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Citrus limetta (Musambi) PEELS: AN EFFICIENT CHEAP SUBSTRATE FOR TANNASE PRODUCTION IN SOLID STATE FERMENTATION

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ABSTRACT

The present study mainly executed to optimize various factors for tannase production employing agro-industrial waste. Ten bacterial strains isolated from fish gut were revived on nutrient agar and then cultivated on tannin-agar plates for holozone and tannase assay. Of all, *Klebsiella oxytoca* was selected due to highest enzyme production (24.52 U/ml) potential among other strains. The medium ingredients (musambi peels (2%), trypton (0.2%), potassium nitrate (0.05%) and potassium chloride (0.1%) was selected employing one variable at a time approach. Optimized culture parameters such as initial medium pH of 3, incubation temperature of 30°C, with 1% inoculum size and 24 h of fermentation period yielded highest enzyme production. Partial characterization of the crude enzyme revealed best enzyme activity at pH 5 and 40°C. The optimum time of enzymatic action was 30 minutes with substrate concentration of 1.5%. These finding could be utilized for further scale up studies.

Key words: Fish gut, *Klebsiella oxytoca*, tannase, optimization, SSF

INTRODUCTION:

Tannin is the polyphenolic substances commonly present in plants. In addition to phenolic reactions, they have distinctive features like alkaloids precipitation ability and as gelatin along with various proteins (Haslam 1996). Tannins mostly precipitate proteins by their chelating feature and are recognized as enzyme inhibitors. They are also liable to bitter taste that reduces the feed intake. Such characters prevent tannin rich plants usage as animal feed source (Selinger *et al.*, 1996). The low concentrations of tannin in food stuffs enhanced milk production and growth rates in animals (Batra and Saxena 2005).

Tannase (E.C.3.1.1.20) is an inducible enzyme, catalyze the ester linkage of tannin, yields glucose and gallic acid (Batra and Saxena 2005, Mahendran *et al.*, 2006). It causes hydrolysis of ester, hence break bonds in diverse substrates like gallo tannins, epicatechin gallate and epigallocatechin-3-gallate to produce gallic acid and glucose (Lekha and Lonsane 1997). In general, the tannase is used in food stuffs, brews, medicinal and organic industries for producing gallic acid, tea and coffee flavor, energizing drinks and acorn wine. Furthermore, tannase is utilized in beer and various fruit juices clarification, enhancement of grape wine flavor and animal feed formation (Mohapatra *et al.*, 2009). It was well-recognized that several bacterial strains utilized tannin and produce tannase.

The current trend is to utilize the potential of different microbes for the manufacture of industrially vital complexes like organic acids, enzymes, secondary metabolites, single-cell proteins etc. from wastes. This practice not only reduces the cost of valuable products but also overcome the burden of wastes on environment. In present context, agro-industrial wastes which is excessively available in Pakistan could be employed for tannase production. The present study is mainly focused on screening of tannase producing bacteria, selection of suitable agro-industrial wastes and optimization of various production factors for tannase production and effect of different factors on tannase activities.

MATERIALS AND METHODS

Substrate: Agro-industrial wastes *i.e.*, musambi peels, banana, grape fruit and pomegranate were collected from local market of Lahore, Pakistan, washed properly, then dried and ground in grinder machine, sifted twice to get fine particles and finally kept in air tight bottle to avoid humidity till their use as substrate during the experimental work.

Source and revival of bacterial strains: Ten bacterial strains isolated from gut of fish (Shakir 2013) were revived on the nutrient agar and then in nutrient broth. From broth, these bacteria were inoculated again on nutrient-agar plate and then used for screening of tannase producing bacteria.

Screening of tannase producing bacteria: Tannin agar medium (TAM) comprising of 1.3% nutrient broth, 1.5% agar-agar and 0.5% tannic acid was used to screen the tannase producing bacteria. A

greenish holozone around the colonies by tannase producing bacteria was obtained after incubation at 37°C for 96 hours. These bacteria were subjected to tannase assay. The strain yielding highest tannase was selected for further study.

Preparation of Inoculum: The selected bacterial strains were inoculated in the nutrient broth and then incubation was carried out for 24 hours at 37°C. The culture was further employed for medium inoculation (1% inoculum size). For inoculum preparation of tannase producing bacteria, growth conditions were kept persistent during whole study.

Enzyme Assay: In order to estimate tannase, the respective inoculated medium after addition of sterilized distilled water (10 ml), was then centrifuged at 8000 rpm for 15 minutes at 4°C. The supernatant containing crude enzyme was further used for enzyme estimation following Miller (1959) method. Briefly, 0.5 ml of supernatant was added to 0.5 ml of substrate solution (tannic acid 0.5% in 0.1M acetate buffer solution), incubated at 37°C for 30 minutes then kept in boiling water bath for about 15 min at 100°C for deactivating the enzyme substrate reaction. Added 1.5 ml of di-nitro-salicylic acid (DNS) and then placed in boiling water bath for about 10 min. The mixture was diluted up to 5 ml using distilled water. The blank solution was proceeded in the similar way but just added distilled H_2O instead of supernatant (enzyme). Absorbance was then noted at 540nm against the blank using a spectrophotometer. For estimation of tannase, glucose was used as standard. One enzyme unit was defined as the amount of enzyme that release 1µmol of glucose during enzyme assay conditions. Every experiment was carried out in triplicate.

Screening of enzyme production medium: The initial medium comprising of KH_2PO_4 0.05%, K_2HPO_4 0.1%, $CaCl_2$ 0.1%, $MgSO_4$ 0.2% (Kallel *et al.*, 2016) was used for screening of carbon, organic nitrogen, inorganic nitrogen and metal salts.

Effect of carbon source: Briefly, initial medium supplemented with different carbon sources (grapefruit, musambi, banana and pomegranate) with 2% concentration was inoculated with 1% inoculum. After incubation at 37°C for 24 hours, the estimation of enzyme was carried for each carbon source. The carbon source yielding maximum enzyme activity was chosen and then incorporated in initial production medium.

Effect of organic nitrogen source: For screening of organic nitrogen source, cultivations of inoculum (1%) were carried out in selected carbon source incorporated initial medium supplemented with organic nitrogen sources like peptone, yeast extract, meat extract and tryptone with 0.2% concentration. After its incubation at 37°C for 24 hours, the estimation of enzyme activity was carried out corresponding to each nitrogen source. The organic nitrogen source producing maximum enzyme activity was then selected and incorporated in the medium.

Effect of inorganic nitrogen source: For screening of inorganic nitrogen sources, selected carbon and organic nitrogen incorporated medium supplemented with 0.05% of NH_4Cl, $NaNO_3$, KNO_3, $(NH_4)_2SO_4$ and NH_4NO_3. After incubation for 24 hours at 37°C, the selection of inorganic nitrogen source following enzyme assay was carried out as defined earlier.

Effect of metal source: To screen the metal salts, selected carbon, organic and inorganic nitrogen incorporated medium supplemented with 0.1% of NaCl, $FeSO_4$, KCl, $MnSO_4$ and $FeCl_2$. After incubition for 24 hours at 37°C, the enzyme estimation was carried corresponding to each metal source. The source producing maximum enzyme activity was chosen to incorporate in respective medium.

Optimization of physical parameters: Physical factors optimization including temperature, pH, inoculum size, inoculum age and incubation period was done for the identification of optimal conditions of the respective strain for maximum tannase production.

Optimization of pH: The optimal medium for enzyme was prepared in various flasks and their pH 3, 5, 7, 9 and 11 was maintained. Then medium was autoclaved and further inoculated by 1% inoculum of twenty-four hours. Afterwards, incubation was done at 37ºC for 24 hours and after that enzyme estimation was carried out. The pH regarding highest tannase value was observed.

Optimization of temperature: The optimal medium for tannase was prepared, optimum pH adjusted and then autoclaved it. This medium was then inoculated with 1% of 24 hours inoculum under sterilized circumstances and incubated at various temperatures *i.e.*, 30, 37and 45°C for 24 hours. After that, the enzyme assay was performed and then the outcomes were assembled. Temperature conforming maximum enzyme production was finalized for future work.

Optimization of inoculum size: For optimization of inoculum size, the optimal medium having optimal pH was prepared. This medium was then inoculated with 1, 2, 3, 4 and 5% inoculum and incubated these flasks at optimal temperature for 24 hours. Then the enzyme assay was done and the inoculum size conforming highest enzyme value was selected for future study.

Optimization of inoculum age: For inoculum age optimization, optimal medium with optimal pH was prepared and autoclaved. That medium was then inoculated with optimal inoculum size of various ages (24, 48 and 72 hours) and kept at optimal incubation temperature. Enzyme assay was further performed to confirm inoculum age yielding highest enzyme value.

Optimization of incubation period: Optimal medium was prepared along with optimal pH. The medium was inoculated with optimal inoculum size and then incubated for various periods *i.e.*, 24, 48, 72 and 96 hours. After their incubation, enzyme estimation was carried out.

Characterization of Crude Tannase

Effect of various pH on tannase activity: Effect on the bacterial tannase activity using various pH *i.e.*, 4, 5, 6..... 11, was studied by 0.5% of tannic acid as a substrate solution, prepared in 0.1M of each of these buffer *i.e.*, acetate buffer (pH 4-5), phosphate buffer (pH 6-7), tris-HCl (pH 8-9) and glycine NaOH (pH 10-11) buffer. For tannase assay, the reaction mixture containing 0.5ml enzyme solution from specific bacteria and 0.5ml of 0.5% tannic acid solution in above mentioned buffers of different pH were continued as defined previously.

Effect of temperature on tannase activity: Effect of different temperatures on tannase activity was determined by incubation of reaction mixture (0.5 ml of bacterial culture supernatant along with 0.5 ml of 0.5% tannic acid solution in optimal pH buffer) at 30, 35, 40, 45, 50, 55 and 60°C for 30 minutes. The enzyme activity was observed as explained above.

Effect of different substrate concentrations on tannase activity: Various concentrations of tannic acid as substrate (0.5, 1, 1.5, 2, 2.5 and 3%) were prepared in the optimized buffer solution in order to determine the effect of substrate concentration on enzyme activity. For this aspect, reaction mixture was incubated at optimal temperature for 30 minutes and enzyme assay was continued as stated previously.

Effect of incubation period on tannase activity: In order to study the impact of incubation period, the reaction mixture containing 0.5 ml of enzyme (supernatant) of selected bacteria and 0.5ml of optimal substrate concentrations *i.e.*, tannic acid solution in optimal pH buffer was incubated at optimized temperature for different time intervals *i.e.*, 30, 60, 120, 150 and 180 minutes. Further, tannase assay was performed as mentioned above.

RESULTS

Strain selection: Ten bacterial strains already isolated from fish gut were screen to study their tannase producing potential. Colonies of *Roultella ornithinolytica*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Bacillus amyloliquefox* were produced greenish zone on tannase agar plate and proceeded for enzyme assay (Table 1). The highest enzyme production up to 24.53 U/ml was yielded by *Klebsiella oxytoca* and selected for further study.

Table 1: Screening of tannase producing potential of bacterial strain based on zone formation and enzyme assay

Sr. #	Strains	Zone formation	Tannase (U/ml)
1	*Bacillus pumilus*	-	
2	*Bacillus amyloliquefaciens*	+	21.78[b]
3	*Enterobacter aerogenes*	+	17.28[c]
4	*Bacillus flexus*	-	
5	*Roultella ornithinolytica*	+	20.56[b]
6	*Aeromonas hydrophila*	-	
7	*Aeromonas bestiarum*	-	
8	*Klebsiella oxytoca*	+	24.52[a]
9	*Aeromonas allosaccharophila*	-	
10	*Aeromonas media*	-	

Abbreviation: +: zone formed, -: zone not formed.
Means that do not share a letter in column are significantly different (P<0.001)

Effect of carbon source: Highest enzyme production by *K. oxytoca* with musambi peels that was 11.24±1.28 U/ml and least value with pomegranate that was 9.93±1.16 U/ml. So musambi peels were selected as carbon source and incorporated in medium (Fig. 1).

Effect of organic nitrogen source: Highest results (13.97±2.69 U/ml) were obtained by *K. oxytoca* when medium supplemented with tryptone and lowest value obtained with peptone which is 9.21 ±1.24 U/ml (Fig. 2).

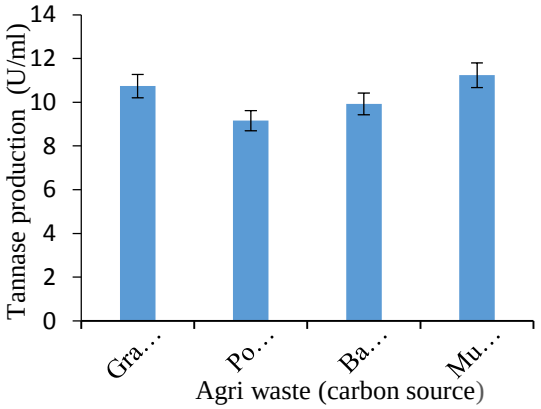

Figure 1: Effect of carbon source on tannase production in SSF.

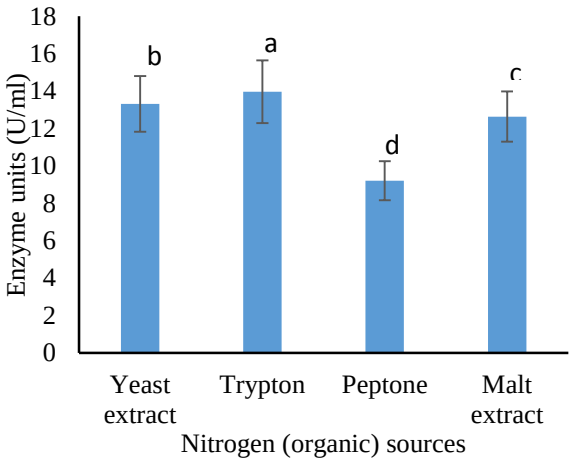

Figure 2: Effect of organic nitrogen source on tannase production in SSF.

Effect of inorganic nitrogen source: Enzyme production (12.37±0.86 U/ml) was low with $(NH_4)_2SO_4$. The best inorganic nitrogen for enzyme *oxytoca* (Fig. 3).

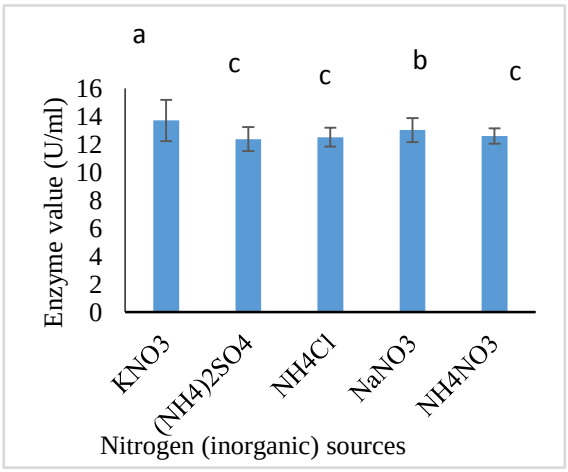

Figure-3: Selection of inorganic nitrogen source for tannase production

Effect of metal salts: Enzyme production (12.78±0.20 U/ml) was maximum by *K. oxytoca* when medium supplemented with KCl and lowest value (11.56±0.42 U/ml) with $MnSO_4$ (Fig. 4). Based on results, KCl was selected for further work.

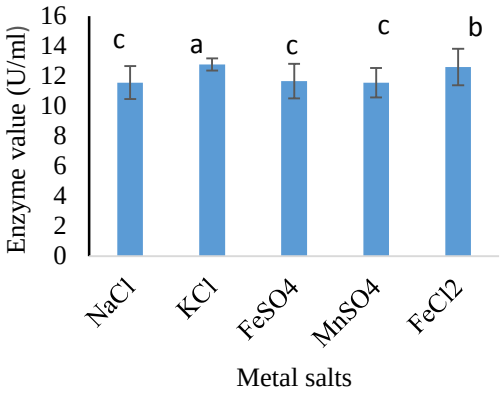

Figure 4: Effect of metal salt for tannase production

Optimization of physical parameters

Optimization of pH: Highest results (9.93±2.99 U/ml) by *K. oxytoca* were obtained when pH of medium was 3 (Table 2). The results showed that at low pH, enzyme production is high and the value decrease by increasing the pH but at pH 9 the value again increase and suddenly at high pH again decrease.

Table 2: Optimization of physical parameters for tannase production by *Klebsiella oxytoca* in SSF

Parameter		Tannase (U/ml)
pH	3	9.93^{a}±2.99
	5	6.98^{c}±2.42
	7	5.70^{d}±1.06
	9	7.85^{b}±3.72
	11	5.56^{e}±1.61
	Significance	P<0.01
Temperature (^{0} C)	30	10.06^{a}±2.46
	37	6.54^{b}±1.70
	45	6.16^{b}±3.32
	Significance	P<0.01
Inoculum size (%)	1%	16.70^{a}±4.19
	2%	6.71^{b}±3.35
	3%	6.71^{b}±3.35
	4%	11.19^{ab}±3.43
	5%	11.55^{ab}±1.77
	Significance	P <0.01
Incubation time (hours)	24	16.76^{a}±1.78
	48	11.98^{ab}±2.98
	72	8.55^{b}±2.00
	96	7.23^{b} ±1.81
	Significance	P< 0.001

Values that do not share an alphabet in a column are significantly different from each other.

Optimization of temperature: Highest enzyme production up to 10.06±2.46 U/ml at 30°C and lowest (6.16±3.32 U/ml) at 45°C by *K. oxytoca* was recorded (Table 2). The results show that the enzyme production has inverse relation with temperature. By increasing temperature, the value of enzyme decreases.

Optimization of inoculum size: Optimum inoculum size for *K. oxytoca* is 1% (Table 2) because it gives best results *i.e.,* 16.70±4.19 U/ml while 2% and 3% both inoculum size gives least results *i.e.,* 6.71±3.35U/ml. These results revealed that the value of enzyme was high at low inoculum size and by increasing the inoculum size the value will be decrease but at 4% to onward the value of enzyme production again increases.

Optimization of incubation period: Conferring to the findings obtained at various time, it was observed that the optimal time of incubation was 24 hours for *K. oxytoca* (Table 2). These outcomes presented that the relation between enzyme production and incubation period is inverse as by increasing the incubation time the value of enzyme is decreases.

Characterization of tannase activity

Effect of various pH on enzyme activity: Enzyme activity was found to be best at pH 5 and gave best results (14.88±1.50 U/ml) (Fig. 5). While lowest results (3.47±2.85U/ml) were appeared corresponding to pH 10. The enzyme activity showed random trend as firstly by increasing the pH the enzyme activity increases, at pH 6 the value was decrease and at pH 7 a minute value is increase and again moves toward high value the enzyme activity was decrease but again at highly basic pH 11 the activity again increases.

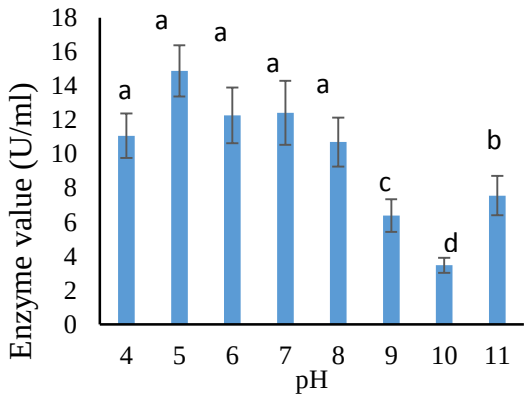

Figure 5: Effect of pH on tannase activity.

Effect of temperature on enzyme activity: Tannase activity (20.34±3.70U/ml) was best at 40^{0}C and least activity (13.41±2.10U/ml) at 55^{0}C (Fig. 6). These results firstly depicted direct relationship between temperature and the tannase activity but at highest temperature, the value of enzyme activity decreases then abruptly at 60^{0}C the enzyme activity showed high value.

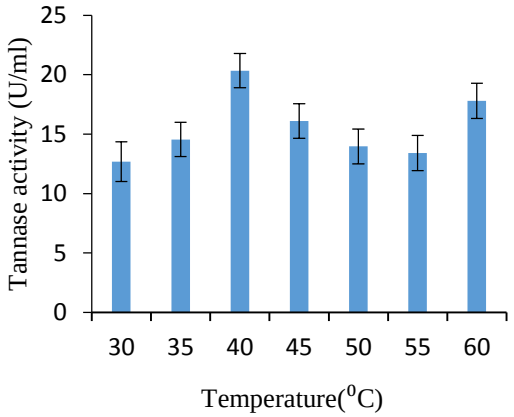

Figure 6: Effect of temperature on tannase activity.

Effect of different substrate concentrations on enzyme activity: Enzyme activity was minimum when 3% substrate concentration was used. Best suitable substrate concentration for maximum enzyme activity (30.81±2.52 U/ml) by *K. oxytoca* was found at 1.5% at which enzyme showed best results (Fig. 7). With the increase in substrate concentration value the enzyme activity also increased thus showing direct relationship but with further increase in concentration, the enzyme activity decreases.

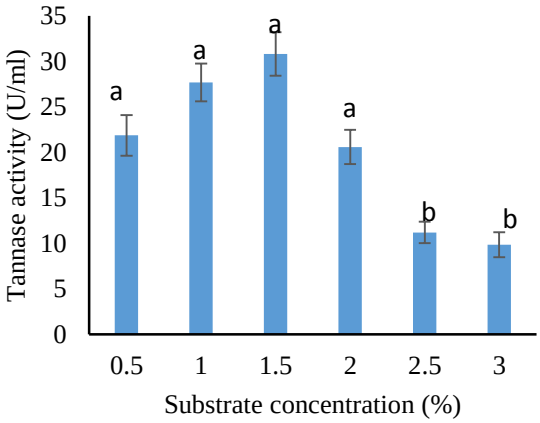

Figure 7: Effect of substrate concentration on tannase activity

Effect of incubation period on tannase activity: Enzyme showed minimum activity after 180 minutes of incubation. For maximum enzyme activity (30.60±1.16U/ml), best incubation time was found to be 30 minutes (Fig.8).

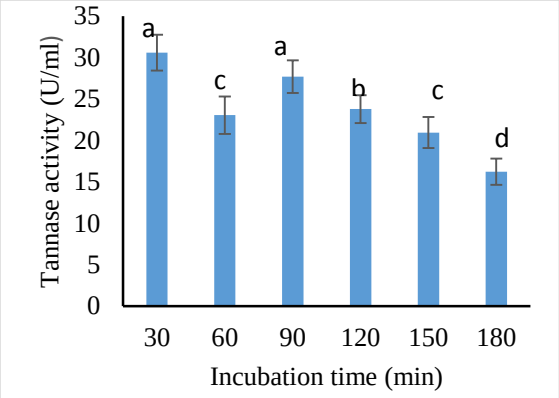

Figure 8: Effect of incubation time on tannase activity.

DISCUSSION

In present investigations, bacterial strains isolated from fish gut were screened for tannase production. Several plants, animals and microbes are reported to produce tannase. In general, tannase producing potential of fungus is comparatively high but its slow growth rate is the major hindrance in industrial usage. The bacterial strains normally preferred in industries because of its high growth rate, resist high temperature and relatively easy genetic modifications (Beniwal *et al.,* 2013, Beniwal *et al.,* 2015). In recent years, concern about tannase production from bacteria has increased and novel bacterial strains from different sources were isolated. *Lactobacillus plantarum* strain isolated from waste of olive mill was experienced for production of tannase (Ayed and Hamdi 2002, Wilson *et al.,* 2009). Numerous researches on degradation of tannin by tannase producing bacteria isolated from soil have been described (Ilori *et al.,* 2007, Dave *et al.,* 2011, Ingole *et al.,* 2012).

In present study, nutrient agar supplemented with 0.5% tannic acid medium was used to screen tannase producing bacteria. The change in medium color from light to blackish green indicates progressive tannase production capability of microbes. Darker green colored zones formation was because of hydrolysis of tannic acid into glucose and gallic acid. Four strains *R. ornithinolytica*, *K. oxytoca*, *E. aerogenes* and *B. amyloliquefox* showed positive results for plate test. The *K. oxytoca* yielded highest enzyme production (24.52 U/ml). Different researchers used different media for isolation and screening of tannase producing bacterial strains (Dave *et al.,* 2011). In present study, tannase production (11. 24 U/ml) by *K. oxytoca* on medium (musambi peels, tryptone, KNO_3, KCl) was considered better because of biodegradation of natural tannins present in peels. The similar spectacle happened with orange pomace that is agitated by *P. variotii* (Madeira *et al.,* 2012). In other study, medium having tannic acid 1%, sodium nitrate 3.0%, potassium chloride 0.05%, magnesium sulphate 0.05% and K_2HPO_4 0.1% was used for *K. pneumonia* growth with enzyme value 3.9 U/ml (Sivashanmugam and Jayaraman, 2011). Tannase production (0.6 U/ml) on MSM medium by *Enterobacter cloacae* was also reported (Beniwal *et al.,* 2010). Tannase production (0.4 U/ml) in MSM medium using *S. ficaria* was obtained (Belur *et al.,* 2010). The medium consists of KH_2PO_4, $AgNO_3$, and $MnSO_4$ activated the tannase production while $Fe_2(SO_4)_3$ and $FeCl_3$ had negative effect (Cavalcanti *et al.,* 2018). In another report, $MgSO_4$ and K_2HPO_4 enhanced the tannase production (Wu *et al.,* 2018). The optimization factors for production of tannase revealed evidently the impact of such parameters on the enzyme yield along with their autonomous nature in impelling the of *K. oxytoca* ability to produce enzyme.

In present study, the highest tannase production (9.93 U/ml) by *K. oxytoca* were shown at pH 3. This result depicts that *K. oxytoca prefer* acidic environment. In another study, bacterial strains showed better. Similar findings for other member of *Klebsiella* was reported by other researcher (Kumara *et al.,* 2015). Optimal growth and tannase production by *K. oxytoca* is at 30ºC during the present study. At low and high temperature, tannase production was low. The similar results were described at 30ºC for *Enterobactor* species (Sonia *et al.,* 2017). Nearly comparable findings described 3.25-fold rise in tannase production by *K. pneumonia* KP715242 when medium was optimized at 34.97 ºC (Kumar *et al.,* 2015).

During present study, better results for tannase production (16.70 U/ml) were achieved by using 1% inoculum and high inoculum concentration reduced tannase production. An increase in cell number (inoculum size) raise the level of biomass but enzyme value decreases after certain limit because of increased competition for uptake of nutrients and nutrients depletion due to greater biomass that results in the reduction of their metabolic activity (Kashyap *et al.,* 2002). A balance must be kept retained among biomass proliferation and the substrate for maximum enzyme production (Pandey *et al.,* 2000). Contrary to this, the greater tannase activity for *Bacillus megaterium* was detected using 10% inoculum size (Tripathi *et al.,* 2016).

In present study, *K. oxytoca* showed 24 hours of optimal incubation period with enzyme value up to 16.76U/ml. In contrast, 92 hours optimal incubation time for *K. pneumoniae* was reported (Kumar *et al.,* 2015). For *K. pneumoniae* MTCC 7162, optimal time of incubation was 20-24 hours (Sivashanmugam and Jayaraman, 2011). Optimal enz-

yme production by *Bacillus sphaericus* was noted after 36 hours incubation (Raghuwanshi *et al.,* 2011). For *Aspergillus oryzae,* 96 hours incubation period is reported for maximum tannase production.

Tannase produced by *K. oxytoca* showed maximum activity at pH 5. However, in various studies, optimal pH ranged from 3–6 pH has been described for tannase activity (Arshad *et al.,* 2019, Farag *et al.,* 2018). For tannase, best pH for enzyme activity was 5.5 by *K. pneumonia* (Sivashanmugam and Jayaraman, 2011). For *K. pneumonia KP715242*, tannase activity was highest at pH 5.2. The tannase activity was low at lowest and highest pH. Mostly acidic pH favored greater activity while less in alkaline range (Raghuwanshi *et al.,* 2011, Ramirez-Coronel *et al.,* 2004, Kumar *et al.,* 1999). The influence of pH on tannase activity occurred because of amino acids protonation/deprotonation and their active sites; in addition, it might be ascribed by conformational alterations induced by amino acids ionization (Kumar *et al.,* 2015). Tannase from *B. cereus* gave better results at pH 4.5 (Mondal *et al.,* 2001). Optimal temperature for tannase activity (20.34U/ml) from *K. oxytoca* was observed 40^0C. Similarly, enzyme activity by *K. pnemuniae*, optimized temperature was found 40^0C (Sivashanmugam and Jayaraman, 2011).

In present study, maximum tannase activity (30.81U/ml) is described for 1.5% substrate concentration. The enzyme activity increased with increased in concentration of substrate. The activity reduced at lower concentration of substrate which might be because enzymes active sites were not properly interacted with substrate. Therefore, few enzyme substrate complex were designed that might result in low product yield. With increase in concentration of substrate, many substrate molecules fix at enzyme active site, producing various products. The enzyme activity raised by increasing concentration of substrate until a saturation point is achieved. The concentration of substrate having greater enzyme activity was the optimal substrate concentration (Battestin and Macedo, 2007). However, further increase in tannic acid concentrations led to reduction in tannase activity as greater concentrations of inducers may have lethal impact *i.e.,* they may yield toxins that might elucidate why low concentrations of tannic acid was effective at inducing tannase enzyme production (Madeira *et al.,* 2012). *K. oxytoca* showed 30 minutes of optimum incubation time and gave best results (30.60 U/ml) for this period.

Conclusion

The growth of *Klebsiella oxytoca* on musambi peels (agri-wastes) and tannase production revealed that this strain have potential to utilize natural tannin. This character depict that it is potential candidate to reduce natural tannin contents in plant for the preparation of low tannin based animals feed at industrial level. It is also valuable for treatment of industrial especially tannery effluents containing tannin contents. The tannase production of select strain under acidic environments showed its utilization in clarification of fruit juices.

REFERENCES

Arshad R., Mohyuddin A., Saeed S. and A. Al-Hassan, Optimized production of tannase and gallic acid from fruit seeds by solid state fermentation. Tropical J Pharma. Res. 18(5): 911-918 (2019).

Ayed, L. and M. Hamdi, Culture conditions of tannase production by *Lactobacillus plantarum*. Biotechnol. Lett. 24(21): 1763-1765 (2002).

Batra, A. and R.K.Saxena, Potential tannase producers from the genera *Aspergillus* and *Penicillium*. Process Biochem. 40(5): 1553-1557 (2005).

Battestin, V. and G.A. Macedo, Tannase production by *Paecilomyces variotii*. Biores Technol. 98(9): 1832-1837 (2007).

Belur, P.D., Mugeraya, G. and S. Subbalaxmi, Studies on the extracellular tannase from newly isolated *Bacillus thurangiences* BN2. In: *Chemical, Biological and Environmental Engineering* Pp. 379-384 (2010).

Beniwal, V., Chhokar, V., Singh, N. and J. Sharma, Optimization of process parameters for the production of tannase and gallic acid by *Enterobacter cloacae* MTCC 9125. J. American Sci. 6(8): 389-397 (2010).

Beniwal, V., Kumar, A., Goel, G. and V. Chhokar, A novel low molecular weight acidothermophilic tannase from *Enterobacter cloacae* MTCC 9125, Biocatal. Agric. Biotechnol. 2: 132–137 (2013).

Beniwal, V., Sharma, A., Marwah, S. and G. Goel, Use of chickpea (*Cicer arietinum* L.) milling agrowaste for the production of tannase using co-cultures of *Aspergillus awamori* MTCC 9299 and *Aspergillus heteromorphous* MTCC 8818, Ann. Microbiol. 65: 1277–1286 (2015) doi:http://dx.doi.org/10.1007/s13213-014-0965-1.

Cavalcanti, R.M.F., Jorge, J.A. and L.H.S. Guimarães, Characterization of *Aspergillus fumigatus* CAS-21 tannase with potential for propyl gallate synthesis and treatment of tannery effluent from leather industry. J. Biotech. 8(6): 270 (2018).

Dave, A., Modi, H.A. and N.Chavada, Study and isolation of tannase enzyme production bacteria from tea waste dump soil site. Int. J. Pharm. Appl. Sci. 2(1): 20-22 (2011).

Farag, A.M., Hassan, S.W., Asmaa, M. and K.M. Ghanem, Purification, Characterization and application of tannase enzyme isolated from marine *Aspergillus nomius* GWA5. J. Pure Appl. Microbiol. 12(4): 1939-1950 (2018).

Haslam, E. Natural Polyphenols (Vegetable Tannins) as Drugs: Possible mode of action. J. Nat. Prod. 59(2): 205–215 (1996).

Ilori, M.O., Adebusoye, S.A., Amund, O.O. and B.O. Oyetoran, A study of tannic acid degradation by soil bacteria. Pakistan J. Biol. Sci. 10(18): 3224-3227 (2007).

Ingole, S.P., Kakde, A.U. and R.C. Maggirwar, Biodegradation of tannery effluent by using tannery effluent isolate. Int. Multidiscipl. Res. J. 2(3): 43-44 (2012).

Kallel, F., Driss, D., Chaari, F., Zouari-Ellouzi, S., Chaabouni, M., Ghorbel, R. and S.E. Chaabouni, Statistical optimization of low-cost production of an acidic xylanase by *Bacillus mojavensis* UEB-FK: its potential applications. Biocatal. Agricul. Biotechnol. 5: 1-10 (2016).

Kashyap, P., Sabu, A., Pandey, A., Szakacs, G. C.R. and Soccol, Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. Process Biochem. 38(3): 307-312 (2002).

Kumar, M., Rana, S., Beniwal, V. and R.K. Salar, Optimization of tannase production by a novel *Klebsiella pneumoniae* KP715242 using central composite design. Biotechnol. Repor. **7**: 128-134 (2015).

Kumar, R.A., Gunasekaran, P. and M. Lakshmanan, Biodegradation of tannic acid by *Citrobacterfreundii* isolated from a tannery effluent. J. Basic Microbiol. 39(3): 161-168 (1999).

Lekha, P. and B. Lonsane, Production and application of tannin acyl hydrolase: state of the art. Adv. Appl. Microbiol. 44: 216-260 (1997).

Madeira, J.V., Macedo, J.A. and G.A. Macedo, A new process for simultaneous production of tannase and phytase by *Paecilomyces variotii* in solid-state fermentation of orange pomace. Biopro. Biosys. Engin. 35(3): 477-482 (2012).

Mahendran, B., N. Raman, and D.J.Kim, Purification and characterization of tannase from *Paecilomyces variotii*: hydrolysis of tannic acid using immobilized tannase. Appl. Microbiol. Biotechnol. 70(4): 444-450 (2006).

Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 31(3): 426-428 (1959).

Mohapatra, P.D., Maity, C., Rao, R.S., Pati, B.R. and K.C. Mondal, Tannase production by *Bacillus licheniformis* KBR6: Optimization of submerged culture conditions by Taguchi DOE methodology. Food Res. Int. 42(4):430-435 (2009).

Mondal, K.C., Banerjee, D., Banerjee, R. and B.R. Pati, Production and characterization of tannase from *Bacillus cereus* KBR9. J. Gen. Appl. Microbiol. 47(5): 263-267 (2001).

Pandey, A., Soccol, C.R., Nigam, P. and V.T. Soccol, Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. Bioresource Technol. 74(1): 69-80 (2000).

Raghuwanshi, S., K. Dutt., Gupta, P., Misra, S. and R.K. Saxena, *Bacillus sphaericus*: The highest bacterial tannase producer with potential for gallic acid synthesis. J. Biosci. Bioengin. 111 (6): 635-640 (2011).

Ramirez-Coronel, M.A., Marnet, N., Kolli, V.K., Roussos, S., Guyot, S.C. Augur, Characterization and estimation of proanthocyanidins and other phenolics in coffee pulp (*Coffea-arabica*) by thiolysis− high-performance liquid chromatography. J. Agricul. Food Chem. 52 (5): 1344-1349 (2004).

Selinger, L.B., Forsberg, C.W. and K.J. Cheng, The rumen: a unique source of enzymes for enhancing livestock production. Anaerobe 2 (5): 263-284 (1996).

Shakir, H.A., Enteric bacterial and heavy metals' load and health status of fishes from river Ravi, Pakistan. Ph.D. Thesis Department of Zoology, University of the Punjab, Lahore, Pakistan (2013).

Sivashanmugam, K. and G. Jayaraman, Production and partial purification of extracellular tannase by *Klebsiella pneumoniae* MTCC 7162 isolated from tannery effluent. African J. Biotechnol. 10(8): 1364-1374 (2011).

Sonia, M., Sharma, K.P. and P.J. Johna, Characterization of Pyrogallol Production from Gallic acid by *Enterobacter* spp. J. Microbiol. Biotechnol. Res. 2(2): 327-336 (2017).

Tripathi, A.D., Sharma, K. and B. Lakshmi, Study on tannase producing *Bacillus megaterium* isolated from tannery effluent. Int. J. Adv. Res. Biol. Sci. 3(7): 28-35 (2016).

Wilson, P.A., Rojan, P.J., Kumar, P. and T. Sabu, Tannin acyl hydrolase production by *Citrobacter sp.* isolated from tannin rich environment, using *Tamarindus indica* seed powder. J.

Appl. Sci. Environ. Manag. 13(4): 95-97 (2009).

Wu, C., Zhang, F., Li, L., Jiang, Z., Ni, H. and A. Xiao, Novel optimization strategy for tannase production through a modified solid-state fermentation system. Biotechnol. Biofuels 11(1): 92 (2018).