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## Utilization of fruit wastes for enzyme production in submerged fermentation

**Abstract.** This study was aimed at production of cellulase, amylase, tannase and ethanol by co-culturing of *Bacillus megaterium* and *Saccharomyces cerevisiae* using fruit waste as the sole substrate in submerged fermentation. Maximum amylase production ( $1.208 \pm 0.14$  IU/mL) was observed in fresh melon peel substrate after 48 h of fermentation. Maximum filter paper activity ( $0.645 \pm 0.02$  IU/mL) and tannase production ( $0.25 \pm 0.01$  IU/mL) was obtained from rotten peach after 24 h of fermentation. Sweet lemon peels yielded highest carboxymethyl cellulase production ( $0.435 \pm 0.02$  IU/mL) after 24 h of fermentation. Maximum reducing sugars ( $2.537 \pm 0.07$  mg/mL) and total sugars ( $17.703 \pm 0.13$  mg/mL) liberation was observed from sweet lemon peels after 24h of fermentation. Maximum ethanol production ( $0.56 \pm 0.03$  mg/mL) was also found from sweet lemon peel after 72h of fermentation. Findings of this study showed that sweet lemon peels had potential for production of biologically active compounds via microbial fermentation.

**Key words:** Fruit waste, *Bacillus megaterium*, *Saccharomyces cerevisiae*, bioethanol, sugars, amylase, tannase, cellulase.

### Introduction

Disposal of massive food, fruits and vegetables waste (FVW) is currently an alarming matter; because of emitting significant amount of greenhouse gases. Hence it has been proved to be a reason of soil and water pollution. About 4.14 tons of CO<sub>2</sub> is counted to be released by per ton of wood waste. Despite that, food and vegetable wastes also seem to be rich in carbohydrates, proteins, fats, antioxidants, some biologically active compounds, natural colorants and in moisture. Due to the biochemical features and size of FVWs, numerous studies have been carried out to get valuable yields by transforming global FVWs [1]. Studies based on microbial processing provide new perspectives by producing organic acids, enzymes, flavoring materials, biomethane, food colorants and bioethanol from FVWs via microbial applications [1].

Enzymes are mostly proteinaceous in nature and found in all living systems in order to catalyze variety of reactions. In production of wine, cheese, beer, bread, vinegar and manufacturing supplies like linen and leather enzymes are substantially being used in the form of plant or bacterial extracts. But the ma-

ior issue associated with enzymes usage in industrial processes is their cost. Wide ranging enzyme production is a matter of high budget utilization and application in manufacturing processes will ultimately affect the prices of the final product. Though, raw materials are contributing 28% of functioning cost. Lignocellulosic material is being used from many decades as a source of inexpensive carbohydrates and can be used as raw matter to produce great valuable yields like organic acids, enzymes, bioethanol, and biodegradable plastics. Processed waste produced via food industries is uneatable and has lignocellulosic nature. Lignocellulose is composed of lignin, cellulose, and hemicellulose, some traces of salts, pectin, minerals, and ash [2].

Lignocellulose is recalcitrant due to its complex structure so we cannot use it directly for microbial processing. Though, lignocellulose is subjected to enzymatic hydrolysis and pretreatment to release fermentable sugars which are utilized for growth and sustenance of enzymes producing microbes [3]. Lignocellulolytic enzymes are being considered very important because of broad range of demand in paper and pulp, detergent, textile, and bioethanol industries. Currently cellulosic-bioethanol is commonly used as

a carbon-neutral technology along with a source of renewable energy. So cellulolytic enzymes demand is increasing day by day [1]. Transformation of ligno-cellulosic matter into significant products like biofuels has a noteworthy global outcome. Lignocellulosic material has a significant role in biofuels production and dropping the world's reliance in overwhelming fossils fuel [4].

Amylolytic enzymes are used to hydrolyze oligosaccharides, polysaccharides, and starch into simple sugars, such as maltose, fructose, and glucose having low molecular weights. The starches are composed of two foremost elements, amylose that is a linear chain of unbranched D-glucose remains linked through  $\alpha$  1-4 bond plus amylopectin, which is well branched D-glucose remains linked by  $\alpha$  1-6 bond. The amylases might be categorized into exo and endoamylases on the basis of their approaches of hydrolysis. Prevailing number of exoamylases attacks  $\alpha$  1-4 bonds, but some of them like glucoamylase can target both  $\alpha$  1-4 and  $\alpha$  1-6 bonds in order to yield simple sugars like glucose and maltose [5].

Endoamylase cleaves  $\alpha$  1-4 bond in starch, while does not interrupt  $\alpha$  1-6 bond in amylo-pectin and its correlated complex polysaccharides. Best illustration of  $\alpha$ -amylase is endoamylase due to production of varying fragments of oligosaccharides by starch. Glucoamylase only targets non-reducing ends while  $\alpha$  -amylase acts on random locations in starch [6].

Tannase (tannin-acyl-hydrolase) is acknowledged for hydrolyzing tannin to glucose and gallic-acid which is a substrate used for the manufacturing of trimethoprim and propylgallate. Tannin is considered to be forth utmost plentiful plant component after celluloses, hemicelluloses, and lignin. Tannase can be used to reduce a stringency of the products so commonly it is used in beverage and food industries, beside that it is valuable in dropping tannic-acid absorption in tannery effluents [1]. All these enzymes had potential application in various industries. This study was designed to produce valuable products (enzymes) from rotten fruits and fruit waste by co-culturing of *Bacillus megaterium* and *Saccharomyces cerevisiae* in submerged fermentation.

## Materials and methods

*Objects.* Microbial strains (*Bacillus megaterium* and *Saccharomyces cerevisiae*) were attained from the microbial collection of Biotechnology laboratory of the Department of Biotechnology, University of Sargodha, Pakistan. *B. megaterium* was revived on nutrient agar (Oxoid, UK) slants and *S. cerevisiae*

was revived on potato dextrose agar (Oxoid, UK) slants.

*Inoculum preparation.* The *Bacillus* culture was taken on loop from nutrient agar slants. Furthermore, it was maintained in sterile nutrient broth vials and harvested for 24 h. The cloudy appearance of *B. megaterium* growth predicted that the successful inoculum was ready to use as stock. *S. cerevisiae* was maintained as stock culture on potato dextrose agar (Oxoid, UK) slants.

*Fermentation technique.* Submerged fermentation was conducted in Erlenmeyer flasks of 250 mL capacity containing 15 mL fermentation media comprised of 2% of substrate separately in each flask and sterilized at 121 °C and 15 Psi for 15 min. Inoculation was carried out in a sterilized environment with 1% of each 24 h old vegetative culture of *B. megaterium* and *S. cerevisiae*. After that inoculated flasks were placed on shaking incubator at 35°C with shaking rate of 120 rpm for 72 h and samples were collected after 24 h, 48 h and 72 h. After each sampling, the sample was centrifugated at 10,000 x g for 10 min at 4°C. The obtained supernatant was further employed for analysis.

*Cellulase assay.* Carboxy methyl cellulase (CMCase) and filter paper activity (FPase) was done as explained in our previous reports [7]. Estimation of CMCase activity was carried out by adding 0.5 mL of crude enzyme with 0.5 mL of 1% CMC (prepared in 0.05 M citrate buffer having pH 5) and reaction was incubated for 30 min at 50 °C. While for FPase 500  $\mu$ L of crude enzyme was added up in a test tube having Whatman No.1 filter paper strips (1x 6 cm) and 0.5ml of 0.05 M Sodium Citrate Buffer (pH 5). Mixture was further incubated at 50°C for 30 min. After incubation 1.5 mL DNS (Sigma, USA) was added in test tubes to stop the reaction and boiled in a water bath for 10 minutes and absorbance was taken at 540nm. Glucose was utilized as standard.

*Amylase assay.* Activity of  $\alpha$ -amylase was calculated using amylase assay conditions [8]. Reaction mixture having 0.5 mL of crude enzyme and 0.5mL of 1% starch solution (pH 7) incubated at 60°C for 30 min. After that 1.5mL of 3,5 dinitro-salicylic acid (DNS) reagent was added to stop the reaction and the mixture was kept in boiling water bath for 10 min. After the cooling of mixture, optical density was recorded at 540 nm. Maltose was taken as a standard.

*Tannase assay.* Tannase activity was calculated by method described by Miller [9]. The enzyme solution volume was taken 1 mL which was added with 1mL tannic acid reagent as substrate. Reagent was prepared in 0.5% tannic acid in acetate buffer. The reactants were incubated for 30 min at 37°C and then placed for 15 min boiling in water bath to stop the en-

zyme substrate activity. From this enzyme-substrate system, 1 mL was taken in test tube and 3 mL of dinitro-salicylic acid (DNS) reagent was added in test tubes and the mixture again boiled for 10 min. After boiling, solution with 10ml distilled water was diluted and absorbance was recorded at 540 nm via above mentioned method against blank solution. Each reaction was carried out in triplicate.

**Analytical methods.** Estimation of reducing sugars released by the hydrolytic action of enzymes was carried out by using dinitro-salicylic acid (DNS) method [9], by taking 0.5 mL sample with 1.5 mL DNS (Sigma, USA). Boiling was carried out at 100°C for 10 min and OD was measured at 540 nm. Total sugars were estimated through phenol sulfuric acid method [10], by taking 0.5 mL sample, 25 mL H<sub>2</sub>SO<sub>4</sub> and 0.5 mL phenol. It was kept under room temperature only for 30 min, after that optical density was measured using spectrophotometer at 490nm. Ethanol was measured by HPLC as described by Irfan et al [11]. BioRad Aminex HPX 87H column (250 mm 9 4.6 mm) was used having mobile phase of 5mM H<sub>2</sub>SO<sub>4</sub>, 0.7 mL/min flow rate and 60°C column temperature. Each sample was passed from sterile membrane filter of 0.2 µl and analysis was done by using 20µl injection volume.

**Statistical analysis.** The whole data generated from experiments was analyzed statistically using Microsoft excel program (version 2016) and values presented as mean of triplicates.

## Results and discussion

Three kinds of fruit wastes were used comprising of fresh melon peel, rotten peach, and sweet lemon peel. Each of the fruit waste medium was inoculated with co-culture of *Bacillus megaterium* and *Saccha-*

*romyces cerevisiae* and incubated for 3 days. After 24, 48 and 72 h, sample was taken and enzyme assays for CMCase, Fpase, amylase, tannase and total sugar, reducing sugar and ethanol were conducted.

Using the fresh melon peel as a substrate, highest enzyme activities of amylase ( $1.208 \pm 0.14$  IU/mL), tannase ( $0.18 \pm 0.01$  IU/mL) and CMCase ( $0.078 \pm 0.01$  IU/mL) was observed after 48 h of fermentation. Highest FPase activity ( $0.43 \pm 0.03$  IU/mL) was observed after 24 h of fermentation (Figure 1). Minimum CMCase, amylase and tannase activities were observed with 24 h of fermentation indicating the importance of the fermentation period.

Maximum reducing sugar  $0.047 \pm 0.001$  mg/mL was obtained after 48 as well 72 h of fermentation by using fresh melon peels, while maximum total sugar  $0.335 \pm 0.004$  mg/mL was estimated after 24 h of fermentation time. No ethanol production was evaluated after 24 h, and  $0.14 \pm 0.01$  mg/mL was experienced after 72 h of fermentation period (Figure 2).

When rotten peach was used as substrate for fermentation, the maximum evaluated enzyme production of FPase ( $0.645 \pm 0.02$  IU/mL), amylase ( $0.45 \pm 0.01$  IU/mL), tannase ( $0.25 \pm 0.01$  IU/mL) and CMCase ( $0.057$  IU/mL) was obtained after 24 h of fermentation time (Figure 3). By increasing the fermentation period up to 48 or 72 h minimum enzyme activity was noted.

Reducing sugar estimated from rotten peach was  $0.013 \pm 0.001$  mg/mL, which remained same after 48 and 72 h of fermentation. Maximum total sugar production  $1.87 \pm 0.05$  mg/mL was optimized after 48 and 72 h of fermentation. Ethanol production by using rotten peach was  $0.051 \pm 0.001$  mg/mL appeared after 48 and 72 h of fermentation, while 24h of fermentation period gave minimum ethanol production (Figure 4).

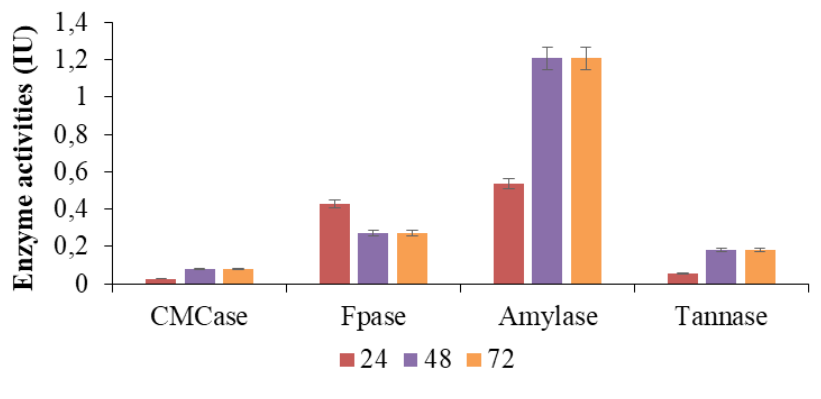
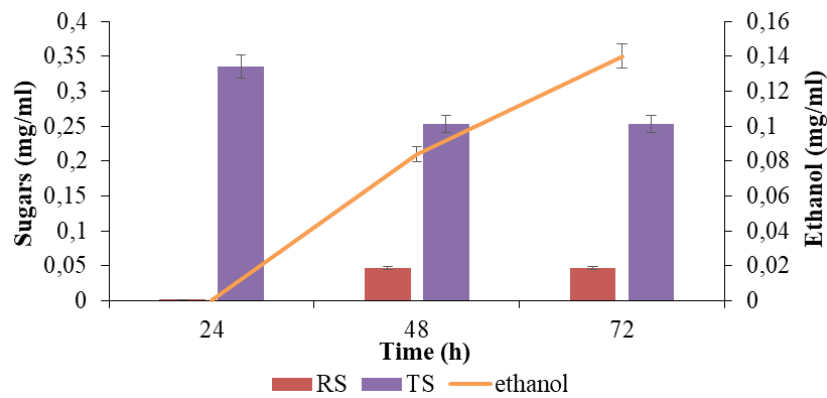
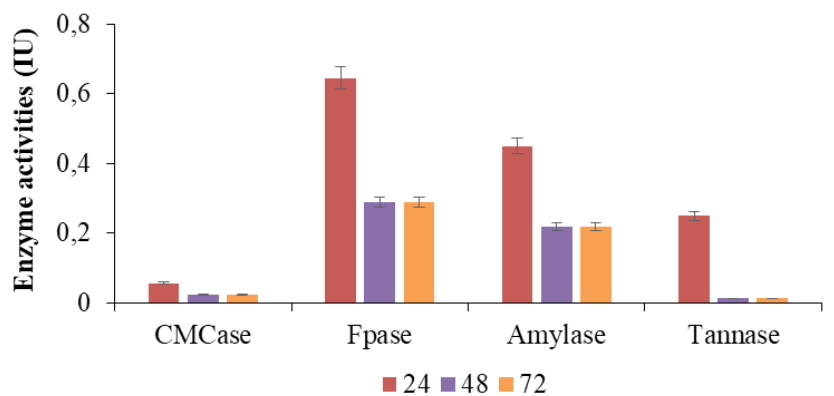


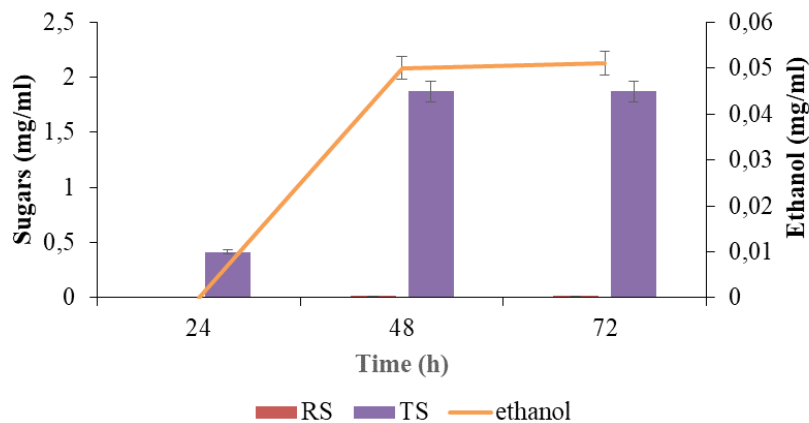
Figure 1 – Enzymes production from fresh melon peels in submerged fermentation



**Figure 2** – Reducing sugars (mg/mL), total sugars (mg/mL) and ethanol (mg/mL) produced in submerged fermentation using fresh melon peels



**Figure 3** – Enzymes production from rotten peach in submerged fermentation



**Figure 4** – Reducing sugars (mg/mL), total sugars (mg/mL) and ethanol (mg/mL) produced in submerged fermentation using fresh rotten peach

Sweet lemon peel also exhibited potential for enzyme production. The maximum production of amylase ( $0.529 \pm 0.03$  IU/mL), CMCase ( $0.435 \pm 0.02$  IU/mL) and FPase ( $0.295 \pm 0.01$  IU/mL) was examined after 24 h of fermentation, while after increasing

fermentation period up to 72 h minimum amylase, CMCase and FPase activities were observed. Tannase production was noted maximum after 48 h of fermentation, which remained same even after 72 h of fermentation (Figure 5).

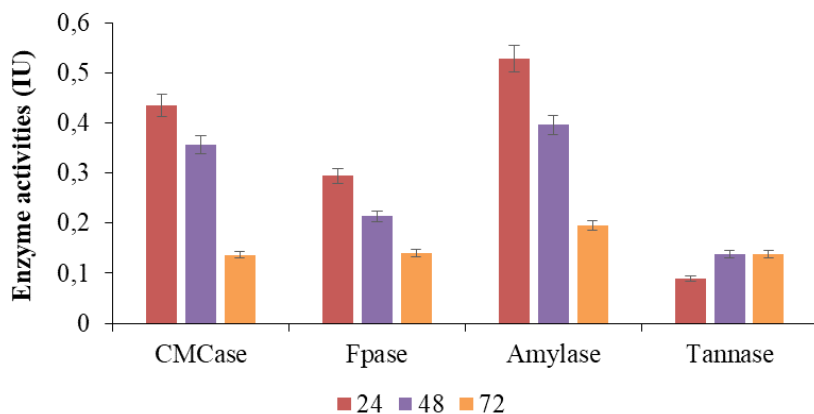


Figure 5 – Enzymes production from sweet lemon peels in submerged fermentation

Reducing sugar and total sugar for the sweet lemon peels were observed to be  $2.537 \pm 0.07$  mg/mL and  $17.703 \pm 0.13$  mg/mL respectively after 24h of fermentation time, by increasing fermentation time to 48 or 72h

a decline in reducing and total sugar was experienced. Maximum ethanol production for this substrate was  $0.56 \pm 0.03$  mg/mL estimated after 72 h of fermentation (Figure 6), while 24h gave minimum ethanol yield.

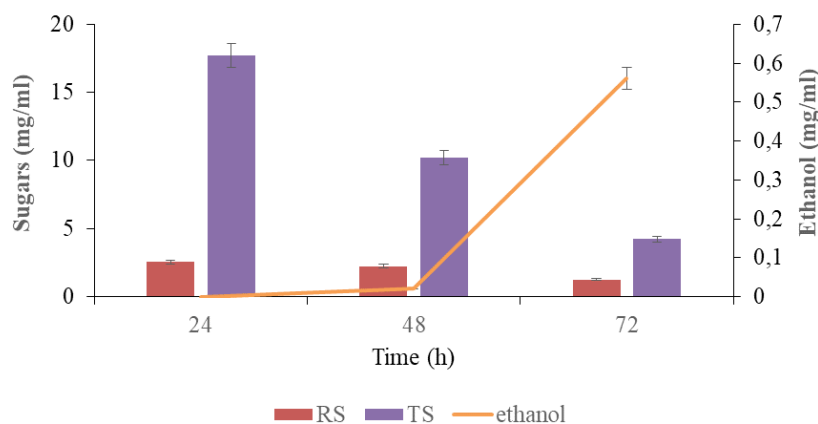


Figure 6 – Reducing sugars (mg/mL), total sugars (mg/mL) and ethanol (mg/mL) produced in submerged fermentation using sweet lemon peels

Norsalwani and Norulaini, [12] reported maximum cellulase activity of 2.65 FPU/ml, produced by palm kernel cakes as substrate. Co-culturing of *Cellulomonas carte*, *B. megaterium*, *P. putida* and *Pseudomonas fleuroscence* on banana solid waste exhibited maximum  $\beta$ , D glucosidase activity of 0.602

U/mL on 25<sup>th</sup> day and FPase of 0.178 U/mL on 20<sup>th</sup> day of fermentation [13]. In a study, decayed fruit waste was taken and novel cellulase producing actinomycetes were isolated and highest enzyme production was observed using fruit waste media as our carbon source [14].

Commonly fungi are known for the production of tannase but some bacterial species like *Lactobacillus* sp. and *Bacillus* sp. were also well known for the production of tannase [15]. A recent study revealed that *Klebsiella oxytoca* had potential of tannase production using peels of *Citrus limetta* in solid state fermentation [16]. Although the findings reported in earlier reports differed with the present study due to substrate, microorganism, and culturing technique.

In a recent study by Cyprian et al [17], maximum concentration of amylase  $259.00 \pm 1.23$  U/mL was reported to be obtained by using Banana waste. It was reported that fermentation period plays an important role in amylase production by using fruit waste. Moreover, decline in amylase production may be caused due to nutrients exhaustion or denaturation of enzyme. Hence short duration of fermentation period may lead to cost effective amylase production. Oshoma et al [18] has reported that Banana peel has a potential of producing amylase due to its easy availability and cost effectiveness. It was further reported that maximum amylase yield can be obtained after 96 h of fermentation. After this period amylase activity gradually decreases due to depletion of nutrients required for microbial growth and amylase production [18]. In a study done for amylase estimation, maximum yield of amylase was reported by using solid waste of banana with microbial strain of *Bacillus subtilis* [19]. Mango kernel was used as a substrate under optimized conditions with microbial strain of *Fusarium solani* and 0.889 U/g of amylase was successfully produced [20].  $\alpha$ -amylase production was optimized to be 8.26 U/mL in submerged fermentation after 5 days of fermentation by *Streptomyces* sp. using orange waste as a sole carbon source [21]. In another study amylase production was experienced with brewer's spent grain hydrolysate as substrate using Catabolite-repressed strain of *Bacillus subtilis* KCC103 through submerged fermentation [22]. Cassava waste has also been utilized for amylase production by *Bacillus* sp. [23].

Zabed et al [24] has reported that *S. cerevisiae* can be employed for ethanol production using fruit and vegetable waste materials and the pH was maintained in the range of 4.0-5.0. In a recent study, it has been reported that maximum bioethanol yield can be obtained by using pineapple waste if fermentation is carried out under low pH range of 3.0-4.0 [25,26]. *S. cerevisiae* has proved to be much better in pineapple waste as compared to other fruit and vegetable wastes. Another study reveals that bioethanol production from Orange peel and Banana was around 2 and 3% [27]. Strain of *S. cerevisiae* with the ca-

shew apple juice has also shown bioethanol production [28]. Banana peel and Apple pomace produced 38% of ethanol yield by *S. cerevisiae* after the 36 h of fermentation [29]. Recent study reported that mango waste yield maximum amylase activity using *Bacillus* sp. in submerged fermentation [30]. Fruit waste of longan had potential of bioethanol production in submerged fermentation [31]. Banana peels has been reported as potential substrate for ethanol production by *Klebsiella* sp. SWET4 [32]. So, these studies have shown that the fruit waste could be utilized as a potential substrate for the production of enzymes and bioethanol.

## Conclusion

Results of this study showed that a huge amount of fruit wastes that are being thrown in open places could be utilized for the production of valuable products through microbial fermentation. Sweet lemon peels were found to be the best substrate for the production of cellulases (CMCase  $0.435 \pm 0.02$  IU/mL and FPase  $0.295 \pm 0.01$  IU/mL) and amylase ( $0.529 \pm 0.03$  IU/mL) in submerged fermentation after 24h. Maximum ethanol production was obtained by sweet lemon peels after 72h of fermentation.

## Acknowledgement

The authors would like to acknowledge the Department of Biotechnology, University of Sargodha, Pakistan for providing facilities to conduct this work.

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