Chapter V

DISCUSSION
Tannases are used for various applications in different industries like food, feed, beverage, brewing, chemical, pharmaceutical and others (Lekha and Lonsane, 1997; Aguilar and Gutierrez-Sanchez, 2001; Boadi and Neufeld, 2001; Aguilar et al., 2007; Darah et al., 2011). However, in the beginning of the 19th century, the practical usage of this enzyme was limited and has not yet been explored to much extent most probably due to its limited substrate spectra and high cost of purification as these are secreted at very low titres by microbes. This thereby increases the cost of the product.

Realizing that tannases have immense applications in different industrial sectors. Hence, these are gaining great market potential. Therefore, it is important and necessary to study and optimize its production process. Microorganisms are becoming the favored choice for the production of industrial enzymes due to its diversity (Underkofler, 1976). Tannases can be obtained in short period of time by microbes through fermentation (Lekha and Lonsane, 1997; Kar et al., 1999; Aguilar et al., 2001a; Belmares et al., 2004; Batra and Saxena, 2005; Purohit et al., 2006; Battestin and Alves-Macedo, 2007). Microbial tannases are more stable than analogous proteins obtained from plant and animal sources (Bhat et al., 1998; Kannan Natarajan, 2009). Moreover, microbes are more amenable to genetic manipulation than animals and plants (Headon and Walsh, 1994; Walsh and Headon, 1994; Lekha and Lonsane, 1997). Amongst microbial tannases, widespread occurrence is found in fungi (Bradoo et al., 1997; Barthomeuf et al., 1994; Hadi et al., 1994; Bajpai and Patil, 1996; Bhat et al., 1998; Aguilar et al., 2007) whereas, it is less common in bacteria and yeasts.

Hence, with a view to obtain a robust tannase producing microorganism, in the present investigation, a series of experiments were planned and executed. Initially, screening of a tannase producing microorganism was carried out. This was followed by process optimization, scale up and its optimization upto a 300 L fermentor. Subsequently, purification, immobilization and characterization of the tannase produced was followed by detailed investigations on its industrial applications. The observations and results for all these experiments are presented in eight sections in Chapter IV: Observations and Results. Experimental details
Discussion

for screening of microorganisms for tannase production are presented in Section I. This is followed by investigations on its identification in Section II. Section III presents in detail all the observations and results obtained on optimized tannase production under both submerged (SmF) and solid state fermentation (SSF) conditions. The translation of any enzyme based process directly depends upon its scalability to pilot scale/size, therefore, in the present investigation, production of this tannase was scaled upto 300 L vessel size and optimized. Results of scale up studies are presented in Section IV. Section V elucidates the different strategies followed for purification of this tannase while the results of tannase immobilization by three strategies are presented in Section VI. The three forms of enzyme i.e. crude, purified and CLEA immobilized were evaluated for various biochemical properties like pH and temperature tolerance and stability, organic solvent stability, effect of metal ions on tannase activity, specificity studies, kinetic studies etc. results of all these experiments are presented in Section VII. The last but one of the most important section, Section VIII investigates in detail the potential of this tannase to carry out different industrially important reactions.

In this chapter, results obtained are discussed in the light of the current status of research on microbial tannases.

Section I: Isolation, screening and selection of microorganisms for tannase production

Isolation of microorganisms from the environment is the microbiologist’s first step in screening for natural products such as secondary metabolites and enzymes (Hunter- Cavaera and Belt, 1999). In this context, in the present investigation, microorganisms which include bacteria and fungi were isolated from nature on NA and PDA plates and subsequently screened on 1% tannic acid agar plates for tannase production. The similar has been reported by Lekha et al. (1993) and Mondal et al. (2000) where potential tannase producers were isolated from soil.

In the earlier reports different fungi, bacteria and yeasts were extensively screened and are reviewed by Lekha and Lonsane (1997) and Bhat et al., (1998). Amongst filamentous fungi many Aspergillus and Penicilli have shown good potential for tannase production (Nishira and Mugibayashi, 1960; Saxena et. al., 1995; Abdel-Nabey et al., 2011). Therefore, in the present investigation, through
quantitative screening, one hundred and fifty fungal isolates and one hundred and fifty bacterial isolates were point inoculated on tannic acid agar plates and were incubated at their respective temperatures i.e. 30/37°C for 24 h for bacteria and 48 h for fungi. Out of these, only 103 fungal isolates and only 5 bacterial isolates were found positive for tannase production.

Subsequently, quantitative estimation of tannase production from these selected fungal and bacterial isolates was carried out in Czapek Dox minimal medium supplemented with 1.0 % tannic acid (Saxena, 1976). Amongst these, on the basis of the tannase titres produced extracellularly, six fungi i.e. *Aspergillus terreus*, *A. flavus*, *Penicillium* sp. SS-1, *Penicillium* sp. (SR-75), *P. chrysogenum* and *P. crustosum* were selected with 4.17, 4.65, 4.96, 4.01, 4.44, 4.70 IU/ml of tannase titres produced respectively when evaluated at pH-5.0 at 40°C.

When these results were compared with earlier reports, it was found that similar results on tannase production were observed by Rajakumar and Nandy (1983); Bradoo et al. (1996); Saxena et al. (2004); Batra and Saxena (2005) while working with *Penicillium* sp. In our earlier laboratory studies, amongst seventy-five *Aspergilli* and *Penicilli* were screened, out of these fifty-two (31 Aspergilli and 21 Penicilli) showed hydrolytic zones on tannic acid agar plates. Amongst these *Aspergilli* produced higher tannase titres when compared with *Penicilli*. Ganga et al. (1978) also reported that *Aspergilli* are more capable of higher tannase production. However, in the present investigation *Aspergilli* and *Penicilli* produced more or less similar titers of tannase.

As the results of the present investigations has shown that 6 potent *Aspergilli* and *Penicilli* produce more or less similar titres of tannase. Hence, the final selection of the fungus was carried out on the basis of some of the important biochemical properties of tannase produced by the organism. Similar approach was also considered for selection of the potent organism by Batra (2004) and Gupta (2010). The evaluation of the tannase produced by the 6 potent fungi was investigated in terms of pH-temperature tolerance and stability. In addition organic solvent stability was also investigated.
Results showed that *Penicillium* sp. SS-1 have shown good biochemical properties amongst the six potent tannase producers. Hence this *Penicillium* sp. SS-1 was selected for the detailed investigation.

**Section II: Identification of selected microorganism/s**

*Penicillium* sp. SS-1 was selected as a potent tannase producing fungus in the present investigation. **Identification up to sp. level of the Penicillium sp. was carried out by 18S rRNA and 600 bp analysis at The Centre for Genomic Applications (TCGA), New Delhi.** The fungus exhibited 100% homology with *Penicillium charlesii* earlier known as *Penicillium waksmanii*. Although many *Penicillium* sp. have been studied and exploited to various extent for the production of many industrially important enzymes such as protease (Djamel et al., 2009; Vamshi et al., 2009), chitinase (Binod et al., 2005; Lee et al., 2009), glucoamylase (Nandi et al., 1989; Sun et al., 2007), lipase (David, et al., 1935; Gulomova et al., 1996), phytase (Zhao et al., 2010) and xylanase (Muthezhilan et al., 2007; Knob et al., 2008). However, *P. charlesii* has not yet been evaluated and exploited for any of the above industrial enzymes. Only in a preliminary screening work of Bradoo et al. (1996) and Batra and Saxena (2005) reported that *P. charlesii* is also a tannase producer. The biochemical characterization of this tannase produced by the fungus has proved to be better than the other selected tannase producers. Hence, the detailed investigation on this fungus was undertaken which includes maximum enzyme production; scale up, its purification and immobilization, characterization and industrial exploitation. The results obtained on these aspects are discussed in this section.

**Section III: Process optimization of tannase production**

The effect of different physiological and nutritional factors influencing tannase production from different fungi under either submerged (SmF) and solid state fermentation (SSF) or by both methods have been reported by several researchers (Pourrat et al., 1982; Deschamps et al., 1983; Barthomeuf et al., 1994; Hadi et al., 1994; Bradoo et al., 1997; Lekha and Lonsane, 1997; Seth and Chand, 2000; Banerjee et al., 2007; Abdel-Nabey et al., 2011; Darah et al., 2011).

In the present investigation, process optimization for maximum tannase production from this strain of *Penicillium charlesii* was carried out by both submerged and solid state fermentation strategies.
A. SUBMERGED FERMENTATION (SmF)

Submerged fermentation is a preferred method for production of most of the commercial enzymes like proteases, amylases, lipases and tannases principally because sterilization and process control are easier to handle in this approach (Lekha and Lonsane, 1997, Pandey et al. 1999).

In the present investigation under submerged conditions, this strain of *Penicillium charlesii* produces only 4.96 IU/ml of extracellular tannase under initial unoptimized conditions.

Similar findings have been reported by many researchers wherein, extracellular tannase production was carried out under submerged fermentation (Seiji et al., 1973; Okamura et al., 1988; Gupta et al., 1997; Aguilar et al., 2001; Batra and Saxena, 2005; Raghuwanshi et al., 2010).

The production of this tannase was now process optimized under submerged shake culture conditions. Different nutritional and physiological parameters were optimized for maximum tannase production using one variable at a time and statistical approaches (Hadi et al., 1994; Bradoo et al., 1997; Sabu et al., 2005).

To begin with, in the present investigation, process optimization was initiated with selection of the most suitable medium for tannase production.

In this context, eight most commonly reported tannase production media were evaluated. It was observed that medium M-1 containing 1.0% tannic acid, sodium nitrate, glucose, potassium dihydrogen phosphate, potassium chloride, magnesium sulfate, ferrous sulfate, zinc sulfate and copper nitrate proved to be the most suitable medium for tannase production from *Penicillium charlesii*. It is a modified Czapek-Dox's minimal medium.

Similarly, Haslam et al. (1961) and Bradoo et al. (1997) reported that Czapek Dox's minimal medium containing 2.0% tannic acid along with the composition as mentioned for medium M1 except glucose was optimal for tannase production from *A. niger* and *A. japonicas*, respectively. Darah et al. (2011) also reported that Czapek-Dox's medium containing 1% tannic acid supports tannase production from *A. niger* FETL FT3.
Depending on the strain and the culture conditions, the enzyme can be either constitutive or inducible showing different production patterns (Belmares et al., 2004). Mostly tannase is an inducible enzyme in nature and the major factor reported for the expression of tannase activity is tannic acid. Results of the investigation showed that this tannase from *P. charlesii* was not at all constitutive in nature. However, tannase is only produced when tannic acid is present in the production medium. On the contrary to our results, Bradoo et al. (1997) had reported that the enzyme produced from *A. japonicus* is constitutive in nature on simple and complex sugar substrates but its enzymatic activity gets doubled in presence of tannic acid as a sole carbon source.

After the confirmation of the inducible nature of tannase in this fungus the concentration of tannic acid was optimized for maximum tannase production using one variable at a time approach.

**One- variable-at-a-time approach**

**Substrate and its concentration:**

In submerged fermentation, the concentration of tannic acid was found to be a crucial factor influencing the levels of enzymes (Lekha and Lonsane, 1997; Mondal et al., 2001). In the present study, 2.0% tannic acid proved to be an optimal concentration for tannase production (9.15 IU/ml). Similarly, several researchers reported that 2.0% tannic acid is optimal for tannase production (Hadi et al., 1994, Lekha and Lonsane, 1997, Bradoo et al., 1997; Banerjee et al., 2001, Paranthaman et al., 2009, Abdel-Nabey et al., 2011). However, it was also observed that any further increase in tannic acid concentration (beyond 2.0%) leads to decline in tannase production. This was supported by Banerjee et al. (2007) who hypothesized that at higher concentrations of tannic acid, it forms complexes with membrane protein of the organism thereby both growth and enzyme production might got inhibited. In another report, Seth and Chand (2000) reported that an increase in tannin concentration resulted in stunted growth and also slowed down the fermentation process due to precipitation of the gallic acid which accumulates on the surface of the mycelial biomass.

Contrary to our results, it is clearly evident from certain reports that a higher concentration of tannic acid (beyond 2.0%) supported an optimal tannase production. Wherein, 3% tannic acid concentration supported maximum tannase
production (Duangban et al., 2007). While, Seth and Chand (2000) reported 3.5% to be the optimum for tannase production while working with A. awamori. In addition, Aguilar et al., (2001) reported maximum tannase production at 5.0% tannic acid in submerged fermentation while working with Aspergillus niger Aa-20.

On the other hand, at lower tannic acid concentration of 1.0% supported an optimal tannase production from A. niger FETL FT3 (Darah et al. 2011). While, Ayed and Hamdi (2002) reported that 1.5% (w/v) of tannic acid is optimal for tannase production.

pH

Initially, tannase production was evaluated in the medium having initial pH of 3.0-12.0. Results showed that though significant tannase production was noted at acidic pH range of 3.0 to 7.0, with an optimal at pH 5.0 (9.15 IU/ml). It was also observed that though acidic pH is more favorable for tannase production; however, this fungus could also produce certain amount of tannase at highly alkaline pH 10.0 (2.16 IU/ml) and 11.0 (0.42 IU/ml). Similarly, Hadi et al. (1994), Kar and Banerjee, (2000), Mondal et al. (2001) and Beniwal and Chhokar (2010) reported that pH 5.0 is optimal for tannase production. Further, in accordance with our results, the optimum initial pH reported for tannase production was in the acidic pH range of 4.5 to 6.5 (Rajakumar and Nandy, 1983; Barthomeuf et al., 1994; Bradoo et al., 1997; Ayed and Hamdi, 2002; Lokeshwari and Raju, 2007; Belur et al. 2010; Darah et al., 2011).

On the other hand, an optimal pH 7.0 was observed by Pourrat et al. (1982) by A. niger LCF 8. Above this pH 7.0, no study has been reported wherein optimal tannase production was observed.

Production of tannase was not attempted below pH 3.0 since it is reported that the enzyme becomes unstable as the protein structure of an enzyme is affected by the pH (Barthomeuf et al., 1994).

Temperature

Here, investigations on the next physiological parameter evaluated i.e. production temperature revealed optimal tannase production (9.18 IU/ml) at 30°C. A significant loss in tannase production was observed when incubation was carried out at temperatures higher than 35°C with minimum tannase
Discussion

titres of only 1.31 IU/ml obtained at 40°C. Since, the growth of this fungus ceases at temperature higher then the optimal temperature. Similar results were obtained by Kar and Banerjee (2000) & Papagianni (2004) where the phenomenon of denaturation and inactivation of enzyme was described.

In the support of our results, Bradoo et al. (1997); Sharma et al. (1999), Aguilar et al. (2001) and Abdel-Nabey et al. (2011) reported 30°C to be the optimal temperature for tannase production.

Contrary to our results, certain researchers have reported an optimal temperature of 25°C for maximum tannase production (Manjit et al., 2008), 33°C (Barthomeuf et al., 1994), 45°C (Lokeswari et al., 2010).

Inoculum density

Inoculum density is another important parameter for tannase production and the size of the inoculum has been reported to play a significant role in the production of metabolites (Sabu et al., 2006). Generally, it has been reported that use of lower inoculum density beyond optimal is not sufficient for initiating growth and thereby less enzyme synthesis occurs (Kashyap et al., 2002). Whereas, a higher inoculum density resulted in decline in the enzyme production due to depletion of nutrients along with an increase in biomass production (Sabu et al., 2005; 2006).

In the present study, an inoculum density of 5 x 10^7 spores/ 50 ml, resulted in significant tannase production of 11.12 IU/ml. However, further increase in the inoculum density beyond this optimal level leads to a decline in the tannase production.

In accordance with our results, Bradoo et al. (1997) and Saxena and Saxena (2004) also reported an inoculum level of 5X10^7 spores/ 50 ml of the production medium being optimal for tannase production from A. japonicus and P. variable, respectively. While, a spore density of 4X10^7 spores/ 40 ml of production medium was employed for A. niger by Sharma et al. (1999) and Aguilar et al. (2001) which is more or less same as that of present optimal concentration.

On the contrary, a higher spore concentration of about 6 x 10^6 spores/ml was reported optimal for maximum tannase production (Darah et al., 2011). In this context Kashyap et al. (2002), reported that an increase in the number of spores
(higher inoculum density), results in the higher biomass production along with an increase in enzyme synthesis.

**Agitation rate**

Agitation rate, is an indirect measure of dissolved oxygen concentration, is a critical factor influencing both the fungal mycelial biomass and tannase production in submerged fermentation.

Seth and Chand (2000) explained that at lower agitation rate there was an inadequate mixing of components present in the broth due to adequate growth and it also affects the enzyme synthesis by accumulation of gallic acid on the surface of the mycelium. While, at higher agitation rates, there was shearing of the mycelium. In the same manner, Barthomeuf *et al.* (1994) and Pourrat *et al.* (1985) found that excessive aeration favors oxidation of tannins and thus inhibits the biosynthesis of the enzyme whereas insufficient aeration impeded growth.

In the present investigation, a decrease in the agitation rate from 200 rpm to 150 rpm resulted in the optimal tannase production of 14.23 IU/ml from this strain of *P. charlesii*. The tannase production was drastically reduced under static conditions (3.16 IU/ml). Further, higher agitation rate of 200 rpm and above reduced tannase titres. Our results are in accordance with the reports of Saxena and Saxena (2004) who also reported 150 rpm as an optimal agitation rate for tannase production from *P. variable*. Similarly, Abdel-Nabey *et al.* (2011) also reported an optimal tannase production from *Aspergillus* sp. at a similar agitation rate of 150 rpm.

Contrary to our results, Yamada *et al.* (1968), Sharma *et al.* (1999) and Aoki *et al.*, (1976a) reported 120 rpm to be the optimal agitation rate while working with *A. flavus*, *A. niger* van tieghem and *Candida* sp., respectively. Further, Selwal *et al.* (2010) and Darah *et al.* (2011) reported 200 rpm as the optimal for tannase production as it could achieve its maximal tannase activity along with high fungal biomass. A higher agitation rate of 220 rpm for *A. niger* Aa-20 (Aguilar *et al.*, 2001a) and 250 rpm for *A. niger* GH1 (Cruz-Hernandez *et al.*, 2006) was found to be optimal for tannase production.

Conflicting results were obtained by Bradoo *et al.* (1997) wherein they reported that there was no difference in tannase production or biomass formation while working with *A. japonicus* under static or shake conditions.
Carbon Source

Carbon is a major component of the cell and the rate at which a carbon source is consumed influences the formation of biomass and/or production of metabolites (Stanbury et al., 1997). Selwal et al. (2010) reported that an additional carbon source promotes initial growth of the biomass since, tannic acid is harder to metabolize than simple sugars. The study on different carbon sources showed that all carbon sources supported tannase production to varied extents. It was also observed that the medium devoid of any carbon source but contains tannic acid could also result in tannase production though to a lesser extent (6.26 IU/ml). This is also supported by Bradoo et al. (1997) where they reported that, microbial degradation of tannic acid, releases glucose and this sugar is then efficiently being utilized as a sole carbon source for the growth and metabolism of the organism (Bradoo et al., 1997).

In our study, the optimal tannase production (21.39 IU/ml) was achieved in a medium containing 2.0% tannic acid along with 0.8% sucrose. On the other hand, minimal tannase production was observed in the production medium supplemented with mannose (8.43 IU/ml) and rhamnose (8.19 IU/ml). Similar to our results, Fumihiko and Kiyoshi (1975), Ganga et al. (1977) and Suseela et al. (1978) found better growth and tannase production on sucrose in comparison to other carbon sources. In accordance with our results, Ganga et al. (1977) reported restricted tannase activity in presence of mannose in the medium. This may be due to the fact that any disaccharide or polysaccharide that releases glucose on degradation proves to be a better carbon source for tannase production (Bradoo et al., 1997).

On the other hand, glucose has been used by several researchers as a carbon supplement in the medium at varied concentrations for enhanced tannase production (Ayed and Hamdi, 2002; Duangban et al., 2007; Goncalves et al., 2011). Further, Vermierre and Vandammme (1988) studied the effect of carbon sources at 3.0% concentration on tannase production from A. niger and found that glucose stimulates tannase production, whereas glycerol showed a negative effect.

Nitrogen Source

In the present investigation, sodium nitrate at a concentration of 0.8% supported maximum tannase production (24.38 IU/ml). Further increase in its
concentration leads to a decline in tannase production. However, a lower tannase yield was observed when corn steep liquor (CSL), casein hydrolysate and arginine were used as nitrogen source.

Similarly, several researchers also reported sodium nitrate as better nitrogen source for fungal tannase production (Hadi et al., 1994; Bradoo et al., 1996; Batra and Saxena 2005; Paranthaman et al. 2009) as compared to other salts and organic nitrogen sources.

Kumar et al. (2007) and Abdel-Nabey et al. (2011) proposed that inorganic nitrogen sources are better as compared to organic nitrogen sources. Since, in presence of complex nitrogen sources or organic nitrogen source/s there is a formation of tannin-proteins complexes, which deprives the microorganism from both the inducer and the nitrogen source and thereby reduces the growth and tannase production. In accordance with this report, Bajpai and Patil (1997) while working with A. niger, A. fischerii, Fusarium solanii and Trichoderma viride also reported ammonium nitrate and potassium nitrate as nitrogen source for tannase production. In a similar trend of results, Aguilar et al. (2001) found ammonium sulfate as the best source for tannase production by A. niger Aa-20. Similarly, Duangban et al. (2007) and Manjit et al. (2008) also reported ammonium sulfate as an selected nitrogen source for tannase production.

On the contrary, Barthomeuf et al. (1994) reported an optimal production from A. niger in presence of urea an organic nitrogen source. Huang et al. (2005) also reported that organic nitrogen source (peptone) is more suitable for tannase production by Aspergillus SHL6 as compared to inorganic nitrogen source.

Divalent cations

Metal ions play a significant role in the production and influence tannase titres. In the present investigation, amongst the different metal ions tested, Mg$^{2+}$ (21.29 IU/ml) and K$^+$ ions (20.73 IU/ml) have a vital role in tannase production. A low tannase yield (14.32 IU/ml) was obtained in medium containing no MgSO$_4$ and KH$_2$PO$_4$. Similarly, Hg$^{2+}$, Ag$^+$, Pb$^{2+}$ and Zn$^{2+}$ were inhibitory for tannase production. Concentration of Mg$^{2+}$ and K$^+$ ions were also studied and it was observed that an increase in MgSO$_4$ from 0.05% to 0.08% along with 0.15% KH$_2$PO$_4$ (29.21 IU/ml) supported tannase production.
Our results are in agreement with the report of Suseela et al. (1985) wherein 0.1% Mg$^{2+}$ ion showed improvement in tannase production by A. flavus and A. oryzae. In the present study, it was observed that trace elements [Iron (Fe$^{2+}$), copper (Cu$^{2+}$) and zinc (Zn$^{2+}$)] in combination present in the production medium optimized so far resulted in 29.21 IU/ml of tannase from P. charlesii. Similarly, Lippitsch (1961) reported that traces of iron, zinc and copper have proved to be essential for tannase production by A. niger. On the other hand, Nishira (1961) reported that iron had no influence on tannase production in case of Penicillium sp.

**Surfactants**

Surfactants and detergents are known to solubilise the membrane proteins that lead to an increase in cell membrane permeability, thereby enhancing the secretion of biomolecules (Ne’eman et al., 1971). Besides this, they may also stimulate tannase production (Goncalves et al., 2011). A wide variety of surfactants like Tweens, Triton, saponins, SDS, have been studied by different investigators for tannase production (Sharma et al., 2008; Naidu et al., 2008; Goncalves et al., 2011).

In this context, our results showed Tween-80 (0.3%) to be the best surfactant for maximum tannase production (35.65 IU/ml). However, a significant loss of enzyme activity was observed in presence of Tween 20, Triton X-100 and saponin.

Similar has been reported by Kar et al. (2003) wherein a decrease in enzymatic activity was observed in presence of Triton X-100. In another report, significant loss of activity in presence of Tween 20 was also reported for tannase produced by Verticillium sp. (Kasieczka-Burnecka et al., 2007) and Paecilomyces variotii (Battestin and Macedo, 2007). Contrary to our present results, Sharma et al. (2008) reported that SDS, tween 60 and tween 80 completely inactivates the enzyme whereas, sodium choleate and sodium taurocholate enhanced tannase production. Similarly, Naidu et al. (2008) reported that in Aspergillus foetidus tannase was completely inhibited by Tween 80 and SDS.

After optimization of both physiological and nutritional factors, the incubation period was now finally optimized. Here, maximum tannase production (35.65 IU/ml) was obtained in 60 h by *Penicillium charlessii*. 
Thereafter, a distinct decline in the tannase production was observed. This yield of tannase is 7.18 fold higher as against the initial unoptimized medium wherein 4.96 IU/ml of tannase was produced.

Incubation period depends on the initial tannic acid concentration and the microorganism used (Lekha and Lonsane, 1997). With an increase in the incubation period there is liberation of higher content of gallic acid which has a catabolite repression on tannase and results in decline in the tannase production (Bradoo et al., 1997; Aguilar et al., 2001; Abdel –Nabey et al., 2011).

A similar incubation period of 60 h has also been reported for tannase production from A. awamori (Seth and Chand, 2000). On the contrary to our results, a lesser incubation period of 36 h was observed as optimal for tannase production in case of A. aculeate DBF9 (Lekha and Lonsane, 1994) and A. niger (Lokeshwari and Raju, 2007). While, Abdel-Nabey et al. (2011) studied two species of Aspergillus and reported that maximum tannase production was in 48 and 72 h of incubation for A. japonicus and A. oryzae respectively.

Response Surface Methodology

Generally, “one-variable-at-a-time” approach is used for optimization of physiological and nutritional factors for maximum production of any bioactive molecule. However, this procedure is time consuming and also has a limitation of non interaction of the most influential factors affecting production. In this respect, statistical methods are of use as they help in evaluating the all possible combinations of most influential factors as obtained by one-variable-at-a-time method. Factorial design makes it possible to take advantage of practical knowledge during the final Response Surface Methodology (RSM) analysis (Kalil et al., 2000). Some of the frequently used statistical designs for maximum production of a desired biomolecule are Plackett-Burman design (Viswanathan and Surlikar, 2001), the Box-Behnken design (Viswanathan and Surlikar, 2001) and the Central Composite Design falling under Response Surface Methodology (Vohra and Satyanarayana, 2002). RSM is widely used to evaluate and understand the interactions between different physiological and nutritional factors (Kaushik et al., 2006; Raghuwanshi et al., 2011). It is a convenient method for developing optimum processes with precise conditions and also minimizes the cost of production of
many biomolecules with an efficient screening of process parameters (Vohra and Satyanarayana, 2002; Kaushik et al., 2006).

The results of the present investigation showed that Face Centered Central Composite Design (FCCCD) falling under RSM a yield of 58.14 IU/ml of tannase was obtained in 60 h in shake flasks under the optimum conditions of 2.0% tannic acid, 1.0% sucrose, 1.0% NaNO₃, 0.08% MgSO₄, Tween 80 0.3%, pH 5 inoculated with 5X10⁷ spores /50 ml and incubated at 30±1°C at 150 rpm. This has resulted in 11.72 fold higher tannase titres as against the yield obtained under initial un-optimized conditions (4.96 IU/ml) of tannase production.

However, there are very few reports of using response surface methodology (RSM) for tannase production. In a similar report the production of tannase by A. niger PKL104 was optimized by response surface methodology (Lekha et al., 1994), which has resulted in 1.34 fold increase in enzyme production. Similarly, the production of tannase and accumulation of gallic acid by A. awamori was optimized in a laboratory bioreactor using response surface methodology (RSM) and Box- Behnken factorial design by Seth and Chand (2000). Wherein, 771 IU of intracellular tannase per gram dry cell weight and 19 g/l of gallic acid was reported after 60 h of incubation.

In a similar trend of results Saxena and Saxena (2004) used D-optimal design in order to evaluate the effect of variables and found significant variables. These variables were then subjected to CCD. This optimization leads to 33 U/ml of tannase i.e. 2.4 fold increase in enzyme production from P. variable. Similarly Sharma et al. (2007) & Beniwal and Chhokar (2010) reported optimized tannase production through statistical approach while working with A. niger and A. awamori MTCC 9299 respectively.

### B. SOLID STATE FERMENTATION

Solid state fermentation is defined as a fermentation process occurring in absence of free flowing water, employing either a natural support or an inert support as a solid material (Van de Lagemaat and Pyle, 2001; Pandey, 2003; Couto and Sanromam, 2006). In most of the reports, fungi have been considered to be most adapted to SSF (Sabu et al., 2005; Battestin and Macedo, 2007; Paranthaman et al., 2008, 2009; Madeira et al., 2011). Since their hyphae can grow
on particle surfaces and penetrate into the interparticle spaces and thereby colonizes on solid substrate (Santos et al., 2003). However, a few studies have also reported satisfactory results in terms of obtaining tannase by SSF using bacterial cultures (Kashyap et al., 2003; Sabu et al., 2006; Prakasham et al., 2006).

The use of SSF presents advantages such as lower power requirements, smaller reactor volume and high productivity due to high cell mass production within short period of time (Bertolin et al., 2003), low capital investment, low waste water output, higher concentrations of metabolites obtained and low downstream processing costs (Kumaran et al., 1997). It also showed similarity with natural habitat of filamentous fungi permits use of wild type microorganisms which showed better performance as compared to genetically modified strains.

In the present investigation, therefore it was considered worthwhile to evaluate tannase production from this strain of *P. charlesii* under SSF using wheat bran as solid substrate. Initially experiments revealed that this strain of *Penicillium charlesii* was completely amenable to solid state fermentation. When grown under unoptimized conditions, this fungus produced 35.49 IU/gds of tannase in 72 h in wheat bran (10 g) supplemented with 10% (w/w) tannic acid, 20.0 ml of distilled water (pH adjusted to 5.0 using 0.05 M citrate phosphate buffer) to achieve a water ratio of 1:2, inoculated with 1x10^6 spores/gds and incubated at 30°C under static conditions with intermittent churning.

**Optimization of tannase production under SSF:**

**a) One variable at a time approach**

All the solid substrates used in SSF have a common feature, *i.e.* their basic macromolecular structure comprises of starch, cellulose, lignocellulloses, pectin or other polysaccharides. Mostly, substrates for SSF are heterogeneous products from agriculture or by-products of agro industries. This basic macromolecular structure confers the properties of a solid to the substrate. In certain cases, this solid serves either 1) as support and as nutrient source or 2) as an inert support.

In first case, these substrates are heterogeneous water insoluble materials from agriculture or byproducts from food industry which have an amylaceous or lignocellulosic nature such as grains and grain by-products, cassava, potato,
Discussion

beans, tamarind seed powder, palm kernel cake, coffee husk and sugar beet pulp (Perez Guerra et al., 2003; Sabu et al., 2005; Aryuman and Hanmounjai, 2006). In the second case, sugarcane bagasse, hemp, resins, inert fibers, polyurethane foam, vermiculite is impregnated with liquid medium which contains all the nutrients such as sugars, lipids, organic acids etc. (Lekha and Lonsane, 1994; Romero-Gómez et al., 2000; Díaz-Godínez et al., 2001; Ramírez-Coronel et al., 2003; Perez-Guerra et al., 2003; Duangban et al., 2007).

Subsequently, in the present investigation on evaluating different substrates for tannase production, results showed that Syzygium cumini (Jamun) leaves was the best substrate which resulted in 74.50 IU/gds of tannase activity at 30 °C in 72 h. followed by Phyllanthus emblica (Amla) leaves which supported 72.25 IU/gds of tannase activity as compared to inert supports i.e. wheat bran, wheat straw, rice bran, corncob (each were supplemented with 10.0% tannic acid) as well as the other support enriched with natural nutrient source (Acacia arabica (Kikar/Babul) bark; Sorghum bicolor (Jowar) husk; Zizyphus mauritiana (Ber) leaves). There was 2.09 fold increase in tannase activity when wheat bran (control) (35.49 IU/gds) was replaced with Syzygium cumini (Jamun) leaves.

However, in an another set of experiment, jamun leaves was supplemented with 5% tannic acid which could support 81.57 IU/gds in 72 h.

Similar to our results, Kumar et al. (2007) examined different solid substrates and observed that jamun leaves proved to be the best substrate for tannase production from A. ruber at 30°C in 96 h of incubation producing 69 U/g. While, Kar and Banerjee (2000) reported 23 U/ml of tannase from R. oryzae using 10% (w/v) of Caesalpinia digyna seed cover powder. Sabu et al. (2005 b) reported 13.03 U/g and 6.44 U/g of dry substrate using palm kernel cake and tamarind seed powder as substrate respectively.

Certain researchers have reported the use of inert support i.e. wheat bran (WB) containing 4.0 % tannic acid as inducer which resulted in a maximum enzyme production of 1367 U/gd mouldy bran (Lekha et al., 1994). In another report, wheat bran (10 g) soaked in 2.5 % tannic acid using Rhizopus oryzae was used by Chatterjee et al. (1996). Gustavo et al. (2001) reported higher tannase titers of 67.5 U/gds using wheat bran enriched with 0.8% tannic acid as substrate. While,
Battestin and Macedo (2007) reported 8.6 fold increase in tannase production from *P. variotii* using **coffee husk and wheat straw** (50:50 w/w) as substrate. Madeira *et al.* (2011) used **castor bean residue** as support for tannase production while working with *Paecilomyces variotii* which resulted in 2600 U/g in 48 h.

In literature, use of different moistening agents for providing moisture to the solid substrate is reported. The moistening agents not only provide moisture to the microorganism but also provide additional supplementary nutrients in the easily accessible form (Pandey *et al.*, 2000; Couto and Sanroman, 2006; Kumar *et al.*, 2007).

In the present study, when different moistening agents were evaluated, it was observed that maximum tannase production (95.82 IU/gds) was obtained in presence of salt solution -I containing 0.6% NaNO₃, 1.52 g/l KH₂PO₄, 0.52 g/l KCl, 0.80 g/l MgSO₄.7H₂O, FeSO₄.7H₂O (trace), ZnSO₄.7H₂O (trace), Cu(NO₃)₂.3H₂O (trace) and 1.0% sucrose as against 81.54 IU/gds in distilled water. This is 1.17 fold increase as against control.

Similar to our results, a salt solution containing (% w/v) NH₄NO₃ (0.5), MgSO₄.7H₂O (0.1) and NaCl (0.1) having a pH 5.0 was used as moistening agent for tannase production from *Lactobacillus sp.* ASR-S1 (Sabu *et al.*, 2006). Similarly, Battestin and Macedo (2007) have reported the use of salt solution containing (g/l): KH₂PO₄ 1.0, NH₄NO₃ 2.0, MgSO₄.7H₂O 0.2, CaCl₂.2H₂O 0.02, MnCl₂.4H₂O 0.004, Na₂MoO₄.2H₂O 0.002, FeSO₄.7H₂O 0.0025 and supplemented with 3.0% tannic acid in 10 g of wheat bran for tannase production from *Paecilomyces variotii*.

On the contrary, Kumar *et al.* (2007) reported that tap water was the best moistening agent when substrate to water ratio was 1:2 (w/v). While, sterilized water has been preferred for tannase production from *A. aculeatus* by Banerjee *et al.* (2007).

In the present investigation, pH of the moistening agent was examined in the pH range from 3.0- 8.0 and it was observed that maximum tannase was obtained at pH 6.0 (100.62 IU/gds) as against 95.80 IU/gds at pH 5.0 which served as control.

Contrary to our results, a lower pH was reported by several researchers for optimal tannase production. Kumar *et al.* (2007) reported that tap water was the best moistening agent with pH 5.5 in a ratio of 1:2 (w/v) with substrate. However,
further increase in the pH of the moistening agent, enzyme production decreased since tannases are acidic proteins with optima around 5.5. Similar has also been reported by Aguilar et al. (2001), Sabu et al. (2005a) and Paranthaman et al. (2008) for tannase production from A. niger Aa-20, A. niger ATCC 16620 and A. oryzae respectively. However, a higher pH of 6.5 was optimal for tannase production from A. niger PKL104 (Lekha et al., 1994).

Moisture ratio plays an important and critical role in solid-state fermentation. Several reports discussed the moisture content and water activity (aw), for both SSF and submerged culture, as critical variables that limit microbial growth, metabolite production and product efficacy (Kumar et al., 2007). Filamentous fungi are known to grow at water deficient substrates like barks of trees, dry leaves etc. (Holker et al., 2004). Since, higher enzyme production was observed at lower moisture ratio of 1:1 or 1:2 might be due to lower water activity needs of fungi. (Kumar et al., 2007).

In the present investigation, variation in initial moisture content of substrate showed that the enzyme synthesis was related to the availability of moisture. The optimal moisture content for tannase production is in the ratio of 1:3 [substrate: salt soln. I (pH 6.0), w/v] which resulted in 129.93 IU/gds. At higher moisture ratio of 1:4, the tannase titres declined to 60.29 IU/gds. It was also observed that high or low moisture ratio as against control leads to decline in tannase production. This may probably be due to the low initial moisture level of the substrate which thereby hinders mass transfer and may cause loss of functional properties of enzymes necessary for cell metabolism. On the other hand, at higher moisture ratio, there might be poor oxygen supply thereby resulting in lesser biomass and enzyme production. At the lowest and the highest water content the decomposition rate of the total organic matter was also found to decrease and this in turn affected the enzyme production (Pandey, 1994). Kumar et al. (2007) reported an optimal substrate to water ratio of 1:2 w/v.

The optimal moisture for tannase production was determined as 60% (Battestin and Macedo, 2007), 62% (Lekha et al., 1994) and 65% (Aguilar et al., 2001; Sabu et al., 2005). Maximal tannase production was obtained from Rhizopus oryzae with wheat bran as solid support and a humidity level of 72% (Chatterjee et al., 1996). Similarly, a higher moisture content of 80% was optimal for tannase production from A. aculeatus DBF9 (Banerjee et al., 2007). A moisture
content of 93% was optimal for tannase production from *Rhizopus oryzae* (Kar *et al.*, 1999).

**Water activity** \((a_w)\) is a measure of the energy status of the water in a system (Grajek and Gervais, 1987). It is defined as the vapor pressure of water divided by that of pure water at the same temperature. Therefore, pure distilled water has a water activity of one. In solid-state fermentation (SSF), it plays a major and important role as it indicates the level of accessible moisture for the microorganism (Pandey, 2004). In the present study, the water activity levels were varied in the range from 0.95 to 0.75 using glycerol. It was observed that \(144.42\) IU/gds of tannase were produced in \(72\) h on lowering the water activity to 0.92. This tannase yield is **11.37% higher** as compared to \(128.53\) IU/gds obtained with 0.95 \(a_w\) (control). Similarly, Troller and Stinson, (1978) reported that low \(a_w\) support extra cellular enzyme production. Pectate lyase activity from *Erwinia chrysanthemi* increased when \(a_w\) was lowered from 0.998 to 0.990 (Mildenhall *et al.*, 1981).

**Inoculum density** plays an important role in tannase production under SSF (Sabu *et al.*, 2006). Tannase titres increased to \(163.13\) IU/gds when a higher concentration of \(1\times10^7\) spores/gds was used for tannase production by this strain of *P. charlesii* as against \(1\times10^6\) spores/gds (144.42 IU/gds). This is 1.129 fold higher in tannase production as against control. Aguilar *et al.* (2001) reported an inoculum density of \(2\times10^7\) spores/ml for inoculating 10 g of dry inert support PUF for tannase production from *A. niger* Aa-20.

In SSF, tannase production depends on the availability of both carbon and nitrogen sources in the medium. Both have a regulatory effect on enzyme synthesis (Patel *et al.*, 2005). **On evaluation of different carbon sources (additives) to the moistening agent showed that sucrose (1.0%) which also serve as control could support tannase production titres (163.21 IU/gds) from this strain of *P. charlesii* in the present investigation.** Similarly, Lagemaat and Pyle (2005) reported that the presence of simple sugars *i.e.* glucose in the medium is utilized rapidly and this thereby leads to the partial induction of tannase from *P. glabrum*. Sabu *et al.* (2006) while working with tamarind seed powder as substrate (tannin) reported an enhancement in tannase production from *Lactobacillus* sp. ASR-S1 when maltose or sucrose was used as an external carbon source in the production medium. While, glycerol followed by maltose at 1.0% (w/v) was found to be optimal for tannase production from *A. niger* ATCC 16620 using the same substrate.
Bradoo et al. (1997) reported that microbial degradation of tannic acid releases glucose and this sugar is readily utilized as the sole carbon source for growth and metabolism of the microorganism. However, in the present study, the use of sucrose as an external carbon source promotes the initial growth of biomass, as the initial presence of tannin (in the support) or external tannic acid in the medium is hard to metabolize as compared to simple sugars. Similar has been reported by Selwal et al. (2010).

Contrary to our results, several researchers have reported that supplementation of carbon source in the optimized medium have no effect (Lekha and Lonsane, 1997; Battestin and Macedo, 2007) or resulted in decline in tannase production (Mudgett, 1986; Aguilar et al., 2001; Kumar et al., 2007).

In microorganisms, nitrogen is metabolized to produce primarily amino acids, nucleic acids, proteins and cell wall components. In the present investigation, sodium nitrate was the selected nitrogen source which could support tannase production to 163.21 IU/gds of tannase titres at 0.6% concentration. It is the same nitrogen source which also supported maximum production of tannase in SmF. All the other nitrogen sources evaluated, supported lower tannase production with minimum tannase yield of 86.43 IU/gds obtained with arginine. On evaluating the best concentration which supports maximum tannase yield was 0.8% (w/v) which resulted in 182.63 IU/gds as against 0.6% (w/v).

Sabu et al. (2006) reported potassium nitrate at 1% concentration as the most effective nitrogen source for tannase production from A. niger ATCC 16620. While, Battestin and Macedo (2007) reported ammonium nitrate as the most suitable nitrogen source at 1.2% concentration. The presence of additional nitrogen source along with the nitrogenous compounds present in the substrate promoted enhanced growth and consequent enzyme production (Chandrasekaran et al., 1991).

In SSF, addition of surfactants (i.e. Tween-20, Tween-40, Tween-80, Triton-X100 and Saponin) to the production medium has often been shown to enhance extracellular secretion of proteins/enzymes. This phenomenon has been attributed to changes in the permeability of the cell or the cell bound tannase (Vijayabaskar et al., 2012). In the present investigation, supplementing the moistening agent with Tween-80 at 0.1% v/v or without any surfactant showed
same tannase titres to 182.63 IU/gds. However, Tween-80 supported maximum production of tannase in SmF, failed to support tannase production in SSF in the present investigation. Therefore, surfactant was not added in the final optimized medium.

Supplementation of surfactants (Tween 80) has resulted in an enhancement of tannase titres as reported by Sabu et al. (2005). A spore suspension of *P. glabrum* was prepared in 10 ml sterile 0.2% tween-80 after five days of incubation at 30°C and was used as inoculum for tannase production (Van de Lagemaat and Pyle, 2005).

Finally, after optimizing various parameters tannase production from this strain of *Penicillium charlesii* under SSF by one variable at a time approach resulted in **182.63 IU/gds** of tannase i.e. **5.16 fold** higher than the tannase units (35.4 IU/gds) as obtained under initial unoptimized conditions.

II) **Response Surface Methodology (RSM)**

The RSM gave the insight on the interactions between factors and significant improvements in enzyme production. Hence, it forms a vital basis for optimizing a process at every stage from laboratory to pilot scale until ready for commercial applications (Lekha et al., 1994). In SSF approach, very few workers have reported using RSM for optimizing the medium variables (Lekha et al., 1994; Kar et al., 2002; Battestin and Macedo, 2007), in spite of the resurgence of interest throughout the world (Stein Kraus, 1984).

In the present study under SSF, after optimization by one variable at a time method, the significant factors obtained were (i) tannic acid, (ii) moisture ratio, (iii) sodium nitrate and (iv) inoculum density. When RSM using the FCCCD model was used, the tannase titres increased to 324.37 IU/gds. This was **9.16 fold** higher as compared to the initial unoptimized yield. The tannase yield increased with lower moisture ratio (1:2.5), increase in sodium nitrate concentration (1.0%) and tannic acid concentration (5.0%). However, any change in inoculum density (1x10⁷ spores/gds) did not show any effect on tannase titers.

Lekha et al. (1994) reported enhancement in tannase production from *A. niger* PKL104 while using response surface methodology (RSM) under solid state fermentation. The statistically significant variables along with the optimized
conditions obtained were: inoculum level (5%), initial pH (6.5), fermentation temperature (28°C), initial moisture (62%) and fermentation time (3 days). This resulted in 1.34 fold more enzyme production then that obtained before optimization. Battestin and Macedo (2007) in a similar work using response surface methodology for tannase production by *Paecilomyces variotii* reported that under optimized conditions of 12.0% tannic acid and % residue (coffee: wheat straw) of 50:50 w/w, the medium was supplemented with 1.2% of ammonia nitrate (external nitrogen source). The tannase production was monitored after five days at 32°C. Results showed 8.6 fold increase in tannase activity as against initial unoptimized conditions.

**Section IV: Scale up studies under submerged fermentation**

In the present investigation, results have shown that 58.09 IU/ml of tannase was produced in 1 L of optimized production medium contained in 5.0 L of conical flask. This result indicated the possible scalability of the system. In order to evaluate this hypothesis, production was now attempted in a 30 L fermentor with 20 L working volume of optimized production medium obtained after RSM. Initial experiments resulted in production of only 51.72 IU/ml of tannase in 60 h under uncontrolled conditions of dissolved oxygen and agitation i.e. 11% lesser as compared to shake flask studies.

Thus, the tannase production was was further optimized in the bioreactor/ fermentor wherein, dissolved oxygen (DO %), agitation (rpm) and air flow (vvm) were optimized this has resulted in the production of 64.32 IU/ml of tannase in 54 h with 5x10^7 spores/ 50 ml of medium. The major controlling factors obtained were 200 rpm, 20% D.O. saturation and 0.5 vvm airflow. Subsequently, the production was further scaled up in a 300 L fermentor (working volume of 200 L) wherein, the conditions which were optimized in a 30 L fermentor were identically translated for this fermentor.

Observations clearly reveal that the tannase yield (64.19 IU/ml) in 54 h was produced by this strain of *P. charlesii* being similar as compared to previous yield as reported in 30 L fermentor. Furthermore there was no need to optimize physiological parameters for 300L fermentor. A fed of tannic acid (1.0%) to the optimized medium after 36h resulted in 68.48 IU/ml of tannase.
While, a fed of (0.5%) NaNO₃ and (1%) tannic acid had no further increase in tannase yield.

In most of the reports, the use of conical flasks was studied in order to carry out submerged fermentation. Very few laboratory studies have been carried out in fermentors for tannase production (Pourrat et al., 1982; Deschamps et al., 1983; Seth and Chand, 2000; Banerjee et al., 2007).

In this respect, production of tannase from *A. niger* (Pourrat et al., 1982; Vermierre and Vandamme, 1988) in a 20 L capacity fermentor, which was properly aerated at a constant airflow rate (0.4 vvm), showed progressive decrease of dissolved oxygen (from 100 to 0% saturation) after 15 h of fermentation. The tannase activity increased during exponential phase of growth to reach a maximum concomitant with an increase in pH. The exponential phase has to be limited by lowering the dissolved oxygen level (D.O. = 0%) to favor enzyme production. The yield of mycelium and specific enzyme activity was best at a constant airflow rate of 0.4 vvm than at a constant dissolved oxygen saturation of 30% (Pourrat et al., 1982). Pourrat et al. (1987) suggested that a critical level must be maintained for aeration as high oxygenation causes oxidation of tannins and catabolism of gallic acid. Tannase production from *A. niger* was carried out in a 6.0 L fermentor, where the initial stirring rate was maintained at 300 rpm and then increased to 450 rpm after 24 h, to offset an increase in viscosity of the medium resulting from mycelial growth. The dissolved oxygen level was regulated at 30-40% by means of Ag-Pb electrode (Barthomeuf et al., 1994). They further reported that under favorable conditions, the fungal mycelia simultaneously synthesized antioxidant which presents tannin oxidation. Seth and Chand (2000) reported tannase production in 3.0 L mechanically agitated bioreactor with 2 L of working volume. The parameters studied were agitation rate (300-400 rpm), air flow rate (0.7 vvm), pH (4.0-6.0) and substrate concentration (25.0-45.0 g/l). The maximum tannase produced was 771.10 IU/g DCW at 60 h of fermentation with 19 g/l of gallic acid concentration in presence of 35 g/l of tannic acid at 300 rpm, pH-5.0. While, for maximum gallic acid accumulation, the optimal parameters are: 45 g/l of substrate concentration, 350 rpm and pH 5.0 which has resulted in 37.76 g/l. Banerjee et al. (2007a) reported the use of raw tannin of *Cassia siamea* in 5.0 L fermentor and yielded 4.1 U/ml with 35 w/v of tannins in the synthetic medium with an initial pH 5.5 by *A. aculeatus*-DBF9 at 30°C after 36 h. The conditions used were 4.0 L/min of aeration rate and
an agitation rate of 100 rpm for maximum gallic acid production (6.8 mg/ml) after
36 h. A protocol for gallic acid production in a 5.0 L fermentor at 40 g/l of tannic
acid concentration extracted from *Quercus infectoria* gall nuts using A. fischeri-
MTCC 150 has been reported (Bajpai and Patil, 2008).

Tannase production studies using bacterial strains were mostly carried out
by submerged fermentation in shake flasks (Mondal *et al.*, 2000; 2001; Ayed and
Hamdi, 2002; Das Mohapatra *et al.*, 2006; Belur *et al.*, 2010; Selwal *et al.*, 2010).
However, Deschamps *et al.* (1983) carried out submerged fermentation in 2.0 L jar
fermentor making use of bacterial isolates.

**Section V: Purification**

Mostly microbial tannases are intracellular and some are extracellular during
submerged fermentation. The downstream processing of fermentation broth begins
with the removal of cells from the fermentation broth, either by centrifugation
(bacterial) or by filtration (fungal). Here; we will majorly focused on purification of
fungal tannases. On the basis of the enzyme location, tannase has been purified
from a variety of fungi, namely, *A. flavus* (Yamada *et al.*, 1968), *A. oryzae* (libuchi
*et al.*, 1968; Fumihiko and Kiyoshi, 1975), *P. chrysogenum* (Rajakumar and Nandy,
1983) and *A. niger* (Barthomeuf *et al.*, 1994) using either culture filtrate (Fumihiko
and Kiyoshi, 1975) for extracellular tannase or mycelial extract obtained by
crushing/grinding of the mycelial cells with sand or glass beads or pulverizing with
homogenizer or osmotic shock or ultrasonic waves in buffers (Yamada *et al.*, 1968)
for intracellular tannase.

The cell free broth is then concentrated by ultrafiltration (Barthomeuf *et al.*, 1994; Zhong
*et al.*, 2004; Sharma *et al.*, 2008; Mata-Gómez *et al.*, 2009) or
ammonium sulfate precipitation (Yamada *et al.*, 1968; Battestin and Macedo 2007;
Naidu *et al.*, 2008; Chhokar *et al.*, 2010) or acetone precipitation (Dhar and Bose,
Amongst other precipitating agents tested a) rivanol (2-ethoxy-6,9-diaminoacridine
monolactate monohydrate) gave 61% recovery of the enzyme from culture broth
and b) polymers (1–90%) such as, polyethylene glycol (Sharma *et al.*,1999),
polyvinyl alcohol and dextran (Kazuo *et al.*, 1973), were suitable for the
precipitation of tannase from culture broth.
This is generally followed by chromatographic purification procedures to homogeneity (Yamada et al., 1968; Rajakumar and Nandy, 1983; Sharma et al., 1999; Bhardwaj et al., 2003; Ramirez–Coronel et al., 2003; Mahendran et al., 2006; Kasieczka-Burnecka et al., 2007; Battestin and Macedo 2007; Mata-Gomez et al., 2009; Chhokar et al., 2010). Additionally, researchers have used different one step purification techniques like aqueous two phase systems (ATPS) (Albertsson, 1986; Lekha and Lonsane, 1994; Gupta et al., 1997; Naidu et al., 2008) or liquid-liquid extraction of biomolecules employing reverse micelles (Castro and Cabral, 1988) this has been used by Barthomeuf et al. (1994) for the purification of tannase from A. niger LCF 8. The advantage of one step purification technique is rapid mass transfer, the ease of operation under continuous mode, rapid and selective separation and an easy and reliable scale-up of bench scale results to higher levels of production.

In the present investigation, three strategies of concentration, precipitation and chromatography were followed sequentially for purification of tannase from this strain of P. charlesii. Initially, the fermentation broth was filtered using six layers of muslin cloth to obtain mycelial free culture broth. The filtrate was then centrifuged to remove any mycelial debris. Concentration of the tannase rich culture broth thus obtained was carried out by ultrafiltration using different molecular weight cut off cartridges to remove excess water and smaller contaminating proteins. Using a 50 kDa membrane cartridge, maximum fold purification of 5.21 with 96.25% recovery was achieved. Maximum specific activity of 110.71 IU/mg of protein was recorded in the retentate. In the present study, it was also observed that when a membrane cartridge of 100 kDa was used, tannase passed through the membranes resulted in a comparable tannase activity in both retentate and permeate and resulted in lower fold purification to only 1.87.

Similarly, the use of 50 kDa cut off membrane for concentrating tannase from A. niger has been reported by Ramirez-Coronel et al. (2003). On the other hand, Barthomeuf et al. (1994) carried out ultrafiltration using a membrane of a high molecular weight cut-off (200 kDa), followed by filtration through a membrane facilitating smaller molecular weight cut-off (100 kDa) membrane. In this way, the lower and higher molecular weight cut-off proteins (contaminants in cell free broth) were removed and the enzyme was collected in the retentate. This permitted a purification factor of 14.9 and an approximate enzyme recovery of 80%. Later,
Sharma and coworkers reported the concentration of *Penicillium variable* tannase through ultrafiltration; wherein they concentrate the crude extract to 5.4 times using 100 kDa membrane and could achieve a recovery of 97% (Sharma *et al.*, 2008). Both these studies have reported a higher fold purification when a high molecular weight cut off membrane was used (100 KDa) for ultrafiltration as compared to only 1.87 fold purification achieved in the present investigation by this cut off membrane.

Ultrafiltration was also attempted for concentration of crude tannase from recombinant *Aspergillus oryzae* (Zhong *et al.*, 2004) and *Aspergillus niger* (Mata-Gómez *et al.*, 2009). Subsequently, this concentrated retentate containing tannase was subjected to precipitation using salts or solvents, an established procedure for eliminating other proteins and thus effectively concentrating and partially purifying the enzyme (Dhar and Bose, 1964; Yamada *et al.*, 1968; Ganga *et al.*, 1977; Beverini and Metche, 1990; Lekha and Lonsane, 1997; Sharma *et al.*, 1999; Battestin and Macedo 2007; Naidu *et al.*, 2008; Chhokar *et al.*, 2010).

Therefore, in the present investigation, precipitation of the tannase was carried out using both salt (ammonium sulfate) and solvent [acetone and rivanol] precipitation. Results show that among these, higher yields were obtained with ammonium sulfate. **91.20% recovery with 7.14 fold purification was obtained with 0–80% ammonium sulfate as against only 42.54% yield and 6.22 fold purification when 0–70% acetone was used. Using rivanol as solvent precipitant, a decreased yield of 36.78% in the precipitate and 9.99 fold purification was achieved. However, this is less than the recovery obtained using ammonium sulfate (0–80%) as precipitating agent.**

Earlier reports showed that ammonium sulfate is the preferred method of precipitating tannase from the fermentation broth as several researchers have used this procedure (Yamada *et al.*, 1968; Battestin and Macedo, 2007; Naidu *et al.*, 2008; Chhokar *et al.*, 2010). Saturation percentages used for enzyme precipitation usually ranges from 80–100%. Similar to our results with ammonium sulfate, a saturation percentage of 80% has been reported by Chhokar *et al.* (2010) for tannase from *Aspergillus awamori* resulting in nearly 1.82 fold increase with 29.05% yield in the precipitate. Battestin and Macedo (2007) also used 80% ammonium sulfate saturation for tannase from extracellular *Paecilomyces variotii* with 34% yield and 1.93 fold purification. A similar saturation percentage (60-80%) has also been used by Paranthaman *et al.* (2009) for tannase from *Aspergillus*
flavus. Nadal and Ghosh (2011) reported a partial purification of *Rhodococcus* tannase by ammonium sulfate precipitation at 80% with 66% yield and 2.23 fold purification. In a rare report, an ammonium sulfate saturation of 100% was used for partial purification of tannase from *Penicillium chrysogenum* with 69% of total recovery by Rajakumar and Nandy (1983).

On the other hand, acetone precipitation has been used by few researchers for partial purification of tannase. **In the present study, a yield of 42.54% was obtained, when the precipitation of tannase was carried out using a saturation of 0-70% acetone.** Lekha and Lonsane (1994) precipitated extracellular tannase from *Aspergillus niger* PKL104 with 50% v/v of cold acetone (pH 3.5). They obtained a 6.2 fold purification but with lower recovery yield of 28% under solid state fermentation (SSF). Mahapatra *et al.* (2005) subjected the supernatant to acetone precipitation using 1:2 v/v and kept at 4°C for 3h. Costa *et al.* (2008) used 75% cold acetone saturation for tannase from *Aspergillus tamari* with more than 70% recovery.

**The other precipitating agent used in the present investigation was rivanol (2-ethoxy-6, 9-diaminoacridine monolactate monohydrate) which resulted in a maximum purification fold of 9.99 fold with 36.78 % recovery.** Sharma *et al.* (1999) reported a higher recovery of enzyme to 61.0 % from the culture broth when rivanol was used as a precipitating agent.

Mostly, a single chromatographic step is not sufficient to get absolutely purified protein. Hence, a combination of chromatographic steps is required (Saxena *et al.*, 2003). **In the present investigation, the precipitated tannase was purified to homogeneity by using DEAE-Cellulose (Ion exchange), phenyl sepharose (Hydrophobic interaction) and Sephadex G-100 (Gel-filtration) column chromatography. Results showed that Sephadex G-100 column supported maximum recovery of tannase upto 63.4% and purification fold of 40.6.**

Several researchers have reported purification using all these chromatographic techniques (ion exchange, hydrophobic interaction and gel filtration) either single or in combination (Yamada *et al.*, 1968; Fumihiko and Kiyoshi, 1975; Rajakumar and Nandy, 1983; Sharma *et al.*, 1999; Ramírez–Coronel *et al.*, 2003; Bhardwaj *et al.*, 2003; Mahapatra *et al.*, 2005; Mahendran *et al.*, 2006;
Discussion

Battestin and Macedo, 2007; Kasieczka-Burnecka et al., 2007; Sharma et al., 2008; Mata-Gomez et al., 2009; Enemour and Odibo, 2010; Beena et al., 2010; Chhokar et al., 2010).

Ion-exchange chromatography is generally the second step employed for tannase production (Yamada et al., 1968; Rajakumar and Nandy, 1983). Since, tannase is acidic protein (Adachi et al., 1971) therefore; anion exchangers such as DEAE-Sephadex and DEAE cellulose were used for the purification of tannase from A. niger (Sharma et al., 1999; Bhardwaj et al., 2003) Paecilomyces variotii (Mahendran et al., 2006), Verticillium sp. P9 (Kasieczka-Burnecka et al., 2007), A. awamori (Chhokar et al., 2010), A. niger GH1 (Mata-Gomez et al., 2009). DEAE-Sephadex A-50 has been used in the purification of tannase from Aspergillus niger van tieghem (Sharma et al., 1999) and involves an acidic column (pH 5.0) and the adsorbed enzyme was eluted using gradient elution with buffers or salts of an increasing ionic strength (Iibuchi et al., 1968; Rajakumar and Nandy, 1983). Ramírez-Coronel and coworkers purified A. niger tannase by a preparative isoelectric focusing followed by anion exchange (Mono Q column) chromatography (Ramírez-Coronel et al., 2003). Battestin and Macedo (2007) reported that purification of extracellular Paecilomyces variotii tannase through ammonium sulfate precipitation followed by ionic exchange chromatography (DEAE-Sepharose) could led to 19.3 fold purification with a yield of 3%.

The adsorption and subsequent desorption of tannase from hydrophobic matrices such as Q sepharose Ff and phenyl sepharose is well known and forms part of hydrophobic interaction chromatography, the most popular technique for purification of tannase. In this context, Enemour and Odibo (2010) reported that tannase elution pattern showed two major peaks corresponding to two different kinds of tannase produced by A. awamori IM1388810 and identified as Tannase-I and Tannase-II. The culture filtrate containing tannase was subjected to dialysis against 6M sucrose followed by Q sepharose Ff and then phenyl sepharose 6Ff which resulted in Tannase I with a % yield and specific activity of 0.061% and 0.117 U/mg and tannase II with 0.53% and 0.144 U/mg of protein respectively.

The last step employed in the purification of tannase is gel-filtration chromatography, since tannase is a high molecular weight protein (200 kDa) (Iibuchi et al., 1968; Yamada et al., 1968). In this Sephadex G-200 (Yamada et al., 1968; Aoki et al., 1976 a; Rajakumar and Nandy, 1983; Mahendran et al., 2006;
Discussion

Sharma et al., 2008; Beena et al., 2010) Sephadex G-100 (Iibuchi et al., 1968; Fumihiko and Kiyoshi, 1975; Ramirez-Coronel et al., 2003; Mahapatra et al., 2005) and Sephadex G-150 (Sharma et al., 1999) have been used.

Barthomeuf et al. (1994) used a different protocol for purification of tannase from a submerged culture of A. niger. The crude enzyme extract was obtained in presence of concanavalin-A through physical disruption of the mycelial mass. The insoluble material was removed by centrifugation and the supernatant was filtered in nylon membrane and by tangential ultrafiltration. The purified enzyme was obtained by high pressure liquid chromatography using column protein pak at 30°C followed by ultrafiltration in centricon of 100 kDa. An increased tannase recovery from 22.5 to 34.5% was observed due to the addition of concanavalin-A to the medium by facilitating desorption of enzyme from its binding site.

The tannase from this strain of P. charlesii exhibited a single band of tannase which showed a molecular weight of 70±1 kDa on SDS-PAGE. According to earlier reports, tannase purified from A. niger was a monomeric protein of 55 kDa as determined by gel filtration (Parthasarathy and Bose, 1976). Similarly, the purified tannase from Paecilomyces variotii is having molecular mass of 87.3 kDa (Battestin and Macedo, 2007). Sabu and coworkers reported that tannase from A. niger ATCC 16620 have as a single monomeric unit of 149 kDa. They also reported that tannase from A. flavus and A. niger N888 also has a single peptide of 80-85 and 165 kDa respectively (Sabu et al., 2005).

The purified tannase in the present study exhibited an isoelectric point of 4.3. Similar pl of 4.3 has been reported for tannase from A. niger LCF8 by Barthomeuf et al. (1994). Beena et al. (2010) reported a pl of 4.4 for A. awamori tannase. Yamada et al. (1968) reported a tannase from A. flavus IFO5839 with a pl of 4.0. Ramirez–Coronel et al. (2003) reported a lower pl of 3.8 for tannase from A. niger Aa20. On the other hand, a higher pl of 5.8 and 6.2 has been reported for tannase from Verticillium sp. (TAH 1) and Verticillium sp. (TAH 2) respectively (Kasieczka-Burnecka et al., 2007).

Section VI: Immobilization

There are several reasons for immobilizing an enzyme. In addition to more convenient handling of the enzyme, it provides for its facile separation from the product, thereby minimizing or eliminating protein contamination of the product.
It also facilitates the efficient recovery and re-use of costly enzymes, and enables their use in continuous fixed bed operation (Sanderson et al., 1974; Gotoh et al., 1998; Varavinit et al., 2002).

Various techniques and support materials have been studied and consequently many immobilized tannase preparations with a wide range of efficiency, stability and activity have been reported (Gloria et al., 2007; Guangjun et al., 2012 ;).

Therefore, in the present investigation, immobilization of this *P. charlesii* tannase was carried out.

Two strategies were followed for immobilization of this tannase.

- **Conventional immobilization** (Physical adsorption, ionic binding, entrapment, covalent binding)
- **Cross linked enzyme aggregates (CLEA)**

Initially, immobilization in the present investigation was carried out by the conventional method of adsorption (chitosan, celite, silica, chitin), entrapment via alginate and polyacrylamide gel and ionic binding (DEAE-sephadex; Amberlite XAD-7; DEAE-cellulose). Amongst these supports, DEAE-cellulose exhibited maximum immobilization of 76.45 %. Whereas, among the physical adsorbents, maximum immobilization of 62.0 % occurred on chitosan. On the other hand, through entrapment (Ca-alginate and polyacrylamide) minimal amount of tannase was immobilized with 28.73 and 18.76% immobilization efficiency respectively.

Contrary to our present results, Mahendran et al. (2006) reported that *Paecilomyces variotii* immobilized on alginate beads (entrapment) resulted in an efficient tannase activity upto third cycle and thereby a gradual decline was observed.

It was observed that covalent binding formed using chitosan and chitin contained 70.46 and 63.54 % of tannase activity. Wherein, on physical adsorption using these supports there is decline in immobilization to 62.93 and 28.42 % respectively.

In this context, the immobilization of tannase using a carrier free method was an interesting alternative. In this method, the enzyme is first precipitated and cross
linked to form either aggregates (CLEA) or crystals (CLEC). These cross linked enzyme aggregates (CLEA/CLEC) are reported to be more stable to denaturation by heat, organic solvents and proteolysis as against the corresponding soluble enzyme or lyophilized powder. Their operational stability and ease of recycling, coupled with their high catalyst and volumetric productivity make them ideally suited for industrial biotransformations.

Therefore, in the present investigation, the tannase was cross linked to form CLEAs. Glutaraldehyde was used as a crosslinking agent while ammonium sulfate at 60% saturation was used as the precipitant. This resulted in 75.27 % relative tannase activity.

The activation of tannases by additives such as surfactants is generally ascribed to the tannase adopting a more active conformation. Cao (2005) suggested that immobilization by covalent binding using a cross linking agent (glutaraldehyde) probably increases the local surface area, which contributes to minimize the steric effect round the active site of the immobilized enzyme. Similarly in the present investigation, tannase activity slightly increased to 83.39 % and 79.32% in presence of sodium taurocholate and sodium choleate as against 75.27% when CLEA was made without any surfactant. On the other hand, Triton X-100 slightly inhibited the immobilization efficiency (70.24 %) of this tannase enzyme.

For any industrial application, the stability of the immobilized preparation is of great importance (Lee et al., 2006). Our results have shown that when the reusability of the three immobilized enzyme forms [Ionic binding (DEAE cellulose), Covalent binding (chitosan) and Cross linked enzyme aggregates CLEAs formed using glutaraldehyde and sodium taurocholate] were evaluated with respect to tannic acid hydrolysis, covalent and CLEA forms of immobilized tannase are much more efficient as against conventionally immobilized tannase on DEAE Cellulose. Enzyme immobilized on DEAE cellulose was 100% stable up to the 3rd cycle, covalent binding on chitosan was stable upto the 5th cycle. However, the immobilization through CLEA was 100% stable even upto the 8th cycle.
Therefore, from these results it can be inferred that covalent and CLEAs are better methods for tannase immobilization as compared to the conventional procedures of ionic binding.

Immobilization through covalent binding of tannase by its glycosidic part onto an insoluble support (Nicholas et al., 1997) or on carriers like chitosan, chitin, Dowex 50 W, DEAE- sephadex A-25, were used for tannase from Aspergillus oryzae (Abdel-Nabey et al., 1999). They reported that maximum tannase activity was observed on chitosan with bi-functional agent glutaraldehyde.

Boadi and Neufeld (2001) observed that coating enzyme with high or low molecular weight chitosan (physical adsorption) reduced enzyme release and could retained hydrolytic activity through three successive batch cycles. This property of immobilization and its economic reuse was evaluated in beverages where it could visibly remove the tea cream.

Contrary to our results, Sharma et al. (2008) reported that Amberlite IR 1204 (ionic binding) is the best support to immobilize P. variable tannase with 69% immobilization and could be reused for six cycles without any appreciable loss of enzyme activity (100% RA).

Section VII: Characterization and kinetic studies

Investigations on evaluating the biochemical properties of tannases are important because it offers an insight to the functioning of this enzyme and its suitability in performing certain reactions (Rajakumar and Nandy, 1983; Farias et al., 1994; Sharma et al., 2002) Studies on pH temperature profile and stability along with studies on substrate specificity and solvent stability are important in designing in vitro catalytic reactions of commercial significance. Therefore, it was felt worthwhile to evaluate this tannase from the selected strain of Penicillium charlesii for its different biochemical properties. Three forms of this tannase were selected for these studies, i.e. the crude, purified and the immobilized form.

Initially, the pH tolerance profile was evaluated for the three forms. Results show that all the three forms exhibited activity to variable degrees in the range 3.0-9.0 with optimum at pH 5.0. Though, a significant activity was noted in the range of 3.0-9.0, higher activity was observed in the acidic range of 3.0-6.0. Maximum relative activity was recorded for immobilized tannase.
followed by crude. The purified tannase though showed activity in the similar range, however had considerably lower relative activity.

With respect to stability, all the three tannase forms exhibited stability when evaluated between pH 3.0 to 9.0 over a period of 48 h. Maximum stability was noted between pH 3.0-6.0 with more than 70% relative activity after 48 h of incubation. The immobilized tannase was again more stable with higher relative activity recorded at all pH after 48 h.

Several researchers reported that tannase being a acidic protein is having an optimum pH around 5.0-6.0 (Adachi et al., 1968; Aoki et al., 1976; Rajakumar and Nandy, 1983; Thomas and Murthaug, 1985; Beverini and Metche, 1990; Barthomeuf et al., 1994; Farias et al., 1994; García-Peña et al., 1999; Mahapatra et al., 2005). Similar to present results, the tannases obtained from A. aculeatus DBF9, A. niger and A. awamori Nakazawa had a pH optima of 5.0 as reported by Banerjee et al. (2001); Yu et al. (2003) and Mahapatra et al. (2005) respectively. In 2009, Mata-Gómez and their coworkers observed significant stability in a pH range from 3.0- 6.0 with an optima at 5.0 from A. niger GH1 tannase (Mata-Gómez et al., 2009).

However, in certain reports, a slightly higher pH optima (pH 5.5) was reported to be optimal for tannase obtained from A. flavus (Yamada et al., 1968), A. oryzae (Libuchi et al., 1972), A. awamori MTCC 9299 (Chhokar et al., 2010) and A. ruber (Kumar et al., 2007). While, an optimal pH of 6.0 was recorded for A. niger tannase (Barthomeuf et al., 1994; García-Peña et al., 1999; Ramírez et al., 2003). The optimal pH for P. chrysogenum tannase was 6.0 as reported by Rajakumar and Nandy (1983). Tannase preparation from A. niger van tieghem incubated at 4°C for 24 h was stable at pH 6.0 (Sharma et al., 1999).

Similar to our results, the pH stability of tannase obtained from P. chrysogenum (Rajakumar and Nandy, 1983) and A. oryzae (Yamada et al., 1968) is in a narrow range from 4.5–6.0 and 5.0–5.5, respectively. Aspergillus niger PKL 104 is also stable in a narrow pH range from 4.5 to 5.5 (Lekha et al., 1994). Battestin and Macedo (2007) showed more than 80% stability of tannase in the narrow range between pH 4.5–6.5. Similarly, in a narrow pH range from 5.0–7.0 was found to be stable, for tannase from P. variotii as reported by Mahendran et al. (2006). Contrary to our results, few researchers reported that tannase has exhibited
broad pH stability. Iibuchi et al. (1968) reported enzyme stability in a broad pH range of 3.5-8.0 from A. oryzae. Tannase obtained from Penicillium variable was found to be stable in a pH range from 3.0-8.0 for 24 h (Sharma et al., 2008). In a rare report, tannase from A. niger GH1 was noted to be active even at lower pH-2.0 at this pH; a relative activity of 45% was still exhibited by this tannase.

In the present investigation, tannase exhibited a temperature optimum of 40°C with activity in a broad temperature range of 20-80°C. The crude, purified and immobilized tannase forms were very stable upto 50°C after 48 h with more than 60% of relative activity (RA) observed. While, at 80°C, the immobilized tannase retained 37.19% as against 16.17 and 8.93% R.A. as obtained for crude and purified respectively.

Temperature optima of 30-40°C have been reported for A. niger (Yu et al., 2004; Sabu et al., 2005). Our results are in agreement with the reports of Batra and Saxena (2005) who reported that 40°C as optimal temperature for A. caespitosum, P. charlesii, P. crustosum and P. restrictum. Similar has been reported for tannase production from Enterobacter sp. (Sharma and John, 2010). A slightly lower temperature of 30°C was reported to be optimal for tannase activity from A. oryzae (libuchi et al., 1968, 1972), P. chrysogenum (Rajakumar and Nandy, 1983) Cryphonectria parasitica (Farias et al., 1994), A. awamori BTMF WO32 (Beena et al., 2010), A. awamori MTCC 9299 (Chhokar et al., 2010) and 35°C for A. niger (Barthomeuf et al., 1994), A. awamori Nakazawa (Mahapatra et al., 2005). On the other hand, an higher temperature of 50°C was optimal for Penicillium variable tannase (Sharma et al., 2008), 50-60°C was optimal for A. flavus IFO5839 (Yamada et al., 1968), for A. aculeatus DBF 9 (Banerjee et al., 2001), 55°C for Paecilomyces variotii (Battestin and Macedo, 2007), 60°C for A. niger van tieghem (Sharma et al., 1999) and 60-70 °C for A. niger (Ramírez- Coronel et al., 2003).

In the present investigation, the enzyme in all three forms have significant stability between 20-45°C with more than 60.0% R.A. However, the immobilized tannase was even stable at 50°C with 64.39% relative activity after 48 h of incubation.

Sharma et al. (2008) reported 80% relative activity at 50°C after 24 h in case of P. variable tannase. On the other hand, the enzyme was stable only upto 30°C

The crude and immobilized *P. charlesii* tannase exhibited a thermostability of 17.39% and 30.42% at 80°C even after 1h of incubation. While, the purified forms was inactive at similar temperature and incubation. On the other hand, Sharma *et al.* (2008) reported a higher R.A. of 54.32 and 61.40% from crude and immobilized form of *P. variable* tannase respectively at similar temperature and incubation. Also, 48.89% R.A. was observed in the purified form of tannase.

Abdel-Nabey *et al.* (1999) and El-Tanash *et al.* (2011) reported that the immobilized form of tannase is more thermostable as compared to the free form. According to their report, the immobilized tannase retained 83% R.A. after 90 min at 60°C while, 76.25% R.A. was obtained by free enzyme after same treatment. On the contrary, Sharma *et al.* (2008) reported that immobilization of *P. variable* tannase did not lead to any change in the character of the enzyme. Therefore, the temperature optima for both free and immobilized enzyme were similar.

In the present investigation, when the crude and the immobilized tannases were evaluated for stability in various organic solvents, it was observed that both these forms were stable in most of the organic solvents with more than 70% relative activity tested even after 6 h of incubation. Maximum stability was in ethyl acetate (81.69% R.A.) while significant loss in activity was noted for the crude tannase only, whereas, in the presence of petroleum ether (16.90%) and cyclohexane (24.60%) after 48 h.

Sharma *et al.* (2008) observed that *P. variable* tannase was completely inhibited by acetone and formaldehyde at 60% v/v after 1 h and significant loss in its activity upto 33 %. was observed in petroleum ether at 60% v/v after 1 h of incubation. Similarly, Mata- Gómez *et al.* (2009) reported that at 60% ethanol and acetone completely inhibits tannase activity, whereas, THF and Formaldehyde exhibits inhibitory activity only at 20%. Chhokar *et al.* (2010) reported that complete loss of enzyme activity was observed at 40 and 60% of ethanol. Wherein, the enzyme activity was completely inhibited by acetic acid, isoamyl alcohol, chloroform and isopropyl alcohol at all concentrations. Beena *et al.* (2010) reported that all the solvents recorded a similar pattern of decreasing activity along with an increase in time and concentration.
Among various surfactants evaluated, only sodium taurocholate stimulated tannase activity up to certain extent with 102.0%, 109.0% and 110.0% RA for crude, purified and immobilized forms, respectively. Minimum relative activity (less than 50%) was recorded on incubation with cholic acid for crude and immobilized forms with very less activity in purified tannase. Similarly, Sharma et al. (2008) reported that sodium taurocholate enhanced tannase production while, SDS, tween 60 and tween 80 completely inactivated the enzyme. Significant loss of activity in presence of Tween 20 was reported for tannase produced by Verticillium sp. (Kasieczka-Burnecka et al., 2007) and Paecilomyces variotii (Battestin and Macedo, 2007). Naidu et al. (2008) reported that Aspergillus foetidus tannase was completely inhibited by Tween 80 and SDS. While, Goncalves et al. (2011) reported that there was an enhancement in the enzyme activity from E. nidulans in presence of SDS.

Inhibition studies primarily provide an insight into the nature of the enzyme, its cofactor requirements and the nature of the active enzyme (Whitaker, 1972; Saxena et al., 2003; Battestin and Macedo, 2007). On evaluating different inhibitors in the present investigation for their effect on tannase activity, results show that tannase was completely inhibited by N-bromosuccinamide at a concentration of 1.0 mM and 5.0 mM when incubated for 1 h in purified and immobilized form. PMSF is another inhibitor which completely inhibits tannase activity at 5 mM. While, O-phenanthroline and dithiothreitol at 5 mM showed negligible inhibition.

Inhibition by n-bromosuccinimide indicated that tryptophan residues played an important role in maintaining the active conformation of the enzyme (Battestin and Macedo, 2007). Rajakumar and Nandy (1983) and Suseela et al. (1995) reported that tannase activity of P. chrysogenum was completely inhibited by PMSF; this thereby suggests that the enzyme needs serine residue for its activity. Similarly Beena et al. (2010) reported that amongst the different inhibitors tested PMSF showed maximal enzyme inhibition (4.5% R.A.), followed by sodium deoxycholate (26.4% R.A.) and phenanthroline (61.04% R.A.). The inhibition of tannase activity in presence of PMSF suggests the presence of serine or cysteine residue in the catalytic site of A. niger GH1 tannase (Mata-Gomez et al., 2009). Furthermore, Sharma et al. (2008) reported that N-ethylmaleimide showed strong inhibition.
Incubation of this *P. charlesii* tannase with different metal ions did not increase tannase activity. However, incubation with Fe$^{3+}$, Co$^{2+}$, Cu$^{2+}$ and Fe$^{2+}$ at both 5.0 and 10.0 mM concentration led to decrease in its activity. Beena *et al.* (2010) explained the phenomenon involved behind the decreased activity in presence of divalent cations could be due to non-specific binding or aggregation of the enzyme. Several researchers have previously reported the inhibitory effects of Fe$^{3+}$, Cu$^{2+}$, Zn$^{2+}$ ions on tannase (Iibuchi *et al*., 1968; Rajakumar and Nandy, 1983; Barthomeuf *et al*., 1994; Kar *et al*., 2003; Sabu *et al*., 2005; Kasieczka-Burnecka *et al*., 2007; Chhokar *et al*., 2010). Similarly, Chhokar *et al.* (2010) reported the inhibitory effect of Cu$^{2+}$, Fe$^{3+}$ and Co$^{2+}$ on tannase activity. Further, Kasieczka-Burnecka *et al.* (2007) also reported that Fe$^{3+}$, Co$^{2+}$, Pb$^{2+}$ and Sn$^{2+}$ inhibit the enzyme activity.

Contrary to our results, an enhancement in tannase activity in presence of Co$^{2+}$ was reported by Mata-Gomez *et al.* (2009) and Goncalves *et al.* (2011). In addition, Beena *et al.* (2010) reported a significant enhancement in the tannase activity by Fe$^{3+}$ ions.

In the present investigation, it was observed that this purified tannase preferentially hydrolyzed tannic acid (95%) followed by propyl gallate (76%), and methyl gallate (73%).

The values of kinetic constraints ($K_m$ and $V_{max}$) depend upon the particular substrate used and the enzyme source (Beena *et al*., 2010). In the present investigation, kinetic studies of the tannase carried out by plotting the Lineweaver Burke plot resulted in a $K_m$ of 0.18 mM, $V_{max}$ of 166.7 µmole/ml/min with tannic acid as substrate. The turnover number or $K_{cat}$ was 15.43 sec$^{-1}$. Katwa *et al.* (1981) reported that hydrolyzable tannins and gallic acid esters are commonly used substrates for tannase. In addition, Rajakumar and Nandy (1983) revealed that the enzyme from *P. chrysogenum* had a $K_m$ value of 0.48 X 10$^{-4}$ M for tannic acid. Furthermore, tannic acid with a $K_m$ of 28 Mm for *A. niger* MTCC 2425 tannase is considered as the best substrate, followed by methyl and propyl gallate (Bhardwaj *et al*., 2003). Maltos *et al.* (2011) reported a $K_m$ of 0.4 mM and $V_{max}$ of 0.05 µmol/min for free tannase from *A. niger* using tannic acid as substrate.

Shelf life studies revealed that the crude tannase retained 92% after one year of storage at 4°C. However, the purified tannase retained only 65%
relative activity under similar conditions while lyophilized tannase retained 95% of its activity after one year. On the contrary, shelf life when evaluated at 30°C showed a drastic decline in purified tannase activity with only 63% RA after 90 days of storage. On the other hand, the crude and lyophilized tannase forms were more stable with 87% and 91% RA respectively even after 365 days of storage. However, Sharma et al. (2008) reported 72.0% and 70.0% R.A. of crude and lyophilized *P. variable* tannase, respectively was obtained at 30°C after 365 days of storage. While, at lower temperature of 4°C, a slight lower R.A. of 60.0 and 67.0 % was retained in case of crude and lyophilized tannase from *P. variable*, respectively at similar incubation period.

**Section VIII: Potential industrial applications**

Tannases are biocatalysts capable of carrying out different reactions of industrial importance. Though, tannases find usage in a wide range of industries, in the present investigation, studies on the industrial applicability of this tannase from *Penicillium charlesii* are aimed at:

- Gallic acid production
- Synthesis of gallic acid esters
- Tea quality improvement
- Animal feed improvement
- Degradation of tannery effluents
- Removal of tannin stains

The results of evaluation for all these experiments are presented in separate sections with respect to the industry they represent *i.e.* food, feed, beverage, brewing, effluent treatment and pharmaceutical.

The tannase from this strain of *Penicillium charlesii* was concentrated and lyophilized. This lyophilized tannase (4.23 IU/mg) was used for evaluating various applications of industrial significance. Enzymatic esterification/transesterification reactions can be accomplished by carrying out the reactions in suitable organic solvents and to shift the reaction equilibrium toward esterification/ transesterification rather than hydrolysis. Therefore, in the present investigation, these reactions were carried out in presence of biobutanol, isoamyl alcohol and ethyl alcohol.
Food and Pharmaceutical industry

1. Gallic acid production

Gallic acid (3, 4, 5-trihydroxybenzoic acid) and esters of gallic acid, also known as gallates, are widely employed as antioxidants by food and pharmaceutical industries (Kar et al., 2002; Yu et al., 2004; Yu and Li, 2006; Aguilar et al., 2007). Besides the antioxidant activity, other biological activities have been described for this group of molecules, mainly anticancer, antibacterial and antifungal properties (Garcia-Najera et al., 2002). Gallic acid is principally involved in the synthesis of trimethoprim (TMP), an antibacterial agent (Hadi et al., 1994).

In the present investigation, tannase could carry out 92.3% conversion of tannic acid to gallic acid in 100 ml of the reaction volume with 20 g of tannic acid in presence 1000 IU of tannase. In order to check the scalability of the reaction, it was scaled up to 2500 ml of the reaction volume. Results showed that more or less same amount of gallic acid was formed (90.1%) at 40°C and 150 rpm. The gallic acid formed through the process of crystallization under low temperature was evaluated for its purity through high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy (both $^1$H and $^{13}$C). The results obtained confirm that the gallic acid formed crystals were 98.99% pure.

Earlier results from our laboratory, (Batra, 2004) reported 89% gallic acid formation at 37 °C, pH 6.0, in 24 h in a 5L of the working volume. Kar and Banerjee (2000) also reported 90.9% yield of gallic acid from tannin by R. oryzae under solid state fermentation. On the other hand, the same research group, under submerged fermentation reported a lower yield of only 27.5% of gallic acid by R. oryzae using 58% tannins of Caesalpinia digyna seed cover. Sharma and Saxena (2012) reported a significant gallic acid production of 92% in 8 h by A. niger and 60% in 8 h by P. variable.

On the other hand, Misro et al. (1997), reported gallic acid production from tannic acid using immobilized cells, yielded 83.5% and 78.5% when immobilized spores and tannase of R. oryzae were used, respectively.
2. **Synthesis of gallic acid esters**

a) **Synthesis of methyl and propyl gallate**

Gallic acid and its derivatives are used as antioxidant in the food industry, dyes and inks, photographic developers and have also been used as an astringent. Propyl gallate, a gallic acid ester, is used commercially as an antioxidant in foods (Weetal, 1985) and is also involved in the synthesis of pyrogallol. Pyrogallol is used in staining fur, leather and hair and also as a photographic developer (Hadi, 1993).

In the present investigation, this tannase from *P. charlesii* carried out efficient esterification of gallic acid with respective alcohols (methanol/propanol) in presence of 10 ml of n-hexane. Results showed an efficient conversion of gallic acid at 0.3 M concentration to methyl gallate and propyl gallate with 60.25 and 85.63 % conversion, respectively after 24 h of incubation.

Weetal as early as 1985 reported that the use of variety of alcohols and diols not only act as substrates in the esterification of gallic acid but also as a solvent for the reaction system. However, addition of n-hexane, a non polar solvent in the reaction mixture proves to be efficient for the reaction which resulted in higher product yield and reduced incubation period as the alcohol (methanol/propanol) and acid (gallic acid) are easily dissolved in presence of n-hexane. It could also be due to enhancement in the stability of the tannase and thereby showed more enzymatic activity in n-hexane system. According to their study, observations revealed that after 120 h of incubation, a total of 85% of gallic acid has been converted to ester as compared to 65-70% in the alcohol system.

Present results are in accordance with their reports where the incubation period to achieve the desired yield (85.63%) has been reduced to a great extent upto 96 h. Similarly, Sharma and Gupta (2003) have reported a yield of 86 % propyl gallate using tannase from *Aspergillus niger van tieghem* with the use of water (1.0%) in non-aqueous media (hexane). On the other hand, Yu et al. (2004) observed 44.3% synthesis of propyl gallate in presence of benzene as reaction solvent as compared to n-hexane wherein only 35.7% was recorded. In this report, microencapsulated tannase was used and the reaction was kept at 40°C for 12 h at 200 rpm. Yu et al. (2004) also reported reduced synthesis of propyl gallate in polar solvents due to the removal of essential hydration water from the enzyme. Further,
it can also be due to strong effect of the solvent on substrate binding as it gets more strongly associated with substrate which could thereby hinders the binding of substrate to the enzyme’s active site. Therefore, most of the researchers have opted for the use of non polar solvents in the reaction system.

The use of salt anhydrates and molecular sieves is a convenient and simple method to maintain a constant water activity ($a_w$) in the reaction system (Halling, 1984 and 1990).

Therefore, in the present investigation, an optimal yield of 82% for methyl gallate and 93% for propyl gallate was observed in 24 h in presence of 5% anhydrous sodium sulfate.

Similar to our results, the use of salt anydrates as been reported by Gaathon (1989) who reported a maximum yield of 50% of propyl gallate in a reverse micellar system comprising of 0.2 M sodium 1, 2 tris (2-ethylhexylcarbonyl) 1-ethane sulfonate (AOT) in heptane, propanol, water, tannic acid and buffering ions. Yu et al. (2004) also observed an increase in the synthesis of propyl gallate from 37.3 (control without salt anhydrate addition) to 45.5% (with MgSO$_4$) and 49.4% (by Na$_2$SO$_4$). Zheng et al. (2006) reported 87.4% synthesis of propyl gallate in the reaction mixture which consists of docusate sodium salt (AOT), isooctane and water. The molar ratio of water to AOT was 15.0. The pH of the reaction mixture was adjusted to 6.0 and the reaction was carried out in presence of tannase (0.6 mg/ml) at 30°C. While, Zheng (2009) recorded a yield of 78.2% of propyl gallate when pH value was 6.0 and the amount of Na$_2$SO$_4$-Na$_2$SO$_4$.10H$_2$O (1:1) was 1.8 g at the temperature 28°C by tannase immobilized in microemulsion-based-gels.

The use of molecular sieves for controlling water activity has been well documented (Kim et al., 1995). The addition of molecular sieves has two advantages: first, it shifts the equilibrium towards ester synthesis and second, it stabilizes the product preventing its hydrolysis by absorbing excess water in the system.

In the present study, addition of molecular sieves resulted in a lowering of yield to 71% of methyl gallate and 84% of propyl gallate by this tannase in 24 h as compared to the use of anhydrite salts as absorbent.
Yu et al. (2004) reported that addition of molecular sieves (0.3 g/ml of reaction media) improves the synthesis of product yield (propyl gallate) by 10% in 8 h of incubation with microencapsulated tannase. Since, addition of molecular sieves shifts the equilibrium towards esterification. Sharma and Saxena (2012) reported 90.7% and 83% of methyl gallate synthesis by A. niger and P. variable respectively in presence of molecular sieves. Similarly, 94.8 and 89.6% of propyl gallate has been reported by A. niger and P. variable tannase.

b) Synthesis of butyl, amyl and ethyl gallate

These compounds are widely used in veterinary medicine against infections of the urinary tract and other bacterial diseases. For humans, it is used in the treatment of typhoid fever and bacterial meningitis.

This tannase could also carry out efficient esterification for biobutanol, isoamyl alcohol and ethyl alcohol in the ratio of 1:10 (gallic acid: biobutanol/isoamyl alcohol/ethyl alcohol) to achieve the esterification and get 51% butyl gallate, 32% Amyl gallate and 63% ethyl gallate after 24 h incubation 40°C at 200 rpm using molecular sieves as water depressor.

Weetal (1985a) reported 85% synthesis of amyl gallate with immobilized A. oryzae tannase. Zheng et al. (2006) reported 92.5% synthesis of amyl gallate in the reaction mixture which consists of docusate sodium salt (AOT), isooctane and water. The molar ratio of water to AOT was 12.5 with an AOT concentration of 0.2 mol/L. The pH of the reaction mixture was adjusted to 6.0 and the reaction was carried out in presence of tannase (0.6 mg/ml) at 45°C. These researchers also reported 90.3% yield of butyl gallate in presence of the reaction mixture which consists of docusate sodium salt (AOT), isooctane and water with the molar ratio of water to AOT (15.0) and an AOT concentration of 0.2 mol/L. The pH of the reaction mixture was adjusted to 6.0 and the reaction was carried out in presence of tannase (0.6 mg/ml) at 40°C. While, Zheng (2009) observed 85% yield of butyl gallate when pH value was 6.0 and the amount of Na₂SO₄- Na₂SO₄·10H₂O (1:1) was 1.8 g at temperature 28°C by tannase immobilized in microemulsion-based-gels.
3. **Tea quality improvement**

“Creaming” is a critical phenomenon in instant tea processing which leads to haziness of the final product. Caffeine and galloyl esters of tea polyphenols are important constituents of this tea cream (Lu *et al.*, 2009). In this respect, Sanderson *et al.* (1974) described instant tea preparation as one of the most promising applications of tannase. In enzyme treated tea infusion on refrigeration resulted in lowering of tea cream formation due to degallegation of gallated catechins and other gallated polyphenols, which thereby prevents their interaction with proteins (Chandni *et al.*, 2011).

In the present investigation, the tannase treated tea infusion showed reduction in tea cream formation at an optimal tannase concentration of 0.1%, and an increase in antioxidant activity to 1.73 and 1.61 fold respectively. Nagalakshmi (1985) also reported 80 and 81% cream solubilization at 45°C during preparation of instant tea by two tannases of *A. oryzae* CLRI 1120 and *A. flavus* CLRI 1121. In our laboratory, Batra (2004) reported 76.62% and 66.88% tea cream solubilization in presence of *A. versicolor* and *A. caespitosum* tannase respectively. Lu *et al.* (2009) reported that at 0.1% tannase concentration there was a reduction in tea cream formation upto 77.7%. In another report, Chandni *et al.* (2011) reported % reduction in tea cream formation to 65–72% from *A. hetromorphous* tannase. Increase in gallic acid on tannin hydrolysis found in tea and tea catechins aids in tea cream solubilization (Lehmberg *et al.*, 1999). Lu and Chen (2008) and Macedo *et al.* (2011) reported an increase in radical scavenging capacity of biotransformed tea to 62.0 % and 55.0 % in green tea respectively.

In the present study, there was no change in the content and concentration of volatile compounds. Moreover, the results also showed that there was an improvement in the quality of tannase treated black tea infusion in relation to color, brightness, strength and flavor as compared to control. These properties of biotransformed tea have thereby led to the establishment of the potential of *Penicillium charlesii* tannase for the production of instant tea (Raghuwanshi *et al.*, 2012). Liang and Xu (2001) reported two polyphenolic products thearubigins (TRs) and theaflavins (TFs) which determines the tea quality. Similarly, Venkateshwaran *et al.* (2002) reported that TRs are responsible for color, body and taste and TFs determines the briskness, brightness and quality of liquor.
4. Animal feed improvement

Tannase has been employed to produce renewable feedstock by biocatalytic conversion of tannins to gallic acid, reduction of hydrolyzable tannin levels in poultry feeds and facilitating efficient feeding through the utilization / digestibility of tannin containing cheaper components in animal feed (Lekha and Lonsane, 1997; Nuero and Reyes, 2002; Aguilar et al., 2007; Graminha et al., 2008; Murugan and Al-Sohaibani, 2010). In the present study it was observed that tannase at a concentration of 0.1% is optimal for tannin degradation, though higher concentration leads to more tannin degradation, but higher amount of gallic acid is produced which thereby lowers the activity of different lignocellulolytic enzymes produced from white rot fungi. Generally, these lignocellulolytic enzymes aids in lignin degradation. However, lower amount of gallic acid was produced in presence of 0.1% tannase concentration which acts as an inducer for these lignocellulolytic enzymes. Similar has been reported by Gnanamani et al. (2006) and Patel et al. (2009) wherein the presence of gallic acid induces laccase (lignocellulolytic enzyme).

Observations clearly reveal that significant decrease in the cell wall constituent of tannin free SSF treated wheat straw (0.1%) i.e. 20.26, 19.47, 17.86, 37.81 and 20.45% was observed in ADF, NDF, hemicellulose, lignin and cellulose of fermented wheat straw after 10 days of incubation. Our results are in accordance with the reports of Hervás and their coworkers wherein they reported that higher degradability of cellulose was obtained as compared to hemicelluloses in presence of lower tannase concentration or higher residual tannins (Hervás et al., 2003).

In the present report, a significant increase in the available crude protein content to 1.27 fold with the lower L/C (0.18) and C/N ratio (81.88) as compared to the control wheat straw (without tannase treated wheat straw) was obtained. Thus, showing the potential for the bioconversion of lignin rich wheat straw into a high energy cattle feed. Srivastava et al. (2011) reported 57.11 % increase in crude protein content on fermenting wheat straw with Ganoderma sp. rckk02 as against raw wheat straw (control). The increase in crude protein content is partially attributed to fungal protein, especially chitin (Hadar et al., 1992) and then decrease in certain cases cannot be fully explained. A lower L/C ratio of 0.18 was obtained in tannin free Ganoderma treated wheat straw at 0.1% tannase. Deshpande et al. (2008) reported that proper C/N ratio promotes good fungal growth and consequently higher enzyme production.
5. Degradation of tannery effluents

During the processing of animal hides for leather, tannins are released in water bodies causing enormous pollution (Van de Lagemaat and Pyle, 2001). Till date, there have been no reports of attempts of biodegradation of tannery effluents using tannases. Though, this enzyme could potentially be used for the degradation of hard and acidic industrial effluent containing tannin rich materials, which will thereby offer a cheap treatment (Banerjee, 2005).

The results of the present study revealed that, there is a considerable reduction in the values of important parameters. The tannery effluent had color equivalent to 4439 U. The treatment of the effluent with the enzyme reduces the color to 3241 U. The pH value of the raw effluent was reduced from 4.3 to 6.0, TDS from 6923 to 5119 mg/l, TSS from 3950 to 2440 mg/l, BOD from 721 to 305 mg/l, COD 3684 to 3006 mg/l and the important pollutant tannin from 10351 to 3224 mg/l. Thus, a significant reduction in the values of TDS, TSS, COD, chloride and tannin by the enzyme was recorded. This aspect of using tannases as agents for bioremediation needs further optimization and subsequent evaluation.

Similarly, Murugan and Al-Sohaibani (2010) reported the biocompatible removal of tannin and associated color from tannery effluents using biomass and enzyme from A. candidus MTCC 9628. Further, Abdulla et al. (2000) also carried out the enzymatic treatment of tannery effluent. The ability of tannase to hydrolyze tannic acid and gallic acid esters may have potential applications in oil wastewater treatment and for partial decolorization processes by utilizing enrichment cultures as reported by Van de Lagemaat and Pyle (2006). Sharma and Saxena (2012) reported 45 and 36% degradation of 15x diluted tannery effluent by A. niger and P. variable.

6. Removal of Tannin stains

Another important result showed that tannin stains caused by tea and betel could successfully be removed from clothes on using tannase. The use of tannase enzyme is beneficial in facilitating the breakdown of extrinsic stain through hydrolysis of tannins which is known to cause discoloration of tooth surface (Laurence- Du-Thumn et al., 2005).

In the present study, cloth pieces having tea and betel stains when washed in solution containing detergent and tannase showed best brightness as compared
Discussion

to other treatments. Increase in percentage reflectance of 72% and 61% as compared to 15.6% and 18.14% for tea and betel stains (control) was achieved. Even the visible observation of cloth pieces after washing, showed better removal of tea stains when tannase along with detergent were used. Therefore, this enzyme can also be used as a tannin stain digester as it has shown the potential to hydrolyze tannins. This is an important attempt to formulate a newer application of tannase as a tannin stain digester. Sharma and Saxena (2012) reported that an increase in temperature from 37 to 50°C enhances the removal of tea/tannin stains in terms of percent reflectance to 15% increase for *A. niger* tannase and 21% for *P. variable* tannase thereby indicating its potential use in tannin stain remover (Tannosol).

To summarize, the results obtained and discussed in the light of current status of research on tannases, have shown that the *Penicillium charlesii* produces a tannase in significant titres under both submerged and solid state fermentation condition. This tannase is a robust enzyme due to its tolerance to a wide range of pH and temperature and stability in aqueous and non-aqueous systems and has a remarkable potential to carry out a variety of reactions which find its usages in different applications in the industries.

Important results obtained in the present investigation have been summarized in the next chapter “Summary and conclusions”. In addition to this, the future course of action and different perspectives for further research on this tannase are presented in the last section of the thesis “Future prospects”.