ISSN 1996-0700

Asian Journal of Biotechnology

Knowledgia SCIENTIFIC
A Place to Publish Outstanding Research

http://knowledgiascientific.com

Asian Journal of Biotechnology 3 (1): 91-97, 2011
ISSN 1996-0700 / DOI: 10.3923/ajbkr.2011.91.97

Release of Cell-associated Tannase of *Serratia ficaria* DTC by Sonication, Surfactants and Solvents

P.D. Belur, G. Mugeraya and B. Nainegali
Department of Chemical Engineering, National Institute of Technology Karnataka Surathkal, Srinivasanagar, Mangalore-575 025, India

Corresponding Author: P.D. Belur, Department of Chemical Engineering, National Institute of Technology Karnataka Surathkal, Srinivasanagar, Mangalore-575 025, India

ABSTRACT

The aim of this study was to find an efficient method to release cell-associated tannase from the biomass of *Serratia ficaria* DTC. In this study ultrasonication, surfactants and solvents treatment was evaluated for the release of cell-associated tannase from the biomass. Ultrasonication could release cell-associated tannase with the concomitant reduction in overall enzyme activity, lead to complete inactivation after 25 min of treatment. Triton X-100, Tween 80, SDS and sodium taurocholate could extract tannase from the biomass. An effort was made to find the optimum concentration of Tween 80 and Triton X-100. Triton X-100 treatment (0.05% w/v) released all the cell-associated tannase with the concomitant release of 4.8% of cellular proteins. Tween 80 (0.05% w/v) treatment resulted in the increase in overall tannase activity by 189% due to unmasking of activity.

Key words: Release, *Serratia,* tannase, Triton X-100, unmasking

INTRODUCTION

Tannin acyl hydrolase (E.C. 3.1.1.20), commonly known as Tannase, hydrolyses the ester and depside bonds in hydrolysable tannins. Tannase finds application in the manufacture of Gallic acid and instant tea. The enzyme is also used as clarifying agent in some wines, juices of fruits and refreshing drinks with coffee flavor. The enzyme has potential uses in the treatment of tannery effluents and pre-treatment of tannin containing animal feed (Belmares *et al*., 2004). Many molds, yeasts and bacteria are known to produce tannase. Tannase is now known to be a ubiquitous enzyme of the microbial world.

Most of the reports on tannase are pertaining to extracellular tannase (ECT) production from bacteria and fungi (Aguilar *et al*., 2007). There are quite a few reports of intracellular tannase activity in microorganisms as well (Lekha and Lonsane, 1994; Seth and Chand, 2000). Perhaps, for the first time we reported Cell-Associated Tannase (CAT) activity in several bacterial isolates. Out of five species we isolated, three were belongs to *Serratia* genus (Belur *et al*., 2010). Production of extracellular enzymes by *Serratia marcescenes* is not uncommon (Bhatti, 1974; Braun and Schmitz, 1980; Gao *et al*., 2004). Mehmood *et al*. (2009) have reported the purification and characterization of a chitinase from *Serratia proteamaculans*. How ever no reports are available on enzyme production from *Serratia ficaria*. This organism is presumed to be non-pathogenic, found mostly in figs and fig wasps (Grimont *et al*., 1979). Cell-associated/cell-bound enzymes are

not uncommon in bacteria. Several reports on cell-associated/cell-bound lipase, protease are available elsewhere (Kopecny and Wallace, 1982; Gibson and Macfarlane, 1988; Pereira-Meirelles *et al*., 2000; Sinsuwan *et al*., 2008).

Extraction of the enzyme from cells and subsequent purification is necessary to characterize the enzyme. Moreover, several applications require cell-free pure enzymes. Purification of the cell-associated enzyme is quite challenging as the removal of an enzyme from the membrane environment often lead to full or nearly complete loss of activity (Duetz *et al*., 2001). The cell-associated products could be released by disrupting the cells using high pressure homogenizer, bead mill, ultrasonicator, or Lysozyme (Harrison, 1991). Treatment of whole cells by organic solvents and surfactants is an attractive alternative to release the cell-associated enzymes. Microbial tannases are glycoproteins having molecular weights of 59-300 KD and the reported number of subunits varies from 2 to 8 (Van de Lagemaat and Pyle, 2006). Releasing enzyme molecules from the cell often leads to full or nearly complete loss of activity (Duetz *et al*., 2001). In view of its structural complexity, releasing tannase with out any loss of activity from the biomass is a challenge.

In the present study, we made an attempt to release the CAT from the biomass of one of our isolates, *Serratia ficaria* DTC using ultrasonicator, surfactants and solvents. Further, we tried to find the optimum concentration of Tween 80 and Triton X-100 for the treatment.

MATERIALS AND METHODS

Organism and cultivation: Several tannase positive bacteria have been isolated and characterized in our laboratory in the year 2007. All these strains were deposited and the identification has also been confirmed from Microbial Type Culture Collection and Gene bank (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India (Belur *et al*., 2010). Among them *Serratia ficaria* DTC (MTCC 8930) was chosen for the current study. It was cultivated in the medium containing (g L^{-1}) Tannic acid (Sigma), 5.0; $MgSO_4$, 0.5; NH_4NO_3, 3.0; KH_2PO_4, 0.5; K_2HPO_4, 0.5; mineral salt solution, 10; with the initial pH of 5.5.

Mineral salt solution was having the composition (g L^{-1}): $(NH_4)_6Mo\ O_{24}.4H_2O$, 0.1; $MnCl_2$, 2.0; $CuSO_4 5H_2O$, 0.2; $CoSO_4$, 2.8. Steam sterilized mineral salt medium and filter sterilized tannic acid were added after sterilization. Fermentation was carried out at 30°C in a rotary shaker at 150 rpm. The Dry Cell Weight (DCW) of the biomass was determined by drying the biomass at 95°C for 36 h.

Sonication: The cell suspension in 0.1 M citrate buffer (pH 3.5) was subjected to sonication using *Sonics* vibra cell, model VCX-130 (Sonics 130 W/50 Hz, USA) in an ice bath with an amplitude of 50% and using pulsed mode of 30:30 sec on: off for different time intervals. Cell suspension of 3.2 g of biomass (DCW) per liter was taken for the study.

After sonication, the suspension was centrifuged at 8000xg for 20 min at 4°C in order to precipitate intact cells and cell debris. The cell-free extract obtained was centrifuged at 30 000xg for 30 min at 4°C. The supernatant was taken to find the intracellular tannase activity. The precipitant thus obtained was employed as a membrane fraction. The precipitant was resuspended in 0.1 M citrate buffer (pH 3.5) and was taken to find the membrane bound tannase activity (Kim *et al*., 2006).

Treatment with surfactants and solvents: Cells were suspended in 0.1 M citrate buffer (pH 3.5) solution of various surfactants and solvents, incubated for 3 h with intermittent mixing at 4°C. The treated mixture was centrifuged at 8000xg for 20 min, the pellet was washed and resuspended in 0.1 M citrate buffer of pH 3.5 and was used to measure CAT activity. The DCW per ml of cell suspension determined was used to calculate the activity and expressed as U g^{-1}. Cell free supernatant was used to measure ECT activity. Overall activity is defined as the sum of CAT and ECT activity.

Tannase assay: Tannase activity was estimated by reaction with tannic acid as substrate followed by the reaction with methanolic rhodanine as described elsewhere (Van de Lagemaat and Pyle, 2001). The quantity of gallic acid released during hydrolysis of tannic acid represents the tannase activity. The sensitivity of the assay was 35.6x 10^{-6} U mL^{-1}. One unit of tannase activity is defined as 1 μM of gallic acid released per minute in the assay conditions. All the determinations were performed in duplicate with an experimental error below 5%.

Protein estimation: Bradford method was used to estimate protein content using BSA as the standard. Total protein content of the biomass was determined by incubating the biomass with NaOH-SDS (0.2 M, 2.5%) at 100°C for 5 min. The mixture was centrifuged at 8000xg for 20 min and supernatant was taken for protein estimation (Guerlava *et al.*, 1998). Total protein of the biomass was expressed as mg of protein/g of biomass (DCW).

RESULTS AND DISCUSSION

Sonication trials: The results are given in Table 1. The biomass suspension had an initial tannase activity of 9.5 U L^{-1}. Sonication treatment resulted in the loss of activity in the initial period. After 5 min of treatment, 82% of initial activity (CAT) was released and recorded as ECT activity. No CAT activity was found after 5 min of sonication. Sonication treatment for 10 min resulted in the release of 86.3% of the CAT activity from the cell with the concomitant release of 62.3% of cellular protein. This suggests that most of the cells were lysed releasing the intracellular contents. The presence of this large quantity of cell proteins makes purification of tannase

Table 1: The release of CAT from the biomass of *Serratia ficaria* DTC by sonication

Time[a] (min)	CAT[b] activity (UL^{-1})	ECT[c] activity (UL^{-1})	% of protein release[d]
0[e]	9.50	0	0
1	2.13	2.1	21.1
2	1.20	3.2	45.4
3	0.64	3.6	47.5
4	0.29	4.2	50.6
5	0	7.8	57.1
10	0	8.2	62.3
15	0	4.1	65.1
20	0	2.1	68.8
25	0	1.8	74.2
30	0	0	88.7
60	0	0	96.7

[a]Duration of sonication, [b]Cell-associated tannase activity measured after sonication, [c]Extracellular tannase activity measured after sonication, [d]Percentage of protein released after ultrasonication. 100% protein content was equivalent to 947 mg L^{-1} for this cell suspension, and [e]Control

Table 2: Extraction of tannase from the biomass of *Serratia ficaria* DTC using surfactant and solvents

Surfactants and solvents (% w/v)	CAT[a] activity (U L^{-1})	ECT[b] activity (U L^{-1})	% of protein release[c]
Control	1.45[d]	0	0
SDS (0.3)	0.04	0.29	48.1
Tween 60 (0.05)	1.43	0	12.5
Tween 80 (0.05)	1.53	1.14	11.2
Triton X-100 (0.05)	0.6	1.74	4.9
Sodium taurocholate (0.5)	0.14	1.31	19.4
Toluene (0.2)	0.13	0	12.5
Ethanol (5)	0.74	0	25.7

[a]Residual cell-associated tannase activity of the biomass, [b]Tannase activity measured in the supernatant, [c]100% protein content was equivalent to 144 mg L^{-1} for this cell suspension and [d]0.5 g biomass (DCW) showing CAT activity of 2.9 U g^{-1} was suspended in 1 l buffer

cumbersome. Sonication tannase (ECT). Release of cell-associated/cell-bound enzymes using sonication is a common technique in laboratory. However, reports pertaining to evaluation of sonication as a technique to release cell-associated enzyme is rather scarce. Present findings are in agreement with this report of Feliu *et al.* (1998). They reported the release of intracellular β-galactosidase enzyme from *E. coli* cells by sonication. They reported that a rapid release occurs in first 5 min and rate of release decreases drastically and reaches a steady value with in another 10 min.

Microbial tannases are glycoproteins having carbohydrate content up to 66% (w/w). Molecular weights of microbial tannases range from 59000-300000 Daltons and the reported number of subunits varies from 2 to 8 (Van de Lagemaat and Pyle, 2006). The sonication for longer duration has possibly altered the complex native structure of the enzyme, which resulted in the loss of activity completely. Reports of the degradation of the released enzyme after cell disruption by sonication are available (Ozbek and Ulgen, 2000). The inactivation of the enzyme may result from ionization and subsequent free radical formation (Harrison, 1991). Hence, sonication can not be used to release functionally active tannase from biomass.

Effect of surfactants and solvents: The results are given in Table 2. Triton X-100, Sodium taurocholate, Tween 80 and SDS could extract, while Ethanol, Toluene and Tween-60 failed to extract tannase from biomass. Treatment with 0.3% (w/v) SDS resulted in the release of 48.1% cellular proteins, indicating the lysis of cells. Present results are in agreement with Chen (2007), who observed that much lower concentration of solvents (<10%) were sufficient to release the cell-associated/intracellular products compared to yeast cells (>50%). Toluene and SDS caused reduction in overall enzyme activity by 90.7 and 76.6%, respectively. Perhaps these substances are inhibitory to the enzyme activity. Except SDS, all the other substances released 3.9-25.7% of total cellular proteins, suggesting that cell wall permeabilization has occurred. Treatment with Tween 80 and Triton X-100 increased the overall activity obtained, possibly due to unmasking of enzyme activity. Unmasking of activity might be due to change in permeability of the membranes which lead to higher access of substrate molecules to membrane bound tannase. Increase in overall activity after the treatment with surfactants has been reported by Kopecny and wallace (1982) in case of proteolytic enzymes of rumen bacteria.

In an effort to find optimum concentration of Tween 80 and Triton X-100, concentration in the range of 0.03-0.30% (w/v) was taken for treatment. The 0.05% (w/v) of Tween 80 treatment resulted in the release of 1.14 units with 1.53 units of unmasking of activity giving an over all activity of 2.67 units, which is 189% higher than the initial CAT activity. With respect to its original

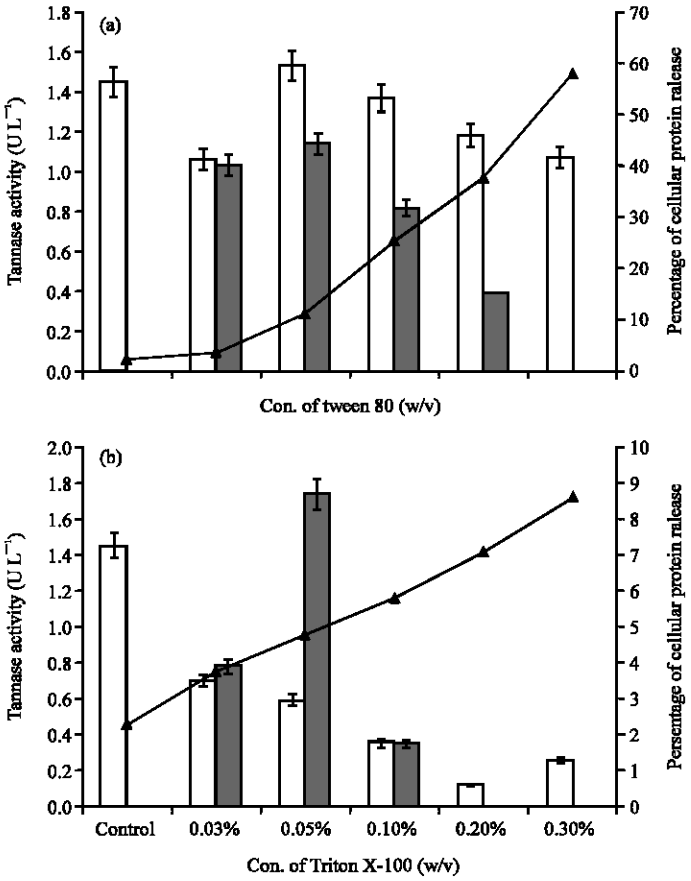

Fig. 1: Extraction of tannase from biomass of *Serratia ficaria* DTC using surfactants (a) Tween 80 and (b) Triton X-100. Cell-associated tannase activity □, tannase activity released ■ and percentage of cellular protein released ▲ are plotted against the surfactant concentration. Surfactant was added to cell suspension in 0.1 M citrate buffer (pH 3.5), incubated for 3 h at 30°C. 100% protein content was equivalent to 875 mg L^{-1} for this cell suspension

activity of 1.45 units, 78.6% was released by 0.05% (w/v) of Tween 80 treatment (Fig. 1a). Tween 80 concentration above 0.2% (w/v) resulted in the decrease in overall activity. Noudeh *et al.* (2008) have compared the effect of Tweens on red blood cells and found that Tween 80 has the lowest hemolytic effect compared to other Tweens.

Similar trend was noticed in case of Triton X-100 also. The 0.05% of Triton X-100 treatment resulted in the release of 1.74 units, which was measured as ECT activity. 0.6 units of the initial CAT activity was still associated with the biomass. If we consider both the CAT and ECT after Triton X-100 treatment, the over all activity was 2.34 units, which was 165% higher than the initial CAT activity (Fig.1b). With respect to the original activity of 1.45 units, all the activity was released 0.05% (w/v) of Triton X-100 treatment. Triton X-100 is known to solubilize proteins of the cytoplasmic membrane and solubilized 40% proteins of the cytoplasmic membranes of *E. coli* (Schnaitman, 1971). Miozzari *et al.* (1978) reported that Triton X-100 of concentration 0.05% (v/v) could permeabilize the yeast cells completely. Despite the of release of only 8.6% cellular proteins, all the CAT activity was released from the biomass, which clearly suggests that cell wall permeabilization had happened.

CONCLUSIONS

In conclusion, this study has demonstrated CAT can be released by sonication, treatment with SDS, Tween 80, Triton X-100 and sodium taurocholate. Among all these methods, treatment with 0.05% (w/v) Triton X-100 was resulted in complete release of initial CAT activity. As it had released only 4.8% of cellular proteins, purification of the tannase enzyme from other proteins becomes much easier. Hence Triton X-100 treatment could be the method of choice for the release of CAT from *Serratia ficaria* DTC. Unmasking of activity to the tune of 165-189%, due to Tween 80 and Triton X-100 treatment is of particular interest from application point of view. This is probably the first report on the release of tannase enzyme from the biomass.

ACKNOWLEDGMENTS

The authors are thankful to the Ministry of Human Resource Development (MHRD), Govt. of India for financial assistance.

REFERENCES

Aguilar, C.N., R. Rodriguez, G. Gutierrez-Sanchez, C. Augur and E. Favela-Torres *et al.*, 2007. Microbial tannases: Advances and perspectives. Applied Microbiol. Biotechnol., 76: 47-59.

Belmares, R., J.C. Conttreras-Esquivel, R. Rodriguez-Herrera, A.R. Coronel and C.N. Aguilar, 2004. Microbial production of tannase: An enzyme with potential use in food industry. Lebensm. Wiss. Technol., 37: 857-864.

Belur, P.D., G. Mugeraya, K.R. Nirmala and N. Basavaraj, 2010. Production of novel cell-associated tannase from newly isolated *Serratia ficaria* DTC. J. Microbiol. Biotechnol., 20: 722-726.

Bhatti, A.R., 1974. Purification and properties of the alkaline phosphatase of *Serratia marcescens*. Arch. Microbiol., 95: 255-266.

Braun, V. and G. Schmitz, 1980. Excretion of protease by *Serratia marcescens*. Arch. Microbiol., 124: 55-61.

Chen, R.R., 2007. Permeability issues in whole-cell bioprocesses and cellular membrane engineering. Applied Microbiol. Biotechnol., 74: 730-738.

Duetz, W.A., J.B. van Beilen and B. Witholt, 2001. Using proteins in their natural environment: Potential and limitations of microbial whole cell hydrosylations in applied bioctalysis. Curr. Opin. Biotechnol., 12: 419-425.

Feliu, J.X., R. Cubarsi and A. Villaverde, 1998. Optimized release of recombinant proteins by ultrasonication of *E. coli* cells. Biotechnol. Bioeng., 58: 536-540.

Gao, L., J. Xu, X. Li and Z. Liu, 2004. Optimization of *Serratia marcescens* lipase production for enentioselective hydrolysis of 3-phenylglycidic acid ester. J. Ind. Microbiol. Biotechnol., 31: 525-530.

Gibson, A.W. and G.T. Macfarlane, 1988. Studies on the proteolytic activity of *Bacteroides fragilis*. J. Gen. Microbiol., 134: 19-27.

Grimont, P.A.D., F. Grimont and M.P. Starr, 1979. *Serratia ficaria* sp. nov., a bacteria species associated with Smyrna figs and fig wasps *Balstophega scenes*. Curr. Microbiol., 2: 277-282.

Guerlava, P., V. Izac and T. Jean-Luc., 1998. Comparison of different methods of cell lysis and protein measurements in *Clostridium Perfringens*: Application to the cell volume determination. Curr. Microbiol., 36: 131-135.

Harrison, S.T.L., 1991. Bacterial cell disruption: A key unit operation in the recovery of intracellular products. Biotechnol. Adv., 9: 217-240.

Kim, S.H., S.H. Song and Y.J. Yoo, 2006. Characterization of membrane-bound nitrate reductase from denitrifying bacteria *Ochrobactrum anthropi* SY509. Biotechnol. Bioprocesses Eng., 11: 32-37.

Kopecny, J. and R.J. Wallace, 1982. Cellular location and some properties of proteolytic enzymes of rumen bacteria. Applied Environ. Microbiol., 43: 1026-1033.

Lekha, P.K. and B.K. Lonsane, 1994. Comparative titers, location and properties of *Tannin acyl* hydrolase produced by *Aspergillus niger* PKL 104 in Solid state, liquid surface and Submerged Fermentation. Process Biochem., 29: 497-503.

Mehmood, M., X. Xio, F.Y. Hafeez, Y. Gai and F. Wang, 2009. Purification and characterization of a chitinase from *Serratia proteomaculans*. World. J. Microbiol. Biotechnol., 25: 1955-1961.

Miozzari, G., P. Nuederberger and R. Hutter, 1978. Permeabilization of microorganisms by triton X-100. Anal. Biochem., 90: 220-233.

Noudeh, G.D., P. Khazaeli and P. Rahmani, 2008. Study of the effect of polyethylene glycol sorbitan esters surfactants group on biological membranes. Int. J. Phramacol., 4: 27-33.

Ozbek, B. and K.O. Ulgen, 2000. The stability of enzymes after sonication. Process Biochem., 35: 1037-1043.

Pereira-Meirelles, F.V., M.H.M. Rocha-Leao and G.L. Jr. Sant'Anna, 2000. Lipase location in *Yarrowia lipolytica* cells, Biotechnol. Lett., 22: 71-75.

Schnaitman, C.A., 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol., 108: 545-552.

Seth, M. and S. Chand, 2000. Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori*-optimization of process parameters. Process Biochem., 36: 39-44.

Sinsuwan, S., S. Rodtong and J. Yongsawatdigul, 2008. Characterization of Ca^{2+}- activated cell-bound proteinase from *Virgibacillus* sp. SK37 isolated from fish sauce fermentation. LWT-Food Sci. Technol., 41: 2166-2174.

Van de Lagemaat, J. and D.L. Pyle, 2001. Solid state fermentation and bioremediation: Development of continuous process for the production of fungal tannase. Chem. Eng. J., 84: 115-123.

Van de Lagemaat, J. and D.L. Pyle, 2006. Tannase. In: Enzyme Technology, Pandey, A., C. Webb, C.R. Soccol and C. Larroche (Eds.). 1st Edn., Springer, New York, pp: 380-397.