CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

MOLECULAR CHARACTERIZATION, PRODUCTION AND OPTIMIZATION OF PECTINASE PRODUCER AND ITS INDUSTRIAL APPLICATIONS

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https://doi.org/10.34302/crpjfst/2023.15.3.4

Article history:
Received: 17 November 2022
Accepted: 1 September 2023

Keywords:
Pectin;
Pectinase Activity;
Soil Bacteria;
Bacillus Sp. ;
Juice Clarification.

ABSTRACT

A naturally occurring component called pectin is present in many fruits, including berries and apples. Almost all plants contain it, where it adds to the structure of the cell. Pectinase is an enzyme product that is used professionally to break down pectin. Pectinases are widely used in the wine and citrus juice industries. It is used in the fruit juice business for clarification because it lowers viscosity, which results in the creation of clear juice. In the current research, soil samples were taken from six different regions in the Ahmedabad district in an effort to isolate bacteria that produce pectinase from those samples. Primary screening produced a total of 41 strains, of which 14 showed pectinase activity. Screening was done by using Vincent's agar medium containing pectin. Best pectinolytic activity was determined by clear zone of hydrolysis on selective media. Among 14 isolates, Isolate 1 was showing highest zone of utilization which was selected for further study. Pectinase was produced by submerge fermentation technique and physicochemical parameters were optimized in which isolate 1 showed highest activity at pH 7.4, temperature 37°C, incubation period 48hrs., inoculation size, substrate, carbon source, nitrogen source. Isolate 1 was characterized by its cultural, morphological, biochemical and molecular basis by 16S rRNA sequencing and designated as *Bacillus subtilis*. This isolate was applied for fruit juice clarification and demucilization.

1. Introduction

Since bacteria are responsible for creating vital goods like enzymes, antibiotics, vaccines, cheese, bread, and many other things, they are very advantageous to society (Antranikian, 1992). From soil bacteria, different enzymes are extracted that are used in a variety of commercial fields. In both technical and commercial processes, microbial enzymes take the place of some harsh chemicals, reducing pollution. Pectinase is one such enzyme that can be produced from various microorganisms, including bacteria and fungi, and is used in a variety of sectors (Kaur *et al.* 2004). Pectic substances are chemicals that are catalysed by pectinolytic enzymes. These are negatively charged and corrosive (Danielle *et al.* 2009). Fruits are an abundant source of pectin, and the mechanical crushing of pectin-containing fruits results in the high viscosity of fruit juices. Juice extraction by mechanical means is challenging (Semenova *et al.* 2006). Pectinase and a few other enzymes take the role of mechanical extraction to clarify fruit juice.

2. Materials and methods

2.1. Sample Collection and Evaluation for Pectinase Producers

Bacteria for the production of pectinase were isolated from soil sample collected from 6 different region of Ahmedabad district including garden area, fruit market and vegetable market. Soil sample was enriched by placing 100 g of soil sample and 1g of pectin powder in a sterile beaker for few days at room temperature. 1g of enriched soil sample was inoculated into enrichment broth containing (g/100ml): Pectin-1, Yeast extract-0.1, Peptone-0.5, $CaCO_3$-0.2 and NaCl –0.2 which was then shook for 10 days at 200 rpm on a rotatary shaker. The enriched soil sample was diluted and inoculated on Vincent's agar medium containing (g/1000ml): sucrose, KNO_3, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, K_2HPO_4, KCl, yeast extract, pectin, and agar. Following 24 h incubation at 37°C, the clearing areas of the medium after the addition of Lugol's iodine solution were used to classify pectinase secretion. Colony with maximum zone diameter was preceded for further studies.

2.2. Identification of the Bacterial Strain

Different staining methods, as well as biochemical and molecular techniques, were used to classify the bacteria that had been isolated.

2.2.1. Sequencing of the Expanded 16S rrna Gene to Identify the Isolated Bacteria

Amplified PCR product was purified using a Helini DNA purification package, and the PCR product was then commercially sequenced. The collected sequence was blasted in the NCBI database, and a Bacillus phylogenetic analysis was performed. FASTA sequence was built using the neighbour-joining (N-J) process.

2.2.2. Genomic DNA Isolation of the bacterial Isolate

The above sequence was blasted against established sequences in NCBI's public libraries, and the findings were shown as a phylogenetic tree. Based on nucleotide homology and phylogenetic analysis, the sample displayed a high degree of resemblance to *Bacillis subtilis*. CLUSTAL W. Performed a molecular phylogenetic study using the Maximum Likelihood approach for pectinase producers. The final dataset included 1434 locations. MEGA7 was used to run evolutionary studies.

2.2.3. Sequences for Scanning Electron Microscopy of bacterial strain

Bacterial isolate were fixed in 6% buffered glutaraldehyde for 24 hours. Scanning Electron Microscope photographs were obtained from mediwave labs, Mumbai**.** The absolute ethyl alcohol was acquired from Hayman Ltd., England. The microbial identification by 16srrna was conducted by Eurofins Genomics India Pvt Ltd. Bangalore 560048, Karnataka, India.

2.3. Optimization physico-chemical parameters for pectinase biosynthesis by *Bacillus subtilis*

Any bacterium species' ability to produce pectinase can vary and be influenced by a variety of physico-chemical factors. Different amounts of these variables may have an impact on the enzyme's secretion. Therefore, in the current study, pectinase enzyme production was optimised at various temperature ranges (28 to 60°C), pH ranges (6 to 12), substrates (sugarcane, paper pulp, tea waste, cassava waste, orange peel, molasses, wheat bran, and agricultural waste), carbon sources (lactose, maltose, mannitol, sucrose, glucose, fructose, cellulose, and starch), and nitrogen source (CTAB, EDTA, triton X-100, SDS, glycerol, and tween 20). The experiment was carried out in 250 ml Erlenmeyer flasks holding the production medium (yeast extract pectin) for the pectinase enzyme (YEP). Following sterilization, flasks were cooled and infused with pure *Bacillus subtilis* cultures that had been separated using selective media.

2.4. Production of Pectinase by Submerged Fermentation (smf)

The best initial ph, temperature, time, nitrogen supply, inoculum age, inoculum size,

incubation period, agitation rate and substrate concentration for pectinase development were determined. Pectinase fermentation medium comprising of Peptone1.0 gl^{-1}, Yeast extract1.0 gl^{-1}, $NaNO_3$ 2.0 gl^{-1}, KH_2PO_4 1.0 gl^{-1}, $MgSO_4.7H_2O$ 0.5 gl^{-1} and substrate 5.0 gl^{-1} was used for submerged fermentation process. After being sterilized, containers were cooled, infused with pure *Bacillus subtilis* cultures, and then incubated for three days at 37 °C with agitation. After centrifuging the production medium, the supernatant was used as a supply of crude enzymes in subsequent research.

2.4.1. Pectinase assay by DNS method (3,5-dinitrosalicyiic acid)(Miller 1959)

Pectin was used as the substrate for the pectinase test (Janani et al. 2011). In a 0.1M acetate buffer with a pH of 6.0, 0.5 ml of the crude enzyme and 0.5 milliliter of pectin solution are combined to create the reaction mixture. It was then warmed for 10 minutes at 40 °C. Following the addition of 1 ml of DNS reagent, the combination was boiled for 5 minutes at 90 °C. Rochelle's salt, one millilitre, was applied to halt the reaction. At 595 nm, the absorption was measured. The creation of a standard graph was done using a normal glucose solution. The amount of enzyme that releases 1 mmol of glucose per minute was used to determine one unit of cellulase activity.

2.4.2. Partial purification of pectinase enzyme Ammonium sulfate precipitation

After adding solid ammonium sulphate to about 20 ml of the crude enzyme solution, the combination was allowed to sit at 40 °C overnight to precipitate. Centrifugation was used to separate the precipitates, which were then dissolved in 10 ml of 50 mm sodium acetate solution with a pH of 5.5.

2.4.3. Dialysis

Enzyme collected after ammonium sulphate precipitation was dialyzed against 30 mm sodium acetate buffer (pH 5.5) at 4°C with three buffer adjustments for partial purification. Enzyme activity and protein content were assessed in the sample that had only partly been purified.

2.5. Statistical evaluation

The data gathered were statistically analyzed using SPSS 16.0. The information is presented as a mean and standard variation (SD). In the one-way ANOVA, p0.05 was found.

2.6. Application of pectinase in fruit juice clarification

Enzymatic treatment is effective way to reduce cloudiness in the fruit juices (Singh and Singh, 2015). In this study enzyme treatment was applied on two fruit juices grapes and oranges. For sample preparation both fruites are washed with purified water and then fine pulp was obtained by use of blender. After that pulp was pasteurized at 85oc for 3 minutes. After cooling different concentration of pectinase (crude enzyme) treatment like 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mg/20g of pulp was given to both fruit pulp separately in a glass tube. Tubes are incubated at $50^{O}C$ at different time interval like 30, 60, 90, 120 and 160 minutes. After that pectinase activity is inactivated by exposed tubes at $95^{O}C$ for 5 minute in water bath. After cooling centrifuge the content of both tubes at 2000 rpm for 10 minutes. After that filter all content through muslin cloth. Extracted juice was analysed for its clarity (%transmission) and yield (%w/w). Juice yield was determined by following formula:

Juice yield % = Weight of clear juice × 100/Weight of sample

(1)

2.7. Removal of Mucilage from Coffee Beans Using pectinase

Manually pulped fresh coffee grounds were used. The mucilage was removed from the pulped beans by soaking them in the enzyme mixture under still circumstances. As per custom, the demucilized coffee beans were ultimately washed and sun dried after being thoroughly felt by hand. The pulped coffee beans were soaked in water without any additional enzymes in order to compare

enzymatic demucilization with spontaneous fermentation.

3. Results and discussions

3.1. Results of pectinase screening, production and optimization

After primary and secondary screening, we identified total 14 isolates from 6 different region of Ahmedabad district including garden area, fruit market and vegetable market. On the premise of having the highest zone of pectin degradation among the 14, one isolate was chosen for additional research. It was named isolate 1 for ease of study. Jaysankar and Graham (1970) stated that pectinolytic organisms are detected by clearing zones on agar plate. Isolate was gram positive rod on the basis of its gram rection. Biochemical characterization was also done in which it gives positive result in starch utilization test, citrate utilization test, Gelatine hydrolysis test and carbohydrate fermentation test (Glucose, Lactose, Maltose, Mannitol, Mannose and Fructose). Identity and phylogeny of the isolate was analysed using 16S rdna analysis. The amplified PCR product was then commercially sequenced after being purified using a Helini DNA purification kit. The collected sequence was blasted in the NCBI database, and a Bacillus phylogenetic analysis was performed. The 16S rdna nucleotide sequences were identified, and a phylogenetic tree based on the 16S rdna FASTA sequence was constructed using the neighbour-joining (N-J) method to categorise the strain. The gene sequence was sent to NCBI, and the genbank library assigned it the accession number MK034151. The sequence was blasted against established sequences in NCBI's public libraries, and the findings were shown as a phylogenetic tree. The bacterial isolate was identified as *Bacillus subtilis* based on the 16s rrna sequence (Figure 1). The majority of bacterial pectinase producers were classified using morphological and biochemical tests, with *Bacillus sp.* Being the most common. Among the many microorganisms, *Bacillus subtilis* is recognized for producing a broad range of extracellular enzymes and has a number of industrial uses.

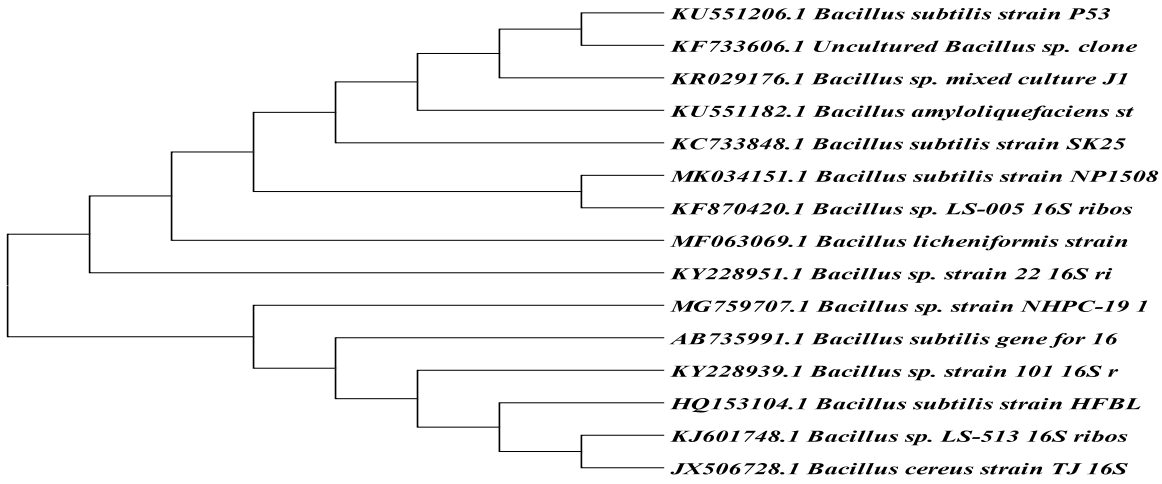

Figure 1. Maximum Likelihood Molecular Phylogenetic study of Isolate 1 reveals *Bacillus subtilis.* The FASTA sequence was BLASTed against established sequences in NCBI's public libraries, and the findings were shown as a phylogenetic tree.

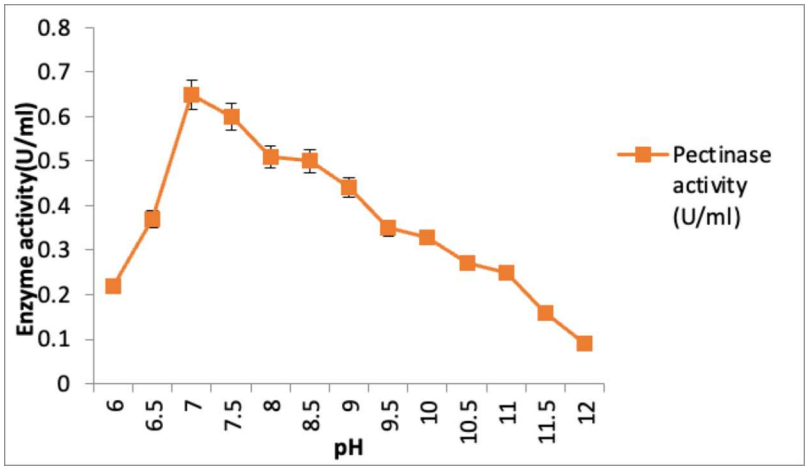

Figure 2. enzyme activity of *Bacillus subtilis* NP1508 With respective to different pH

Error bar in the graph represents standard deviation of the mean value. Enzyme activity was optimized by providing different incubation temperature in production media. SPSS 16.0 was used to do statistical analysis on the data collected. The data are provided in the form of a mean and standard deviation (SD). $p<0.05$ was obtained in one-way ANOVA.

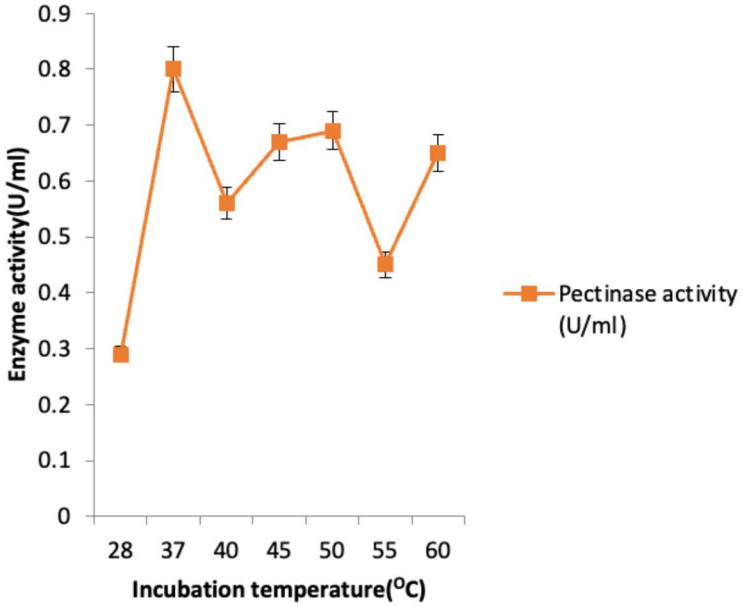

Figure 3. Enzyme activity of *Bacillus subtilis* NP1508 With respective to different incubation temperature

Error bar in the graph represents standard deviation of the mean value. Enzyme activity was optimized by providing different incubation temperature in production media. SPSS 16.0 was used to do statistical analysis on the data collected. The data are provided in the form of a mean and standard deviation (SD). $p<0.05$ was obtained in one-way ANOVA.

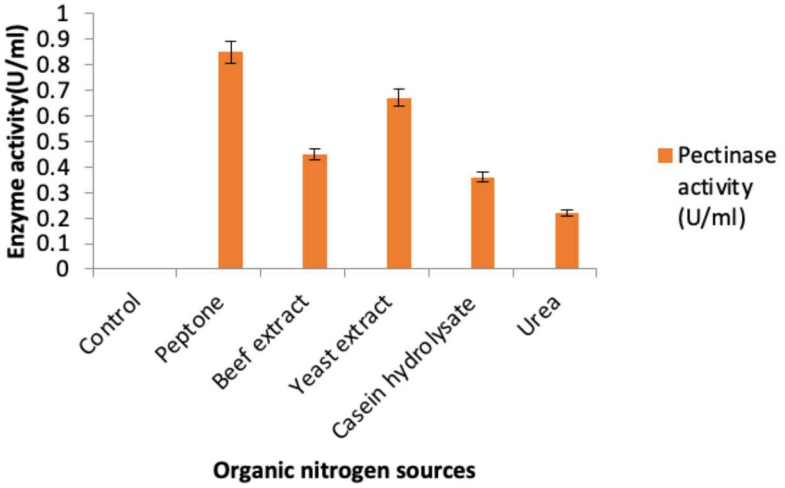

Figure 4. Enzyme activity of *Bacillus subtilis* NP1508 With respective to different organic nitrogen source

Error bar in the graph represents standard deviation of the mean value. Enzyme activity was optimized by providing different incubation temperature in production media. SPSS 16.0 was used to do statistical analysis on the data collected. The data are provided in the form of a mean and standard deviation (SD). $p<0.05$ was obtained in one-way ANOVA.

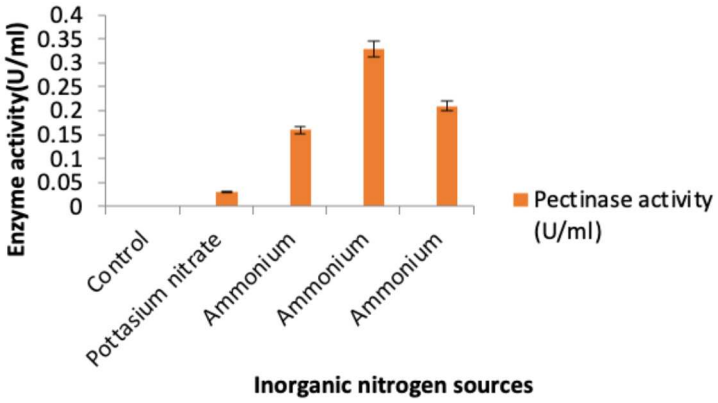

Figure 5. Enzyme activity of *Bacillus subtilis* NP1508 With respective to different inorganic nitrogen source.

Error bar in the graph represents standard deviation of the mean value. Enzyme activity was optimized by providing different incubation temperature in production media. SPSS 16.0 was used to do statistical analysis on the data collected. The data are provided in the form of a mean and standard deviation (SD). $p<0.05$ was obtained in one-way ANOVA.

Bacillus subtilis was studied in detail for the pectinase production with respect to development of low-cost and easy available medium ingredient by submerged fermentation. As per the swain, submerged fermentation is the preferred method for the production of pectinase. Production medium was optimized for different physicochemical parameters. Among raw substrate, cassava wastes showed highest pectinase activity 0.94±0.07 U/ml (Table 1). The use of cost effective agro-residue for pectinase production in submerged fermentation would cause a substantial reduction in the cost of enzyme (Smith and Aidoo, 1988; and Pilar *et al.,* 1999).

Table 1. Enzyme activity of *Bacillus subtilis* NP1508 With respective to different raw substrates

Raw substrates used	Enzyme activity (U/ml)	P value
Sugarcane	0.44+0.06	0.12
Paper pulp	0.67+0.08	0.13
Cassava waste	0.94+0.07	0.05
Tea waste	0.90+0.06	0.12
Orange peel	0.77+0.07	0.19
Molasses	0.84+0.08	0.01
Wheat bran	0.67+0.06	0.17
Agriculture waste	0.31+0.08	0.15

Pectinase production was higher at pH 7(Fig. 2), 37^{O}C temperature(Fig. 3), 2.5% inoculum, 24 hrs old culture, 48 hrs of incubation period, 150 rpm agitation rate. As shown in Table 1, cassava waste, tea waste and molasses shows highest activity. According to Kashyap *et al.* (2003), the pH and temperature conditions at which the soil strain Bacillus sp. DT7 produces the most extracellular pectinase are 7.2 and 37 °C. According to Sunnotel et al. (2002), using Bacillus sp. At 37 ^{O}C and pH 7.2 increases pectinase output. Our study shows similar results of optimum temperature and pH. This study shows that peptone and ammonium sulphate gives maximum pectinase activity as an organic and inorganic nitrogen source respectively (Fig. 4 & 5). Optimization parameters are represented in graphical form (See Fig. 2 to 5). According to Galiotou-Panayatou and Kapantai (1993), ammonium phosphate and ammonium sulphate did have a beneficial impact on pectinase production.

The greatest amount of pectinase could be produced, according to Phutela *et al.* (2005) when yeast extract and ammonium sulphate were present in the growth medium. According to Sarvamangala and Dayanand (2006) pectinase synthesis in submerged fermentation is increased by glucose and sucrose.

3.2. Application of pectinase in fruit juice clarification

Fruit juice clarification method used crude enzyme. According to this study, juice recovery increased along with rising enzyme concentration and incubation time. By combining these juice extraction methods with different pretreatments, such as cold, hot, and enzymatic extraction, the yield of juice can be improved (Chadha *et al.* 2003). Enzymatic treatment, when compared to cold and heated extraction methods, significantly increases juice recovery (Joshi *et al.* 1991). Juice that had been clarified by enzymes experienced a decrease in viscosity and the creation of clusters, which makes centrifugation or filtration easier. Because of this, the juice has greater clarity and more intense taste and colour (Abdullah *et al.* 2007). The sort of enzyme, incubation duration, temperature, enzyme concentration, agitation, ph, and use of various enzyme combinations all play a role in the biomaterial's enzymatic degradation (Baumann 1981). Grape and orange juice yield increased by increasing enzyme concentration and incubation time up to 180 min. (Table 2 and Table 3).

Table 2. Grape juice yield (%w/w) optimization by enzyme concentration and incubation time

Enzyme concentration mg/20g of pulp	30 min.	60 min.	90 min.	120 min.	180 min.
0.5	63	62.5	63.5	63.5	64.5
1.0	63	63	63	64.5	64.5
1.5	64	64	64	65.5	65.5
2.0	64.5	64.5	65	65.5	66
2.5	64.5	65.5	65.5	66.5	66.5
3.0	65	65.5	66.5	66.5	66
3.5	65.5	66	66.5	66	68

Table 3. Orange juice yield (%w/w) optimization by enzyme concentration and incubation time

Enzyme concentration mg/20g of pulp	30 min.	60 min.	90 min.	120 min.	180 min.
0.5	52	53.5	54.5	55	56
1.0	52	53	54.5	56	56.5
1.5	52.5	53.5	55	55.5	56
2.0	53	54.5	55	56	57
2.5	53.5	53.5	55	56.5	57.5
3.0	53.5	54	55	56	57.5
3.5	54	54.5	55	56.5	58

According to Yusof and Ibrahim (1994), the yield of juice increased with the volume of enzyme used and the length of the incubation period. Juice clarity increases after enzymatic therapy. As long as the temperature is below the enzyme's denaturation temperature, the temperature speeds up enzymatic processes and, consequently, the rate of clarification. In my study percentage transmission of grape and orange juice increase by increasing enzyme concentration and incubation time up to 180 min. (Table 4 and 5). When the incubation period changed for bananas, the brightness exhibited a similar pattern of behaviour (Lee *et al*. 2006).

Table 4. Effects of enzyme concentration and incubation time on the clarity of grape juice (%Transmission)

Enzyme concentration mg/20g of pulp	30 min.	60 min.	90 min.	120 min.	180 min.
0.5	2.2	2.2	2.3	2.4	2.5
1.0	2.9	2.8	2.8	2.9	3.0
1.5	3.1	3.3	3.3	3.4	3.3
2.0	3.8	2.6	2.8	2.9	3.0
2.5	4.2	4.3	4.3	4.5	4.4
3.0	4.4	4.5	4.5	4.8	4.9
3.5	4.7	4.7	4.9	5.0	5.3

Table 5. Effects of enzyme concentration and incubation time on the clarity of orange juice (%Transmission)

Enzyme concentration mg/20g of pulp	30 min.	60 min.	90 min.	120 min.	180 min.
0.5	0.7	0.7	0.8	0.8	0.8
1.0	0.9	1.0	1.2	1.2	1.1
1.5	1.4	1.5	1.5	1.6	1.6
2.0	1.5	1.5	1.6	1.7	1.7
2.5	1.8	1.8	1.9	2.0	2.2
3.0	1.9	2.0	2.1	2.1	2.4
3.5	2.1	2.2	2.2	2.5	2.8

3.3. Use of pectinase for the removal of mucilage from coffee beans

Manually pulped fresh coffee grounds were used. The crude pectinase-infused water was used to soak half of the pulped legumes, while the other half underwent spontaneous fermentation. Within 24 hours of incubation, fully demucilized coffee seeds with pectinase treatment were visible. Demucilization, however, was not finished in the instance of natural fermentation within 36 hours of fermentation. These findings are consistent with the research conducted by Duy et al (2016). In the fermentation of coffee, pectinolytic bacteria are used to remove the mucilaginous coat from the coffee beans (Carr,1985; Kashyap et al., 2000 and Jayani et al., 2005).

4. Conclusions

Bacillus subtilis NP1508 produced pectinase in considerable amounts after 48 hours of fermentation medium incubation at 37 °C and 7.0 ph. With glucose and sucrose as the carbon source, peptone and yeast extract as the nitrogen source, and cassava waste, tea waste, and molasses as the substrate, enzyme output was at its highest. This bacterial enzyme was used to demucilate coffee beans and clarify fruit liquid. Increases in enzyme concentration and incubation time resulted in increases in fruit juice output and clarity. On coffee seeds that had received pectinase treatment, complete demucilization was seen within 24 hours of incubation.

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Acknowledgment

This work was carried out at Parul institute of applied science and research, Parul University. I am very thankful to the management of Parul University for supporting and providing necessary requirements to complete my work.