Pectinolytic Activity of Mutagenic Strain of Leuconostoc Mesenteroides Isolated From Orange and Banana Fruit Waste

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Abstract
The aim of this study was to isolate pectinolytic bacteria from fermented banana and orange peels. The bacterial isolates were identified using standard biochemical method. The bacteria isolates were screened on pectin agar plates. All the isolates showed pectinolytic activity in terms of making zone surrounding their colony on pectin agar medium. Pectinase activity was determined by dinitrosalicylic (DNS) acid method while protein concentration in the fermentation broth was quantified by Lowry method. The screened isolate designated OP6 tentatively identified as Leuconostoc mesenteroides with highest pectinase activity was subjected to mutagen (Ethidium bromide). The mutants of Leuconostoc mesenteroides generated were screened for pectinase production in comparison with the parent (wild) type in submerged state fermentation. All the mutant strains generated from Leuconostoc mesenteroides had their pectinolytic activities repressed in comparison with the wild strain. Out of mutants screened, mutant designated AB4 have the highest pectinolytic activity 1.54 U/mg. The pectinase activity produced by AB4 was approximately 32% lower than the wild strain. The pretreatment of Leuconostoc mesenteroides with Ethidium bromide caused enzyme repression. The appreciable yield in pectinase activity displayed by the mutant strains when compared with the wild type suggests its industrial relevance. Therefore, use of other chemical mutagens can be tested for further strains improvement.

Keywords: Chemical mutagens; Pectinase; Leuconostoc mesenteroides; Agricultural wastes; Submerged fermentation

Introduction
Many countries today, especially Africa depend on agriculture as sole bedrock of its economy [1]. Human involvement in various agricultural practices with a motive to supply foods, employment opportunities and source of income has resulted in the accumulation of wastes in our environment, posing havoc to bio-lives and aquatic creatures [2,3]. Wastes generated from indiscriminate disposal to the environment could be as raw materials for various industrial processes [4,5]. The wastes generated from our homes and agricultural farms with adequate nutrient could serve as alternative and cheaper source for microbial growth, production of single cell protein and industrial enzymes [6].

Fruits peels constitute major agricultural biomass are produced through human activities [7]. Most fruits peels are prone to microbial spoilage; there by-products could be of use as functional ingredients in the production of functional foods, since they are good sources of dietary fiber and bioactive compounds [8]. Fruit peels are good source of phenolic compounds which may potentially be used in food formulations or when extracted can be used as natural antioxidants – scavenging the oxidation stress [9]. The citrus peel and seeds are very rich in phenolic compounds, such as phenolic acids and flavonoids, the peel is richer in flavonoids than seeds [10,11]. These peels contain macro and micronutrients with several therapeutic, antiseptic, analgesic, anti-inflammation and nutritional values. They can be processed as ingredients and essential additives to many preparations like as juices, cocktails and cooking’s [12].
Pectin is a soluble complex polysaccharides primarily present in
the plant tissue [13]. It contains D-galacturonic acid in the form of macromolecules linked with α-1-4-glycosidc bindings which can easily degraded by pectinolytic enzymes [14].

Pectinases are a group of enzymes that contribute to the degradation of pectin by various mechanisms enzymes in an industrial process [15,16]. Pectinase enzyme produced by microorganisms which include fungi, bacteria and higher plants which has been harnessed for its uniqueness in the biotechnological sector due to their potential application in paper-pulp industry, food and feed industry [17,18], textile industry [19] fruit juices processing and biofuel production. The choice and types of enzymes produced by the microorganisms depends on variety of growth parameters like inoculum size, pH value, temperature, inducers, medium additives, aeration, growth, time and fermenting substrates [20] and presence of various metal ions as activators and inhibitors [21] and other growth factors to obtain high pectinase productivity [22].

Pectinases production on solid or submerged fermentation medium containing inexpensive substrates (apple pomace, citric peels, pectin or other agricultural wastes and pectin) as carbon source can be achieved by optimally adjust the growth factors [15,23]. Pectinase have been vastly used in clarification of fruit juice, pulp and paper industry, retting of fibers, pestic waste water treatment and oil extraction [24-26].

Materials and Methods

Samples collection

The samples (orange and banana peels) were obtained from Oja-Oba, Akure, Nigeria in a clean polyethylene bag and then transported to the Microbiology laboratory for analyses.

Isolation of microorganisms

Microbial analysis was carried out by weighing 1 g each of the samples, then dispensed into 9 ml of sterile distilled water, serially diluted up told 10^6 dilution factor for the isolation of lactic acid bacteria. Zero point one milliliter (0.1 ml) from the diluents were aseptically introduced into the sterile Petri dishes, pour plated with sterilized molten de Man Rogosa and Sharpe agar. The plates were inverted, incubated at 35°C for 36-72 hours in an anaerobic chamber. After incubation, the bacteria growth were counted and recorded as colony forming unit per milliliter (cfu/ml). Pure culture of the isolates were obtained by repeated streak on the bacteriological medium and then stored on the slant for further use.

Screening of bacteria for pectinase production

The culture broth containing the bacteria cells was serially diluted and spread on bacteriological medium supplemented containing 1% pectin, incubated for 24 hours at 35°C. The observable zone of clearance around the colonies indicates pectinase production (activity) and the ratio of diameter of clear zones to colonies were calculated.

Chemical mutagenesis using ethidium bromide (etbr) for pectinase production

The screened bacteria isolates were subjected to chemical mutagenesis using Ethidium bromide as described by [27]. Four millilitre (4 ml) of the bacteria broth culture of each strain was pipette aseptically into 15 ml of ethidium bromide solution and incubated at 37°C for 15 minutes in water bath. At interval of 20-260 minutes, 2 ml of the sample solution was taken and centrifuged at 6000rpm at 37°C for 15 minutes. The supernatant obtained from the centrifugation, was decanted and the cell pellet was re-suspended in 5 ml saline to stop the reaction. Zero point two milliliters (0.2 ml) of the washed cell suspension was serially diluted in saline and plated on pectin agar medium. The plates were incubated for 24 hours at 37°C and the number of colonies in each was counted. Mutants for hyper production of pectinase were detected by the intensity of their zone of clearance.

Pectinase production

The mutant strain **Leuconostoc mesenteroides** with highest pectinase activity was grown in a fermentation medium containing (g/l): glucose (0.1 g); peptone (0.5 g); MgSO₄⋅7H₂O (0.5 g); KH₂PO₄ (0.5 g) and FeSO₄⋅7H₂O (0.01 g), Sucrose (0.1 g), KNO₃ 0.6, MgSO₄ (0.25 g), CaCl₂ (0.1 g), NaNO₃ (0.2 g), KCl (0.5 g), (NH₄)₂HPO₄ (0.7 g), cassava and orange peels (2 g) for pectinase production. The initial pH of the medium was adjusted to 7.0. Then, 50 ml culture medium with 2% cassava and orange peels were autoclaved, cooled and inoculated with one ml (1 ml) of overnight grown mutant strain of the bacteria culture and incubated for 24 hours at 37°C on a rotary shaker at 120 rpm. The supernatant recovered by centrifugation of the culture medium was used as crude enzyme source.

Pectinase assay

The quantitative screening of mutant strain of bacterium for the pectinase production was done according to method of [28] using pectin as substrate. The reaction mixture containing 0.5 ml of the crude enzyme and 0.5 ml of pectin in 0.1 M acetate buffer with pH 6.0 was incubated at 37°C for 15 minutes. The reaction mixture was then added with 1 ml of Dinitrosalicylic acid (DNS) reagent and boiled for 10 minutes at 37°C. The reaction mixture was terminated by adding 1 ml of Rochelle’s salt. The enzyme activity was observed spectrophotometrically at wavelength 540 nm. One unit of pectinase activity was defined as the amount of enzyme, liberating one micromole of galacturonic acid per unit volume under assay condition. The protein content was determined using Bovine Serum Albumin as standard.

Results

**Table 1** shows the total bacteria counts from the fermented samples. The microbial counts from banana peels increased from 25 × 10^6 cfu/ml to 83 × 10^6 cfu/ml while fermented orange peels increased from 11 × 10^6 cfu/ml to 63 × 10^6 cfu/ml from orange peel respectively. The high microbial loads were observed in fermented banana peels with increase in fermentation time.

The bacterial isolated from the fermented samples showed
the occurrence of lactic acid bacteria with the occurrence of Lactobacillus delbrueckii, Lactobacillus bulgaricus, Lactobacillus brevis, Lactobacillus acidophilus, Lactobacillus megaratarum, Lactobacillus bulgaricus, Leuconostoc mesenteroides, Lactobacillus lactis Lactobacillus casei Lactobacillus plantarum were isolated from the samples. Lactobacillus acidophilus was isolated only from banana peels towards the end of the fermentation period (Table 2).

The primary screening of pectinase-producing bacteria by plate assay showed the halo zones around the colonies indicates extracellular pectinases production (Table 3). The bacteria isolates showed varied zone of clearance on pectin medium after incubation. The highest zone of clearance exhibited by L. megaratarum was 38.76 mm while L. fermentum showed the lowest zone of clearance of 25.75 mm. L. casei showed the highest colony diameter.

The screened bacterial isolates exhibited varied pectinase activities in media supplemented with pectin. The highest specific pectinase activity of 13.13 U/mg was produced by Leuconostoc mesenteroides. Protein content ranged from 0.16-7.81 mg/ml (Table 4).

The bacteria isolate designated OP5 showed the highest pectinase potential, it was then selected for mutagenic studies (using Ethidium bromide as mutagen). The mutants generated exhibited varied pectinase activity. Mutant strain AB4 showed the highest pectinase specific activity 1.54 U/mg while the mutant AB10 showed the lowest pectinase specific activity of 0.55 U/mg when compared with the parent wild strain (Table 5).

### Table 1. Total bacteria counts (cfu/ml) from fermented cassava and orange peels.

| Samples          | Fermentation period (hours) × 10^6 (cfu/ml) |
|------------------|-------------------------------------------|
|                  | 24  48  72  96  120                       |
| Banana peels     | 25  43  68  83  61                        |
| Orange peels     | 11  26  52  63  37                        |

### Table 2. Occurrence of bacterial isolates from the samples.

| Isolate code | Fermentation time (hours) | Identified microorganisms          |
|--------------|---------------------------|-----------------------------------|
| BP1          | 24  48  72  96  120       | Lactobacillus fermentum           |
| BP2          | 24  48  72  96  120       | L. plantarum                      |
| BP3          | 24  48  72  96  120       | L. casei                          |
| BP4          | 24  48  72  96  120       | L. acidophilus                    |
| BP5          | 24  48  72  96  120       | L. delbrueckii                    |
| BP6          | 24  48  72  96  120       | L. bulgaricus                     |
| OP1          | 24  48  72  96  120       | L. brevis                         |
| OP2          | 24  48  72  96  120       | L. acidophilus                    |
| OP3          | 24  48  72  96  120       | L. megaratarum                    |
| OP4          | 24  48  72  96  120       | L. bulgaricus                     |
| OP5          | 24  48  72  96  120       | Leuconostoc mesenteroides         |
| OP6          | 24  48  72  96  120       | L. lactis                         |

### Table 3. Pectinase screening of the bacterial isolates.

| Identified microorganisms | Colony (mm) | Clear zone (mm) | Ratio of clear zone/ colony |
|--------------------------|-------------|----------------|------------------------------|
| Lactobacillus fermentum  | 25          | 25.75          | 1.03                         |
| L. plantarum             | 30          | 35.40          | 1.18                         |
| L. casei                 | 37          | 38.48          | 1.04                         |
| L. acidophilus           | 30          | 31.80          | 1.06                         |
| L. delbrueckii           | 28          | 28.28          | 1.01                         |
| L. bulgaricus            | 29          | 32.48          | 1.12                         |
| L. brevis                | 33          | 35.64          | 1.08                         |
| L. acidophilus           | 26          | 26.52          | 1.02                         |
| L. megaratarum           | 34          | 38.76          | 1.14                         |
| L. bulgaricus            | 31          | 35.96          | 1.16                         |
| Leuconostoc mesenteroides| 29          | 30.45          | 1.05                         |
| L. lactis                | 27          | 28.89          | 1.07                         |

### Table 4. Production of extracellular pectinase by bacterial isolates from different samples.

| Identified microorganisms | Pectinase activity (U/ml) | Protein content (mg/ml) | Specific activity (U/mg) |
|--------------------------|---------------------------|-------------------------|--------------------------|
| Lactobacillus fermentum  | 3.12                      | 2.64                    | 1.18                     |
| L. plantarum             | 4.08                      | 7.81                    | 1.52                     |
| L. casei                 | 2.14                      | 1.43                    | 1.49                     |
| L. acidophilus           | 6.03                      | 5.88                    | 1.03                     |
| L. delbrueckii           | 5.43                      | 3.38                    | 1.61                     |
| L. bulgaricus            | 3.06                      | 5.46                    | 0.56                     |
| L. brevis                | 3.01                      | 2.00                    | 1.51                     |
| L. acidophilus           | 3.82                      | 1.18                    | 3.24                     |
| L. megaratarum           | 5.11                      | 3.30                    | 1.55                     |
| L. bulgaricus            | 4.87                      | 2.73                    | 1.78                     |
| Leuconostoc mesenteroides| 2.10                      | 0.16                    | 13.13                    |
| L. lactis                | 3.49                      | 1.74                    | 2.01                     |

### Table 5. Pectinase activity of parent and mutant strains of Leuconostoc mesenteroides.

| Mutant strains | Pectinase activity (U/ml) | Protein content (mg/ml) | Specific activity (U/mg) |
|----------------|---------------------------|-------------------------|--------------------------|
| AB1            | 10.03                     | 11.43                   | 0.90                     |
| AB2            | 6.69                      | 6.21                    | 1.05                     |
| AB3            | 6.60                      | 14.63                   | 1.45                     |
| AB4            | 9.81                      | 6.38                    | 1.54                     |
| AB5            | 12.38                     | 10.78                   | 1.15                     |
| AB6            | 4.54                      | 7.81                    | 0.58                     |
| AB7            | 8.33                      | 12.30                   | 0.65                     |
| AB8            | 6.04                      | 8.18                    | 0.74                     |
| AB9            | 8.61                      | 12.61                   | 0.68                     |
| AB10           | 7.91                      | 14.36                   | 0.55                     |
| AB11           | 10.17                     | 10.84                   | 0.94                     |
| AB12           | 8.38                      | 13.62                   | 0.62                     |
| AB13           | 9.62                      | 7.19                    | 1.34                     |
| AB14           | 6.17                      | 9.04                    | 0.68                     |
| AB15           | 7.11                      | 10.11                   | 0.67                     |
| Wild strain    | 5.13                      | 7.64                    | 4.75                     |

Key: BP = banana peels, OP = orange peels
Discussion

Accumulation of agricultural wastes in our environment could pose hazard related to some health issues arising from the obnoxious smell release from processing sites as a result of anaerobic fermentative microorganisms from the dump sites to the environment. Some agricultural wastes contain nutrients supporting the colonization of the microorganisms. Many researchers had reported isolation of microorganisms from various agricultural wastes [23].

The increase in microbial population in the fermenting substrates might be due to the bioavailability and utilization of nutrients required by microorganisms to grow, contamination arising from the indiscriminate disposal of wastes to the environment and favourable growth conditions [29]. The decrease observed in microbial population towards the tail end of the fermentation might be due to the secretion of metabolites into the fermentation medium and growth inhibitors which reduced the microbial potency for the synthesis and production of desirable end products. Generally, the microorganisms associated with wastes could be harnessed for a desirable enzyme production for industrial use. Isolation of microorganisms for enzyme production from soils, rice mill and soil under decaying wood [30,31], agro-wastes [23,32], decaying organic soil [33], abattoir waste [34] and wood waste [35] has been reported. Also, screening of importance industrial enzymes from agro-wastes for lipase production from banana and potato peels extract was reported [36].

The bacteria isolated from different agricultural waste exhibited different activity ratio on pectin agar plates. This could be attributed to the potential capability of the isolates showing different pectinase producing capabilities of the isolates.

Screening of enzymes from microorganisms based on clear zones formation on plate agar had been early reported for Bacillus subtilis [37], Bacillus sp. and Pseudomonas spp [38], Aspergillus flavus [39], Cellulomonas fimii [40], Apergillus niger, Articulaspore infiata, Mucor mucedo, Gonatobotrys simplex, Penicillium itaculum, Aspergillus repen [29]. The differences in diameter of clear zones formed by the bacterial isolates could be as a result of the ability of pectinase produced to diffusion through the medium and/or direct functionality of their genetic makeup to secrete cellulase with high diffusion speed [23] and production of array of extracellular enzymes, including cellulases, hemicellulases, linamarases, pectinases, chinases, amylases, proteases, phytases and mannases in the fermentation medium [32].

The variation in the pectinase activity of microorganisms growing on pectin media could be attributed to the source of isolation and their genetic make-up [32] while the variation in protein content generated by each of the microbial strains in submerged state fermentation could be due to the production of other microbial enzymes (amylases, cellulases, linamarases, protease and xylanases) in the fermentation medium.

Mutant generation from microbial origin for enzyme production caused an improvement in strain development reported a decrease in production of α-amylase of mutant strain of Bacillus amyoliquefaciens UNG-16 when compared to the wild parental strain. The effect of this mutagens has shown the most effective strain improvement for the in the production of α-amylase [41].

The advancement in technology for over 50 years has been utilized in conjunction with the modern techniques in improving the strain of microorganisms for enzyme production in a culture medium depend on its phenotype [42,43] reported mutation inducement in enhancing lipase production from Rhizopus sp. using UV radiation and NTG. More also, Lactobacillus delbrueckii treated with UV light with desirable traits has been successfully employed for the industrial enzyme production [20].

Today, many researchers has geared their efforts focusing on the developing new microbial strains with desirable traits for maximum productivity, this can be achieved through mutagenesis and screening. The use of various mutagens (physical and chemical) to develop better yield strains of enzyme production for various industrial uses had been reported [44]. The use of various mutagens for strains improvement for industrial relevant enzymes production has been reported [29,45] reported strain’s improvement using ethyl methyl sulphonate, n-methyl-N’-nitro-N-nitroso guanidine and UV light for enzyme production. The use of chemical mutagenesis and UV could cause changes in the genetically improved strain of desirable enzymes from microbial source such as cellulase, amylose and pectinase for its maximum production [46]. The variation observed in the pectinase activity exhibited by the mutant strains could be due to DNA damaging factors and differences in their ability to repair damaged genes.

Conclusion

The use of inexpensive substrates to reduce cost for enzyme production is on increase. The pectinolytic activities exhibited the bacteria isolates in response to mutagen for improved enzyme production suggests that it can be exploited for various industrial applications and it is suggested that further molecular studies should be carried out on the improved mutant strains to reveal the mutation.
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