decrease in the pH of the fermentation medium from the initial pH of 6.0 (Table 2). Treatment 2 has the lowest pH value which may be due to the composition of the fermentation medium. This is followed by T1 and T3, respectively. As clearly seen in Table 2, there is a decrease in enzyme activity as the medium pH decreases. This could be due to the fact that as the acidity of fermentation medium increases, the activity or production of the enzyme decreases[23].

The maximum values of total dry weight (%) were obtained for each treatment on the 3rd day of fermentation. Treatment 3, being the highest pectinase producer, also has the highest dry matter(%) of (16.665±0.092), followed by T1 and T2, having (15.779±0.374) and (15.554±0.330) respectively. A high value on the measured dry matter throughout the fermentation period is a result of the microorganism growth. Odetokun[24] and Blandino et.al [11] reported that during fermentation, microorganisms, which degrade the substrate readily, may have secreted extracellular enzymes in the peels which are more direct indicators of increase in the cell concentration.

3　Conclusion

Among the different fermentation medium composition, 6% mango peel powder plus 1% pectin gave better results as compared with the defined medium, which is the most widely used fermentation medium, and pure mango peel powder. Nevertheless, the use of mango peel powder as sole base material of fermentation medium also presents a substantial outcome on the production of pectinase as there is no significant difference observed between this medium and the defined medium statistically. This suggests that there are other components in the mango peel aside from pectin which can serve as food for the fungi. This further enhances the production of pectinase by the fungi during the fermentation period. To achieve more superior pectinase yield, it is recommended that the mango peel powder be supplemented with pectin when used as base material of fermentation medium.

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