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Organic acids production from *Lactococcus lactis* and *Leuconostoc mesenteroides* using a novel citrus and potato waste medium

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Abstract: Two strains of lactic acid bacteria (LAB) i.e. *Lactococcus lactis* and *Leuconostoc mesenteroides* were evaluated for their capability of utilizing inexpensive citrus waste medium (CWM) and potato waste medium (PWM) to produce industrially important organic acids. In CWM amylase activity was found to be 13.55 U/mg (*L. lactis*) and 12.89 U/mg (*L. mesenteroides*). Similarly in case of PWM amylase activity was found to be 14.45 U/mg (*L.lactis*) and 15.25 U/mg (*L. mesenteroides*) as compared to 1.72 U/mg (*L. lactis*) and 1.52 U/mg (*L.mesenteroides*) in MRS supplemented with 2% starch. β-galactosidase activity in CWM was found to be 20.45 U/mg (*L. lactis*) and 19.31 U/mg (*L.mesenteroides*). In case of PWM β-galactosidase activity was found to be 23.63 U/mg (*L. lactis*) and 22.59 U/mg (*L. mesenteroides*) while only 1.98 U/mg (*L. lactis*) and 2.1 U/mg (*L. mesenteroides*) activities were found in MRS supplemented with 2% starch. Further, TLC analysis of samples from *L. lactis* and *L. mesenteroides* grown in CWM and PWM has confirmed the production of acetic acid (Rf value-0.45) and lactic acid (Rf value-0.41). This work suggests efficient utilization citrus and potato waste based medium by LAB to make organic acid production economical by eliminating the need of costly pre-treatment steps (gelatinization, liquefaction, saccharification) and making direct conversion feasible.

Keywords: Organic acid; Lactic acid bacteria; Potato waste; Citrus waste; a-amylase; β-galactosidase, MRS medium

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Organic acids have long history of being utilized as food additives and preservatives for preventing food deterioration and extending the shelf life of perishable food ingredient. The main organic acids in industrial use are Citric, Acetic, Tartaric, Malic, Lactic and Gluconic acid (Suresh, 2013). Raw material cost is one of the major factors in economic production of organic acids. Biological production offers significant advantages over chemical synthesis due to the fact that biological production can use cheap raw materials which mainly include agro-industrial wastes. Agro industrial wastes, both solids and liquids, are generated in large amounts every year. Their uncontrolled disposal (High BOD or COD) not only results in significant environmental and public health problems such as global warming acidification, oxygen depletion, eutrophication, odour, etc. But also represent a loss of valuable biomass and nutrients. Therefore their reuse in processes is of particular interest due to their availability and low cost. Application of agro-industrial residues in bioprocesses provides an alternative way to replace the refined and costly raw materials and the bulk use of such materials also help to solve many environmental hazards (Anuradha, 1999; John, 2006; Pandey et al. 2000; Lowry et al. 1951; Wee et al. 2006; Ray, 2009). Thus Agro-industrial wastes also have a good potential for conversion into useful products of higher value as by-product, or even as raw material for other industries (Roda et al. 2014; Itelima et al. 2013; De Freitas Borghi et al.2009). Organic acids are examples of such valuable by-product of the fermen-

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tation of high carbohydrate containing industrial substrates. (Mudilyar, 2011). Till date various agro industrial wastes such as whey, molasses, starch waste, beet, cane sugar, cassava, bagasse, apple pomace, soybean, potato residue, pine apple waste, wheat bran, kiwi fruit peel, citric pulp have been used for the production of organic acids (Suresh, 2013). But the use of these complex natural starchy raw materials for production of organic acids involve pretreatment by gelatinization and liquefaction followed by enzymatic saccharification to glucose and subsequent conversion of glucose to organic acids by microbial fermentation. Research efforts are focused on looking for new and effective nutritional source and new progressive fermentation techniques enabling the achievement of both direct conversion of substrate to organic acids by bacteria possessing both enzymatic and organic acid producing character which will eliminate the two step process of saccharification followed by microbial fermentation to make it economical (Cheng et al. 1991; Zhang and Cheryan, 1991). The focus of this work was to study organic acids mainly acetic, lactic, citric acids production with emphasis to use potato and citrus waste as substrates to replace sugars and costly nitrogenous materials. Potato processing plants release an appreciable amount of starch in wastewater streams, additionally; potatoes, which do not fit the standard quality criterion, are discarded. They therefore could be utilized as cheap substrate for microorganisms producing intermediate volume high value organic acids (World Bank Group, 2002). Discarded, off-grade potatoes account for as much as 6.75 MT/day in addition to starch (approximately 50g/L) containing effluents (up to 6000L/day) (World bank, 2004; Mudilyar,2011). Both off grade potatoes and processing effluents can be utilized conveniently as a medium for fermentative production of Lactic acid using appropriate strains of amylolytic lactic acid bacteria.

Ripe citrus fruits are used as both fresh fruits and a source of juice. To extract juice citrus fruits are washed, punched without peeling and pressed. Citrus waste which is a byproduct of squeezing the fruits contains 90% liquid and is sent to the waste disposal plant (Yoo et al. 2011). Citrus waste has been found to be a source of pectin, bioactive compounds (anti-oxidative d-limonene, cancer-inhibiting hesperidin and antimicrobial naringin), animal feed stock, dry feed additive (Braddock 1983; Formica and Regelson,1995; Gladine et al. 2007) The solids of this waste can be processed into pellet-type feed. However, disposal of citrus juice waste containing high concentration of organics, (i.e., 50,000 ppm B.O.D.), poses a significant burden to the waste treatment facility (Yan et al. 2009). Lactic acid bacteria are traditionally fastidious microorganisms and have complex nutrient requirements (Fitzpatrick & OKeeffe, 2001). Refined sugars have been more frequently used to produce organic acids (Hofvendahl & Hahn, 1997; Farooq et al. 2012). However, these are economically not feasible due to high cost of pure sugars whereas the product is relatively cheap. Potato and Citrus waste high in moisture and rich in carbon source have been considered as attractive nutrient source for industrial organic acids production. In this view, a search has been made for efficient utilization of renewable agro-industrial potato and citrus wastes as cheap substrates by *Lactococcus lactis* and *Leuconostoc mesenteroids* for organic acids production.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were purchased from HiMedia, Mumbai, (India) or Sigma (USA). Potato starch was purchased from HiMedia, India. Modified MRS was prepared containing starch in place of carbon source at 1.5 % concentration.

Procurement of lactic cultures

Cultures of *Lactococcus lactis* MTCC 440 and *Leuconostoc mesenteroides* (NCIM 2073), were procured from Microbial Type Culture Collection Center, Chandigarh and National Chemical Laboratory, Pune, India respectively.

Collection of citrus and potato waste

Peel and pulp of citrus and pulpy spoiled potatoes were collected from fruit juice shop in market of Thapar University, Patiala and from heap of waste in an agricultural field of Patiala respectively.

Citrus waste medium (CWM) preparation

Peel and pulp of citrus waste were crushed using properly washed mortar and pestle. Crushed peel and pulp were extracted using 0.5 % (W/V) $Ca(OH)_2$ as a solvent. Extracted liquid was filtered using whatman filter paper no 1. The Characteristics and composition of citrus waste medium were measured and are shown in Table 1. A clear solution was obtained following extraction and filtration. The pH of filtered medium was adjusted to 6.5. Medium was sterilized at 121° C/15 lbs for 15 min. by using autoclave. After sterilization it was allowed to cool at room temperature and stored at 4°C in refrigerator before further use.

Table1 Characteristics and Composition of citrus waste medium in g/liter

Parameter	Before treatment	After treatment
Total Solids	45±2.1	30±2.0
Total insoluble solids	4.1±0.5	2.1±1.0
Total soluble solids	26±1.1	23±1.2
Flavonoid	11±1.2	1.5±0.5
Reducing Sugars	1.1±0.8	0.2±0.02
COD	20±1.0	5.0±0.5
BOD	29±1.3	12±0.03

Potato waste medium (PWM) preparation

Pulpy spoiled potatoes were crushed using mortar and pestle. Crushed material was filtered using Whatman filter paper no 1. The pH of filtered medium was

adjusted to 6.5. The medium was sterilized at 121° C/15 lbs for 15 min. by using autoclave. Following sterilization it was allowed to cool at room temperature and stored at under refrigeration condition (4°C).

Potato waste water

The compositional analysis of potato starch waste water including reducing sugar, starch, total solid sand Chemical oxygen demand (COD), Biochemical oxygen demand (BOD) and total nitrogen was done as per APHA standard methods (2005) for water and wastewater before and after analysis (Table-2).

Table2 Characteristics and Composition of potato waste medium in g/liter

Parameter	Before treatment	After treatment
Total Solids	55±2.0	32±2.0
Total insoluble solids	5.1±0.5	2.3±1.0
Total soluble solids	29±1.0	27±1.5
Starch	15±1.2	1.5±0.5
Reducing Sugars	1.2±0.7	0.2±0.01
COD	21±1.0	5.1±0.5
BOD	31±1.5	13±0.05
Total Kjeldhal Nitrogen	2.1±0.1	0.5±0.04

Propagation of lactic cultures in waste medium

L. lactis and *L. mesenteroids* were grown separately in MRS medium, CWM, PWM (prepared as above) and CWM: PWM (different ratio 1:1, 1:2, and 2:1) in 100 ml Erlenmeyer flask and inoculated with 1% (v/v) (10^8 CFU/ml) overnight grown culture and allowed to incubate at 37°C for 16-18hrs.

Enzyme Assay

a-amylase activity of L. lactis and L. mesenteroids grown in waste medium and MRS were determined. For this cells after propagation in waste medium were removed by centrifugation (8000 rpm, 15min, and 4°C) and the supernatant was considered as the crude enzyme solution for assay. a-amylase activity of this crude enzyme solution was determined by DNS method Giraud E (1993). Enzyme activity was measured using 3,5-Dinitrosalicylic acid (DNS) which is an aromatic compound that reacts with reducing sugars produced as a result of glycolytic breakdown of complex carbohydrates in waste medium by amylases and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. The enzyme activity was determined at different pH values (3.5-5-6.5, 0.1 mol/l citrate-phosphate buffer) and temperatures (30-60°C). One enzyme unit is defined as the amount of enzyme that permits the hydrolysis of 10 mg of starch in 30 min under the conditions. Triplicate analyses were performed for all samples.

β-galactosidase was estimated as described by Miller (1959). β-galactosidase activity of *L. lactis* and *L. mesenteroids* grown in waste medium and MRS were determined. For this cells after propagation in waste medium were removed by centrifugation (8000 rpm, 15min, 4°C) and the supernatant was considered as the crude enzyme solution for assay. ONPG (ortho-nitrophenyl-β-D-galacto-pyranoside) assay was used to determine β-galactosidase activity. Enzyme activity was measured by the rate of appearance of yellow color using a spectrophotometer at 420 nm. The enzyme activity was expressed as specific activity (U/ml soluble protein) and one unit of β-galactosidase activity (U) was defined as the amount of enzyme that liberates 1 nmol O-nitrophenol per minute. Triplicate analyses were performed for all samples.

Determination of protein

Protein concentration of β-galactosidase activity in supernatant was determined by the method of Lowry (1951) using bovine serum albumin as the standard.

Thin layer chromatographic analysis

The TLC analysis was carried as described by Lee (2001). TLC plates of 10x10 cm. dimensions using silica gel (Merck) were prepared. A series of 10% (w/v) standard solutions of Lactic acid, Acetic acid, Citric acid, Butyric acid were prepared. Crude enzyme solution (supernatant) of both cultures from different medium and standard solutions of organic acids were spotted 5 mm from one end of the TLC plate. A hand-type hair dryer was used to dry all the spotted samples. The spotted TLC plate was then placed in the bottom of rectangular chamber of dimensions 10×24×24 cm containing mobile phase (Solvent). The percentage composition of the solvent system was acetone–water–chloroform–ethanol–ammonium hydroxide (60:2:6:10: 22). TLC plates were placed in such a way to ensure a sufficient supply of solvent vapour and the chamber was closed. The development of the chromatogram was allowed to proceed until the solvent had travelled 6–7 cm beyond the starting line. Therefore, the total time required to analyze the organic acids using the TLC system was approximately 55 min. The TLC plates were then removed from the chamber and allowed to dry in air. The dried TLC plates with organic acid chromatograms were sprayed with an indicator solution of 0.25 g of methyl red and 0.25 g of bromophenol blue in 100 ml of 70% methanol and were observed for color development by brief heating (1–3 min) in a hot dry oven (165°C).

Statistical analysis

All the experiments were performed in triplicate. Error bars on graphs show the standard deviation. The data were analyzed by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

a-amylase activity assay of *L. lactis* and *L. mesenteroides* grown in different medium in equal and different ratio

a-amylase activity assay was done for both cultures *L.lactis* and *L.mesenteroides* grown in MRS, CWM, PWM

and CWM and PWM in different ratio (1:1, 1:2, 2:1). a-amylase activity was found to be 1.72 U/mg, 13.55 U/mg, 14.45 U/mg, 16.64 U/mg, 14.75 U/mg, 18.68 U/mg for *L.lactis* when grown in MRS, CWM, PWM and CWM and PWM in different ratio of 1:1, 1:2, 2:1 respectively(Fig. 1). For *L. mesenteroides* a-amylase activity was found to be 1.52 U/mg, 12.89 U/mg, 15.25 U/mg, 14.76 U/mg, 13.28 U/mg, and 17.75 U/mg for MRS, CWM, PWM and CWM and PWM in different ratio of 1:1, 1:2, 2:1 respectively (Fig. 2). This data clearly showed that amylolytic potential of both lactic cultures were found to be enhanced when grown in CWM and PWM or a combination of these two as compared to while grown in MRS.

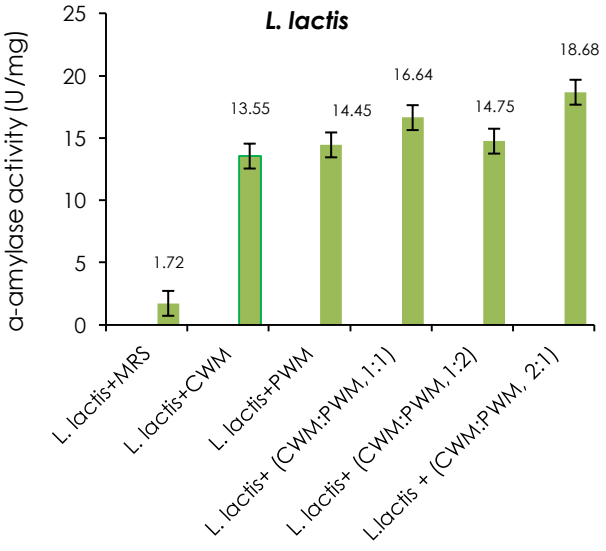

Figure1. Production of a-amylase by *L. lactis* in different composition of CWM and PWM

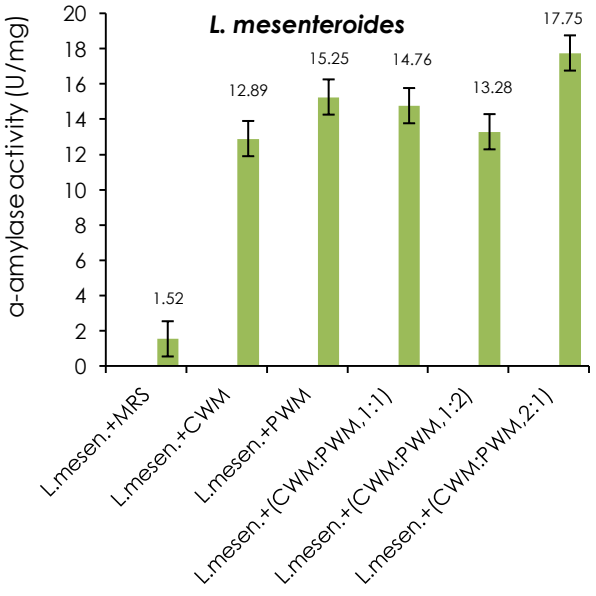

Figure 2 Production of a-amylase by *L. mesenteroides* in different composition of CWM and PWM

β-galactosidase activity assay of *L. lactis* and *L. mesenteroides* grown in different medium in equal ratio

β-galactosidase activity assay was done for both cultures *L. lactis* and *L. mesenteroides* grown in MRS, CWM, PWM and CWM and PWM in equal ratio (1:1). β-galactosidase activity was found to be 1.98 U/mg, 20.45 U/mg, 23.63 U/mg, 26.22 U/mg, for L. lactis when grown in MRS, CWM, PWM and CWM and PWM in equal ratio of 1:1 respectively (Fig. 3). For *L. mesenteroides* β-galactosidase activity was found to be 2.1 U/mg, 19.31 U/mg, 22.59 U/mg, 24.55 U/mg for MRS, CWM, PWM and CWM and PWM in equal ratio of 1:1 respectively (Fig.4).

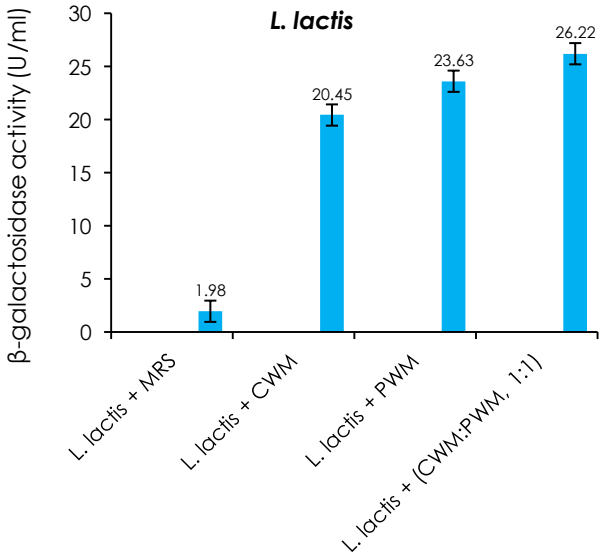

Figure 3 Production of β-galactosidase by L. lactis in same composition of CWM and PWM

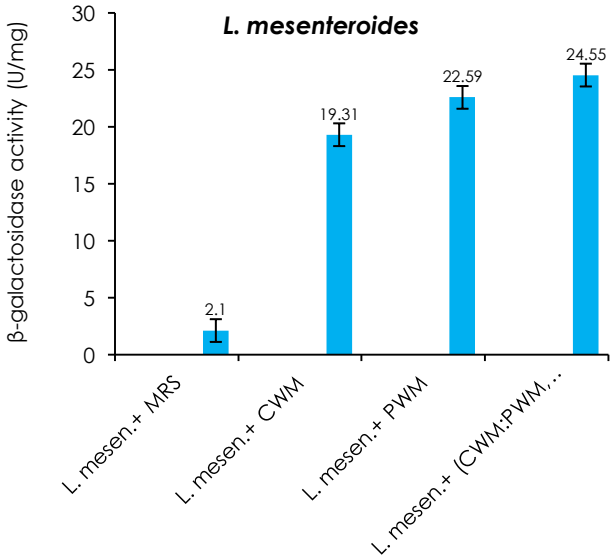

Figure 4 Production of β-galactosidase by *L. mesenteroides* in same composition of CWM and PWM

This data showed that for *L.lactis* β-galactosidase activity was found to be enhanced when grown in CWM and PWM or a combination of these two as compared to while grown in MRS. But the same results were not obtained for *L. mesenteroides* as for this culture maximum β-galactosidase acivity was obtained with MRS.

Organic acid analysis from CWM and PWM

Organic acids produced in CWM and PWM by LAB was analysed using TLC in figure 5 and 6 showed representative chromatograms for the organic acids produced in the culture broth of *L. lactis* and *L. mesenteroides* along with chromatograms which included standard organic acids lactic acid, acetic acid, butyric acid and citric acid. Rf value is determined by using the following formula given as:

$$Rf = \frac{\text{Migration distance of the substance}}{\text{Migration distance of the solvent front}}$$

(Rf values of standards and samples prepared in novel CWM and PWM medium were calculated as shown in Table-3) The Rf values of two standard lactic acid and acetic acid are 0.41 and 0.45, respectively. It has been found that the Rf value of *L. lactis and L. mesenteroides* strain is similar with the Rf value of standard Lactic acid and Acetic acid as shown in the above given table. This study presents a simple and fast method for the identification of organic acid using a thin layer chromatographic in agro waste medium. The chromatogram was sprayed with indicator solution (methyl red–bromophenol blue in 70% ethanol). These organic acids showed different *R*f values. The total time taken to analyze the organic acids in the *L .lactis and L. mesenteroides* culture broths using the proposed method was approximately one hour.

Table 3 Rf values of standards and prepared samples in novel CWM and PWM medium

Sample	Rf
Citric acid	0.36
Acetic acid	0.45
Butyric acid	0.32
Lactic acid	0.41
L. mesenteroides	0.45
L.lactis	0.41

Conclusions

Productions of organic acids require lengthy, laborious and costly pretreatment steps which include gelatinization and liquefaction. The present study was carried out and suggests the use of potato and citrus waste based medium as an attractive alteration for costly sugars for organic acid production. Ganguli (2014) reported α-amylase and β-galactosidase production in potato starch waste by *Lactococcus lactis* subsp lactis isolated from pickled yam. But, yet there is no such report in which different combination of CWM and PWM has been used. It has been seen that when we used different combination of CWM and PWM then activity of both α-amylase and β-galactosidase get enhanced as shown in the graphs. So use of potato and citrus waste based medium can become an attractive alternative for costly sugars and can act as cheap substrate for organic acids production on large scale. Agro waste such as whey, molasses, starch waste, beet, cane sugar and other carbohydrate rich materials are cheap available renewable raw materials.

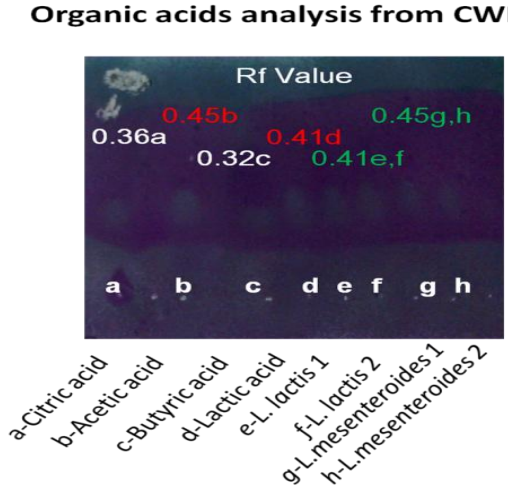

Figure 5 Separation of standard organic acids and samples on Merck Silica gel 60 F254 10×20 cm TLC plate. **(a)** Citric acid **(b)** Acetic acid **(c)** Butyric acid **(d)** Lactic acid **(e)** *L. lactis* 1 **(f)** *L. lactis* 2 **(g)** *L. mesenteroides* 1 **(h)** L. *mesenteroides* 2

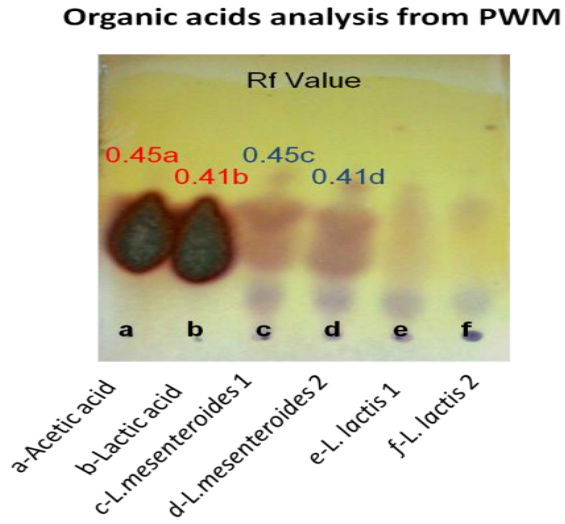

Figure 6 Separation of standard organic acids and samples on Merck Silica gel 60 F254 10×20 cm TLC plate **(a)** Acetic acid **(b)** Lactic acid **(c)** *L. mesenteroides 1* **(d)** L. mesenteroides 2 **(e)** L. lactis 1 **(f)** *L. lactis* 2

In this work, *Lactococcus lactis* and *Leuconostoc mesenteroides* were evaluated for their capability of utilizing inexpensive potato and citrus waste medium. α-amylase and β-galactosidase activity of these cultures were determined and found to be enhanced when cultures were grown in these waste medium as compared to routine laboratory medium MRS. Qualitative estimation of lactic and acetic acid production in citrus and potato waste medium by above said cultures was confirmed by Thin Layer Chromatographic analysis.

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Conflict of Interest
The Author(s) here declare(s) that there is no conflict of interest regarding any financial relationships, personal relationships, academic competition and intellectual passion.

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