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Isolation of Pectinolytic Bacteria from Decayed Orange Peel (*Citrus sinensis*) in Clarification of Tea Extract

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ABSTRACT

Background: The production of microbial enzymes at the industrial scale and their commercialization has attained a lot of value and public focus. The usage of microorganisms as bio-reactors begins due to the evolution of enzymes produced by microorganisms. There are so many industrially significant enzymes are originated from microbes, that include pectinases, lipases, amylases, proteases, xylanases, *etc*.

Methods: The actual work involved the use of microbes isolated from the decaying orange peel and tomato pulp for the extraction of the pectinase enzyme. The organisms were isolated and screened from the samples and were cultured onto a pectin agar plate. Morphological and microscopic identification revealed that the bacteria isolated were *Bacillus sp.* Pectinase production media was later used for the lab-scale production of pectinase enzyme by inoculating the bacteria and incubating them for 48 hours. The enzyme thus produced was purified by Various methods. The pectinase enzymes isolated from *Bacillus sp***.** were used for the improvement of tea leaves quality and clarification. The enzymes were characterized and purified.

Result: The outcome of both purified pectinase enzymes and crude enzyme preparation on the improvement of tea extracts were determined in terms of flavonoids (310 mg/gm), DPPH (49 mg/gm), caffeine content (0.56-0.95), moisture content (7.2-6.7%), ash (4.1-4.6%), total phenol content (285 mg/gm) and pH (6) and the shelf life analysis of the tea produced. The results thus obtained can conclude that the use of *Bacillus sp.* in the industrial production of pectinase is highly beneficial. According to the findings obtained from the study, the enzyme-treated tea extract improves its various quality parameters more than the tea extracts which are non-enzyme treated. And the utilization of microbial pectinase produced from bio-waste was the most cost-effective and yielded good results for industrial use.

Key words: *Bacillus sp*., Clarification, *Microbial pectinase,* Orange peel, Tea leaves extract.

INTRODUCTION

Pectinase refers to a group of enzymes that digest a material called pectin, which can be found in the cell walls of higher plants. A pectic substance is a polysaccharide made up of an alpha-1,4-linked d-galacturonic acid. It is classified into two groups: the first is pectic acid, a polymer of galacturonic acid and the second is pectin, a polymer of galacturonic acid whose carboxyl groups are methyl esterified. By opening glycosidic bonds, these enzymes aid in the conversion of polygalacturonic acid to monogalacturonic acid. It weakens the cell wall and improves the yield of juice extract from the fruits through this procedure. Pectinases are divided into three categories: hydrolase, lyases and esterase (Mojsov, 2016). Enzymes are utilized to speed up the process. Fruit juices are made up of colloids, which are polysaccharides, proteins, tannins and metals.

Pectinases are required for the extraction and clarification of fruit juices and wines, as well as the extraction of oils, flavors and pigments from plant materials, the development of cellulose fibers for linen, jute and hemp production, espresso and tea fermentations and novel applications in the production of oligo glucuronides as functional food elements. Pine apple, papaya and waste obtained from the orange, apple, grape and lemonade industries are employed as enzyme production sources (Kuo *et al*., 2019). The search for pectin hydrolyzing enzymes in biomass from fruit processing waste is gaining traction. With this background the present study is conducted based on the objectives such as the Isolation of pectinase- producing bacteria from degraded fruits and vegetables: Identification of pectinase-producing bacteria by using different methods, Production of pectinase enzyme, Purification of pectinase enzyme, Incorporation of pectinase enzyme to the tea extract, determination of improvements and quality changes in tea extract, Determination of shelf life of the enzyme-treated tea extract (Nadar and Rathod, 2019).

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MATERIALS AND METHODS

Materials

Materials used to complete the whole research were Glass wares, Chemicals, Weighing balance, Laminar air flow, Incubator, Incubator shaker, Water bath, Microscope, Centrifuge, Column chromatography, spectrophotometer.

Collection of sample

The orange peel was purchased from the local market of Gandhipuram, Coimbatore. The sample collected for the experiment was tea leaves which were collected from the hills of Idukki, Kerala. All other chemicals were purchased from Himedia, Mumbai, India. The criteria for purchasing samples are fresh, ripped and dust-free. All the chemical parameters were carried out in Dr. N.G.P. and other assays were performed at CBNR Labs, Eachanari, Coimbatore, Tamil Nadu, India. The entire research work was completed during a course period from year 2017-2019.

Production of pectinase enzyme and optimization

Serial dilution techniques were done for the development of desired micro-organisms. The sample used for serial dilution was a decaying orange peel. It is then incubated for 24-48 hours at 37°C. It is further screened by the spot inoculation method. Screening of the bacteria was done by the use of CTAB. It is then introduced to PSBM (Pectinase selective broth medium) For the large-scale production of pectinolytic bacteria. It is then incubated for 48 hours at 37°C to optimize the incubation time.

Biomass estimation

Pectinase selective agar media was prepared and incubated for 4 days at 37°C and the biomass was estimated to identify the optimum day. Each day 1 ml of the sample was transferred to the pre-weighed tube and centrifuged at 5000 rpm for 5 minutes and the supernatant was separated and the pellet was taken as biomass, the Biomass was calculated by using the following formula (Orduna *et al.*, 2010).

Biomass = Tube with pellet weight - Pre-weighed tube weight

Pectinase enzyme assay

According to Miller. G.L pectinase assay was quantitatively done by using pectin as substrate. The reaction mixture containing 0.5 ml of the crude enzyme and 0.5 ml of pectin in 0.1M acetate buffer with pH 6.0 was incubated at 40°C for 10 min. The reaction mixture was then added with 1 ml of DNS reagent and the mixture was boiled for 5 min at 90°C. The absorbance was read at 595 nm. “One unit of Pectinase activity was defined as the amount of enzyme which liberated 1 µm glucose per min” (Miller, 1959).

Precipitation of enzyme

The fresh media was prepared and after incubation cell-free enzyme was separated by centrifugation. 60% of ammonium sulfate was added to the enzyme and dissolved fully, the sample was incubated at -4°C for 24 hrs for the precipitation (Steward and Lew, 1985).

Dialysis

Dissolved 5 Mm EDTA and sodium bicarbonate in 100 ml distilled water and boil it. Kept the dialysis tube in the above solution for about 5 minutes to activate the membrane. After dialysis collected the sample and stored at -20°C (Sorensen, 2003).

Column chromatography

Column chromatography is a technique to purify enzyme compounds. The stationary phase was prepared by adding 0.5 gm of Sephadex G-75 in the 20 ml of distilled water for complete dissolving and after complete dissolving gel was transferred to the pre-settled column and allowed to set. The sample was added to the column and allowed to elute the enzyme. 1 ml per 10 minutes was set to the sample for purification and the fractions were collected (8-fraction) the enzyme presence was confirmed by taking OD at 280 nm and the fractions were used for further study.

SDS-Page

The unit of electrophoresis was set and 20 µl of a sample (crude and purified) and 10 µl of 2× dye were added to the electrophoresis unit. And after heating at 90°C for 10 minutes the samples were loaded with marker and run at 100V for 30-45 minutes. Molecular weight in kDa was recorded.

FTIR

According to the protocol and research performed by Cruz-(Espinoza *et al.*, 2012) it was revealed that the phytochemical constituents of the tea were estimated.

Flavonoids and phenols

Flavonoids and phenols are a universal group of naturally occurring polyphenolic compounds characterized by the flavan nucleus and represent one of the most prevalent classes of compounds in plant-derived beverages, vegetables and fruits. Flavonoid content was analyzed using the folin ciocalteau method (Sivasakthi and Sangeetha, 2014).

DPPH

2, 2-diphenyl-1-picrylhydrazyl DPPH is a common abbreviation for the organic chemical compound 2, 2-diphenyl-1-picrylhydrazyl. DPPH is a crystalline powder with dark color composed of stable free-radical molecules. Two major applications are importantly described below, both in laboratory research (Wern *et al.*, 2016).

Caffeine

Caffeine is a stimulant that can be seen in the central nervous system included in the class of methylxanthine. It is a psychoactive drug that is widely consumed by the world. It is legal and unregulated in so many other parts of the world, disparate from other psychoactive drugs present. (Gramza-Michałowska, 2014).

Moisture content

Moisture content (MC) is the weight of water contained in the sample expressed in percent. MC is usually referred to as the wet basis meaning the total weight of the grain including the water (MCwb) (Sivasakthi and Sangeetha, 2012).

Ash

The process of heating the substance in the presence of oxidizing agents to remove water and organic matter present in the substance is called ashing (Sivasakthi and Sangeetha, 2012).

pH

pH is the main key parameter in food production. An optimally adjusted pH value determines the degree of enzyme activity. The pH range is from 0 to 14, with 7.0 being neutral. Anything above 7.0 is alkaline and anything below 7.0 is considered acidic (Sivasakthi and Sangeetha, 2014).

Shelf life analysis

PCA (Plate count agar) is the medium commonly used for the detection of microbiological growth. It is used to record the total viable growth of the bacteria in the given sample. PCA is not recognized as a selective medium to detect microbial growth.

RESULTS AND DISCUSSION

Production of pectinase enzyme and optimization

After an incubation time of 24-48 hours at 37°C, bacteria were grown on the agar plate. Observed bacterial isolates can be shown in Plate 1. Isolates be like white, filamentous and sponge-like structures.

The pectinase-producing bacteria obtained from the serial dilution techniques were further screened for pectinolytic activity by the spot inoculation method. The clear zones of pectinase-producing bacteria were obtained in PSAM (Pectinase Selective Agar Media) within 48 hours at 37°C after treatment with CTAB (cetyl trimethyl ammonium bromide) solution (Plate 2). For identifying the resulting bacterial species, gram staining methods are carried out. The purple-colored gram-positive rods are obtained as the result of the gram staining method (Plate 3).

Biomass estimation and enzyme assay

Pectinase enzyme assay was calculated using the equation given below:

$$\text{Enzyme activity} = \frac{\text{Absorbance of enzyme solution} \times \text{Standard factor}}{\text{Time of incubation (Min)}}$$

The biomass was estimated and corresponding enzyme activity was calculated showing that maximum biomass (0.048) and enzyme activity (0.783) observed at 48 hours of incubation time, considered as the optimized factor out of the Pectinase Enzyme Assay (Table 1).

Precipitation and dialysis of enzyme

After precipitation with ammonium sulfate dialysis was done. If the protein sample is not sufficiently buffered, a little pH shift may occur because the ammonium sulphate solution normally has a pH between 5 and 6. The purification process was carried an overnight, spectrophotometer readings has been taken to assess the maximum enzyme activity and the amount of protein (Table 2).

Table 1: Biomass estimation and pectinase enzyme assay.

Incubation time	Biomass	Enzyme activity
24 hrs	0.036	0.446
48 hrs	0.048	0.783
72 hrs	0.041	0.214
96 hrs	0.021	0.130

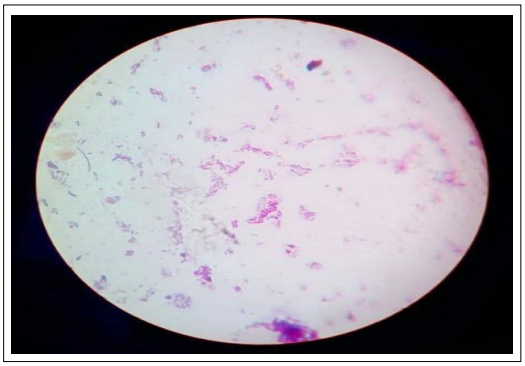

Plate 1: Pectinase-producing bacteria.



Plate 2: Clear zones from spot inoculation.

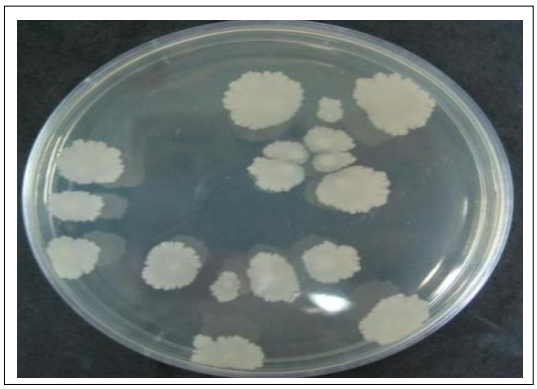

Plate 3: Gram-positive rods.

Column chromatography

Purification was done using column chromatographic techniques. And the fractions were collected (8-fraction) and the enzyme presence was confirmed by taking OD at 280 nm, the fractions were used for further study (Table 3).

SDS page

Molecular masses of enzymes can be estimated by comparing the migration of crude enzymes in interest to the standards of known size. The relative mobilities of the standards are plotted against the log of their molecular masses. The sizes of purified enzymes are then extrapolated from the standard curve. The size of the crude enzyme obtained was 55 kDa. And the molecular mass of the purified enzyme is 57.2 kDa (Plate 4).

FTIR

The control and the sample FTIR chromatograms are presented above with notable peaks in the detection of enzymatic compounds (Plate 5 and Plate 6).

Total flavonoids content

Flavonoid content was determined spectrophotometrically using the method of aluminum chloride assay. The total flavonoid content of the sample was observed as 310 mg/gm. The determination of TFC was then repeated for 3 months. There is no change in flavonoid content were observed till 3 months. Compared to the control, the sample shows higher flavonoid content in it. The table below shows the values obtained from the determination of TFC of both sample and control till three months (Table 4).

According to the study done by Kodama *et al.* (2010) the highest flavonoid content observed in tea was 197 mg. When comparing this value with the enzyme-treated tea, it

Table 2: Dialysis of enzyme.

Enzyme	OD value	Enzyme activity	Protein in mg/g
Precipitated sample	0.946	1.73	13
Dialysis	1.209	2.20	8

Table 3: Results obtained from column chromatography.

No of fractions	OD at 280 nm	Enzyme activity	Amount of protein µg/ml
1	0.376	0.686	24
2	0.508	0.927	38
3	0.763	1.393	16
4	0.712	1.3	11
5	0.528	0.965	41
6	0.846	1.545	9
7	0.609	1.112	6
8	0.347	0.634	20

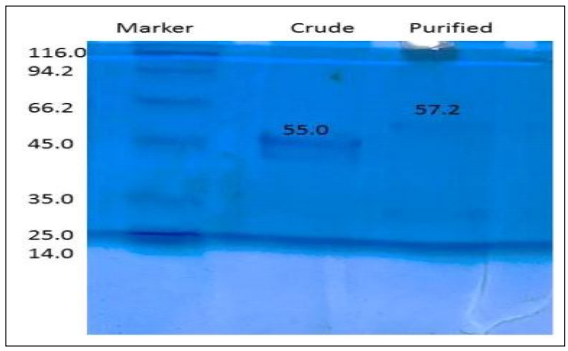

Plate 4: Observed results of SDS PA.

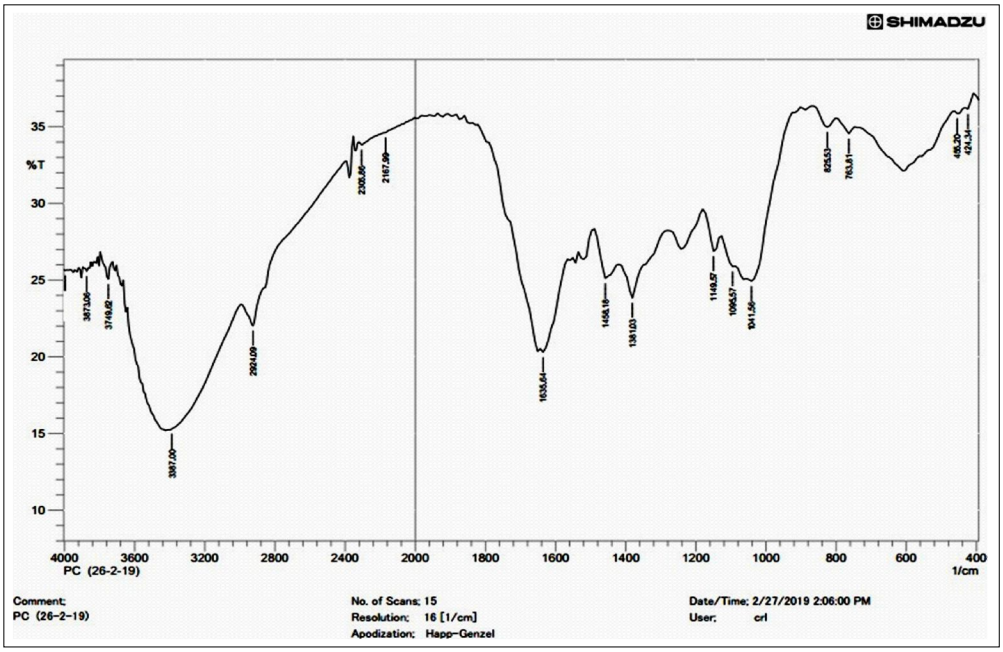

Plate 5: FTIR-control.

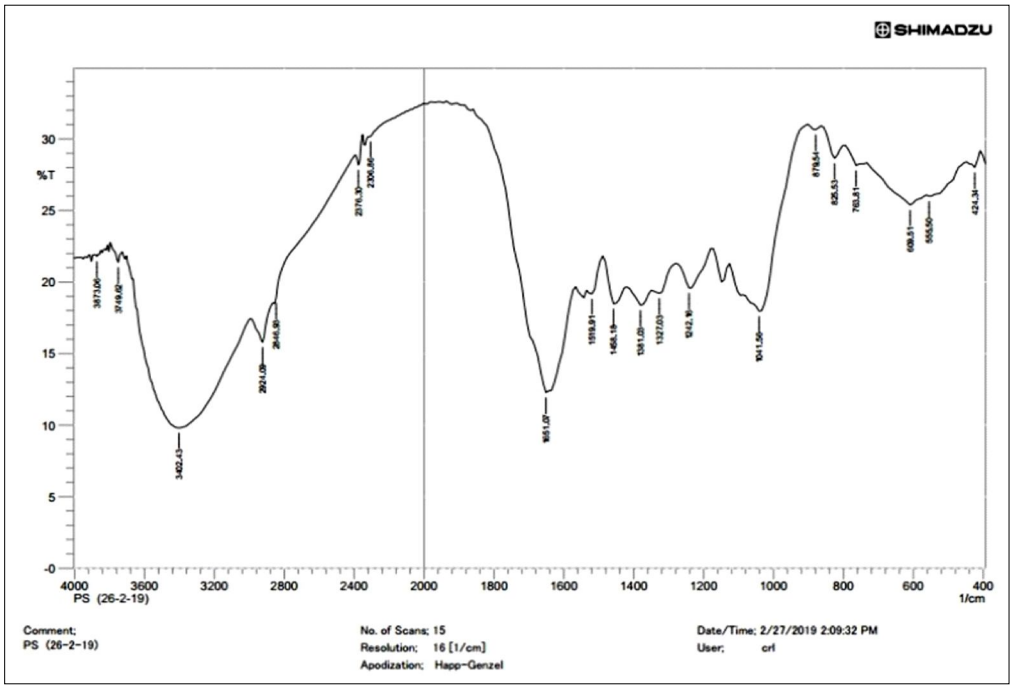

Plate 6: FTIR-sample.

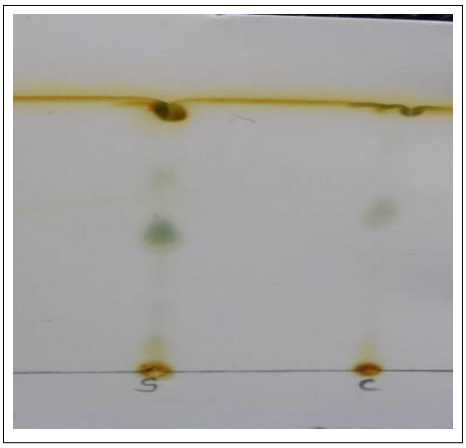

Plate 7: Rf value for control and sample.

can be seen that the TFC values are improved when the treatment with pectinase enzyme.

DPPH

The free radical scavenging activity of the sample was determined by the DPPH assay method. This is mainly used to screen the antioxidant activity present in the plant extract. DPPH is checked every three months.

Table 8 shows the antioxidant activities present in the control (tea extract) and the sample (enzyme-treated tea extract) by the DPPH method. The sample shows 49 mg/g of DPPH. Which is higher than that of the control taken (Fig 1). Likewise study by Kodama *et al.* (2010) found similar results shown in the present study conducted here. It has been observed that fresh tea contains an antioxidant value from 44 to 59 mg/gm.

Caffeine

TLC is performed to determine the caffeine content of the sample and control by quantitative method. Rf value was obtained between 0.56-0.95 in a sample. And for control between 0.64-0.97 obtained as a result (Table 5 and Plate 7).

According to Salihovic *et al.* (2015) content of caffeine was in the range from 33.9 to 110.73 mg/gm. Caffeine can make a person addicted to it and can have adverse effects because consumption of more than 1 gm of caffeine can lead to death. In the present study caffeine content was between 0.56 and 0.98 mg/gm. And comparing the results with the control, it can be seen that caffeine content was slightly decreased after enzyme treatment.

Moisture and ash content of tea

Table 6 presents the moisture and ash content of the control (tea extract) and sample (enzyme-treated tea extract).

About 6% moisture content was observed in tea leaves. Almost similar values are obtained in this study. The moisture content gradually decreases from month to month. In the case of ash, Similar results were coincidental with previous findings of a research team of Imran *et al*. (2018), study manifest 4.95 -5.11% of ash content in tea leaves.

Total phenol content

Total phenolic content was observed as 285 mg/g in a sample (enzyme-treated tea extract), in control (tea leaves) it varies between 258-239 mg/g for 3 months. Table 7 shows the results of total phenolic content.

According to the study conducted by Imran *et al*. (2018), black tea contains 325 m/gm of polyphenols in them (Table 7).

Table 4: Total flavonoid content.

Samples used	0th day (mg/gm)	1 month (mg/gm)	2 months (mg/gm)	3 months (mg/gm)
Control	220	220	216	214
Sample	310	310	310	310

Table 5: Caffeine content.

Criteria	Rf value
Sample	
Spot 1 (2.7/4.8)	0.56
Spot 2 (3.3/4.8)	0.68
Spot 3 (4.6/4.8)	0.95
Control	
Spot 1 (3.1/4.8)	0.64
Spot 2 (4.7/4.8)	0.97

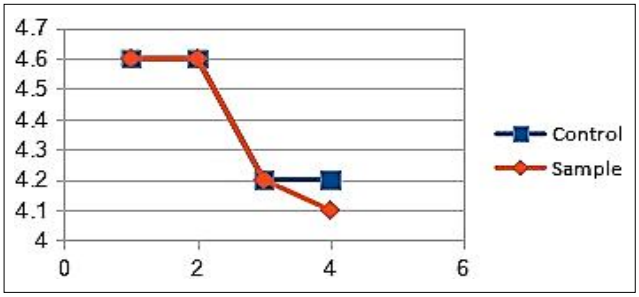

Fig 1: Antioxidant activity of both control and sample for 3 months.

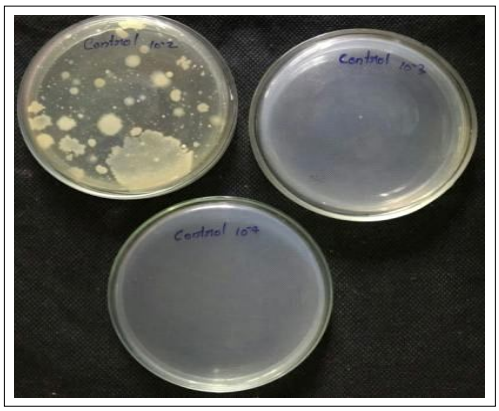

Plate 8: Control.

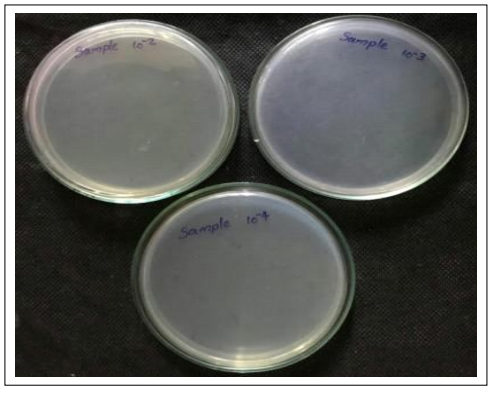

Plate 9: Sample.

pH and shelf life analysis (Microbial load)

The pH and Shelf life of tea samples are discussed in Table 8.

pH value of tea

According to Reddy *et al*. (2016), the pH of tea was observed as 5.06.

Shelf life analysis (Microbial load)

Bacteria

The results of a TPC at 37°C on plate count agar (PCA) (one ml/plate). The microbial growth of the product observed was good 90 days after the production of the sample. Thus it can be concluded that the enzyme-treated tea sample was more resistant to microbial growth than normal tea.

First month

In the present study, raw tea leaves were used as a control and enzyme-treated tea extract was used as the sample. The bacterial diversity was obtained in the first month of shelf-life analysis. And the result shows, there is no notable bacterial growth was observed in the control and sample.

Second month

On the second month of shelf-life analysis, there is no observed growth of bacteria was obtained.

According to the results obtained, it can be concluded that the enzyme-treated tea extract as well as raw tea leaves,

Table 6: Moisture content.

Parameters	0th Day (%)	1 Month (%)	2 Month (%)	3 Months (%)
		Moisture		
Control	6.8	6.8	6.5	6.5
Sample	7.2	7.0	6.9	6.7
		Ash		
Control	4.6	4.6	4.2	4.2
Sample	4.6	4.6	4.2	4.1

Table 7: Total phenolic content.

Samples used	0th Day (mg/g)	1 Month (mg/g)	2 Months (mg/g)	3 Month (mg/g)
Control	258	255	239	239
Sample	285	285	285	285

Table 8: pH and Shelf-life analysis-bacteria.

Samples	0th Day			1 Month			2 Month		3	Month		
used	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}
Sample	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Control	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	pH of tea											
Sample	6	6	6	6								
Control	6	6	6	6								

of them shows a good shelf-life until two months of period (Plate 8).

Third month

At the third month of shelf-life analysis, the control shows notable bacterial growth in the 10^{-2} agar plate and there is no growth observed in the 10^{-3} agar plate. The bacterial growth obtained at 10^{-2} dilution was 32 CFU. In the case of the sample, there is no observed bacterial growth (Plate 9).

CONCLUSION

The present study investigated the isolation, screening and production of pectinase by using bacterial species from degraded orange peel. Through this study reveals that pectinase of natural origin like microbial production (Bacterial and Fungal) could use as a safer and cheaper alternative to existing synthetic enzymes for industrial and medical purposes.

Conflict of interest

All authors declare that they have no conflict of interest.

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