Appl Biochem Biotechnol (2011) 164:68–76
DOI 10.1007/s12010-010-9115-9

Purification of ß-glucosidases from *Pichia etchellsii* Using CIM Monolith Columns

Roopa K. Gaonkar · Saroj Mishra · M. A. Vijayalakshmi

Received: 9 June 2010 / Accepted: 19 October 2010 /
Published online: 1 November 2010

Abstract β-Glucosidases (EC 3.2.1.21) are industrially important glycosyl hydrolases used for cellulose saccharification as well as for synthesis of glyco-conjugates. Crystal structure of only one β-glucosidase of family 3 of the glycosyl hydrolase families is available due to difficulty in purification of these closely related enzymes from a given source. Multiple steps used during purification result in low yield, making it difficult to study their properties. Conditions for purification of two closely related β-glucosidases (BGL I and BGL II) of family 3 from *Pichia etchellsii* were investigated in this study. Two weak anion exchange columns convective interaction media-diethyl amino ethyl (CIM-DEAE) and CIM-ethylenediamine (CIM-EDA) were used for this purpose. The results obtained at 0.34 ml disk (CIM-DEAE) level were scaled up to 8 ml CIM-DEAE tube column wherein BGL I and BGL II were separated from the major contaminants in the cell-free extract. The recovered enzymes were completely resolved in the second step using CIM-EDA. A final specific activity of 9,180 IU/mg and 2,345.3 IU/mg was achieved for BGL I and BGL II respectively with an overall yield of 33%. The system should be applicable to resolution of other closely related enzymes from this family.

Keywords *Pichia etchellsii* · Family 3 β-glucosidases · CIM-DEAE · CIM-EDA · Chromatographic scale up

R. K. Gaonkar · M. A. Vijayalakshmi
Centre for Bioseparation Technology, VIT University, Vellore, Tamil Nadu 632014, India

S. Mishra (✉)
Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz-Khas, New Delhi 110016, India
e-mail: saroj98@hotmail.com

M. A. Vijayalakshmi
Département Génie Biologique, LIMTech.S, Centre de Recherches de Royallieu, UTC, Compiegne, France

Abbreviations

CIM-DEAE	Convective interaction media-diethylaminoethyl
CIM-EDA	Convective interaction media-ethylenediamine
*p*NPG	*p*-Nitrophenyl-β-D-glucopyranoside

Introduction

The enzyme β-glucosidase [EC.3.2.1.21] catalyzes the hydrolysis of glucosides containing residues linked by β (1–4) linkages. These enzymes are classified in to family 1 and family 3 of the glycosyl hydrolase (carbohydrate degrading enzyme) families and occur in animals, plants, and microbes. A number of important physiological processes are mediated through these enzymes. In cellulolytic microorganisms, this enzyme is involved in the terminal step of cellulose hydrolysis by breaking down cellooligosaccharides and cellobiose to glucose [1, 2]. These enzymes have been studied extensively due to their possible role in cellulase induction [3], cellulose breakdown, ability to release aromatic compounds from their flavorless glucosidic precursors, which include monoterpenol, C-13 norisoprenoids, and shikimate-derived compounds linked to mono- or di-glycosides [4]. Many of these properties make it useful in biotechnological applications. Supplementing β-glucosidase in commercial cellulase preparations has been reported to result in increased rate of cellulose hydrolysis and ethanol production. Direct conversion of cellulose to ethanol has been demonstrated in recombinant *Saccharomyces cerevisiae* expressing heterologous genes of exo/endo-glucanase and β-glucosidase [5, 6]. Many β-glucosidases are being increasingly used for carbohydrate synthesis. Compared to glycosyltransferases, which are dependent on cofactors, the ready availability of microbial β-glucosidases, lack of dependence on expensive cofactors makes them enzymes of choice for synthetic purposes. Several factors regulate the synthetic processes such as water activity, presence of organic solvents, and increasing the substrate concentration (for review, see [7]).

Rationale approach of engineering these enzymes for achieving high yield of glycoconjugates requires knowledge of their structures, including groups involved in catalysis. A large amount of information is available for family 1 β-glucosidases. Members of this family have polypeptide chains less than 700 amino acids, organized into a single triosephosphate isomerase barrel. Sequence similarity between 17% to 30% exists among the members of this family. These hydrolyze the β-1–4 glycosidic linkage with an overall retention of anomeric configuration. Some members of this family have been used for carbohydrate synthesis. Although the action of family 3 enzymes is similar (retaining enzymes) to that of the family 1, these do not belong to any one of the known glycosyl hydrolase clans, indicating unusual folds. Members of family 3 share very little sequence similarity. Only one crystal structure of *Hordeum vulgare* [8] has been solved.

Pichia etchellsii is a thermo-tolerant yeast that grows on simple phosphate succinate medium and produces multiple β-glucosidases. Two cell wall-bound inducible β-glucosidases viz. BGL I and BGL II have been reported [9], and both belong to family 3 of the glycosyl hydrolase families [10, 11]. The enzymes have been used for synthesis of alkyl glucosides [9, 12, 13] albeit with low yields. Previous attempts in purification of these enzymes involved multiple chromatographic steps (sepharose-DEAE/sepharose-Q, sephadex G-200, hydroxyapatite chromatography), and low yields. Here, we describe a two-step chromatographic method for obtaining homogeneous BGL I and BGL II from cell-free extract of *P. etchellsii* using CIM monoliths. CIM has emerged as one of the popular monolith supports for purification of macromolecules. Because of the large pores (up to 1.5 μm in diameter), these columns are suitable for binding large biomolecules like

proteins, polysaccharides, and oligosaccharides. Unlike soft gels, mechanical stability is not affected by the increased porosity, and as there is no diffusional limitation, the scale up is linear [14–16]. Because capacity and resolution are flow-independent properties, the flow rates of up to 10-fold higher (than the particle based supports) can be used with negligible pressure drops.

Materials and Methods

All chemicals used in the experiments were of analytical grade. CIM-disks and tubes were obtained from BIA Separations, Slovenia. *p*-Nitrophenyl-ß-D-glucopyranoside (*p*NPG) and *p*-nitrophenol were obtained from Sigma. Cellobiose was obtained from Fluka. The glucose oxidase–peroxidase kit was obtained from Span Diagnostics, India. Amicon ultrafiltration units and the membranes for filtration were obtained from Millipore.

Culturing of *P. etchellsii*

P. etchellsii JFG-2201 (Deutsche Sammlung Von Mikroorganismen) was revived according to standard procedures [17], and the glycerol stocks were made and stored at −80°C. Culturing was done according to the protocol followed by Wallecha and Mishra [9], and the cells were maintained on yeast extract, peptone, dextrose (YPD) slants for routine use. For enzyme production, the yeast was grown in phosphate-succinate medium with the composition, yeast extract 2.5 g, peptone 5 g, succinic acid trisodium salt 8.2 g, $CaCl_2$ 0.3 g, K_2HPO_4 8.7 g, $(NH_4)_2SO_4$ 4 g, and MgSO4 0.5 g/l. The pH of the medium was adjusted to 4.7 using HCl. The primary culture was prepared by inoculating a single colony from a YPD slant into 50 ml medium contained in a 250-ml flask. The culture was allowed to grow at 40 C and 220 rpm for 8 h. Enzyme production was started by inoculating 2% (*v*/*v*) of the primary culture into 400 ml production medium contained in 2 l flasks. The cells were grown at 40 C and 220 rpm for 14 h, and the growth was monitored by measuring OD_{600}. The culture was induced with 10 mM cellobiose and after an additional incubation of 8 h, the cells were harvested by centrifugation at 9,000×*g* and washed with 50 mM sodium phosphate buffer, pH 7.

Cell Lysis and Cell Free Extract Preparation

The cell biomass from 1.8 l media was suspended in 50 mM phosphate buffer, pH 7, and subjected to sonication using Vibracell sonicator (titanium probe diameter of 6 mm) with a pulse length of 10 s and power output of 23 W. Sonication was done in 7 cycles (3 min lysis followed by 2 min of cooling) in the ice bath. After cell lysis, the cell debris was removed by centrifuging the sonicated extract at 5000×*g* at 4 C for 15 min. The cell-free extract was distributed into 50 ml aliquots and stored at −20 C.

Purification of BGL I and BGL II

Step I—CIM-DEAE Chromatography

The extract was taken out from −20 C followed by rapid thawing and filtered through 0.45 μ membrane filters. The purification conditions were optimized using a 0.34 ml CIM-DEAE disk. Different conditions for binding and elution were studied at analytical scale. The optimum binding for BGL I and BGL II was determined by using 25 and 50 mM of

phosphate buffers, pH 7.2. Elution was carried out using NaCl step gradient of 0.1, 0.2, 0.3, and 1 M NaCl in respective phosphate buffers at a flow rate of 10 ml/min. Samples of 1 ml were collected to determine protein and β-glucosidase activity. Once the binding and elution conditions were optimized, the scaling-up was done using 8 ml tube CIM-DEAE column with the same binding and elution conditions. The protein concentration loaded on the column was optimized (from initial values of 10, 14, 32 mg) for reduction of contaminants in the fractions containing BGL I and BGL II. Chromatography was performed on AKTA-Fast Protein Liquid Chromatography (FPLC) system.

Step II—CIM-EDA Chromatography

The 0.2-M NaCl eluted fractions from CIM-DEAE, displaying activity on both *p*NPG and cellobiose, were pooled, concentrated, and buffer exchanged using Amicon 10 kDa ultra filtration units. CIM-EDA disk was equilibrated with 25 mM MOPS buffer, pH 6.5, and the sample, which was in the same buffer, was injected. Washing of the unbound proteins was done with the MOPS buffer, and elution was performed using same buffer and 0–1 M NaCl linear gradient in 10 min. The flow rate of 3 ml/min was maintained. Fractions of 0.5 ml were sampled for analysis. In order to resolve closely appearing peaks of BGL I and BGL II, different initial salt concentrations and different elution conditions were used.

Analysis of Protein and Enzyme Activity

SDS-PAGE Analysis

The proteins were analyzed for purity using SDS-PAGE as described [18] using 8% resolving gel under non-reducing conditions. The gel was stained using silver nitrate fast staining method [19].

Homogeneity and Zymogram Analysis

The homogeneity of the purified enzymes was determined by running on 8% SDS-PAGE. For zymogram analysis, the pure fractions were run on native PAGE (6%) in identical halves of the gel. The gel was cut in the middle, and one half was stained with Coomassie brilliant blue R-250 to detect the proteins. The other half was incubated with 4 mM *p*NPG at 50°C for 30 min. One molar Na_2CO_3 was added to stop the reaction. The appearance of yellow color confirmed β-glucosidase activity [20].

Protein Determination

The OD_{280} was noted down for all the eluted fractions from CIM-DEAE column. Protein was determined with Coomassie brilliant blue G-250 as described by Bradford [21] using bovine serum albumin as the standard.

β-Glucosidase Assay

The progress of purification of BGL I and BGL II was monitored by assaying activity on cellobiose and *p*NPG as described [9]. With cellobiose as substrate, the liberated glucose was measured by glucose oxidase peroxidase kit as per manufacturer's directions. One unit

of activity was defined as the enzyme that liberated 1 μmole of glucose per milliliter per minute. Activity on *p*NPG was measured by following release of *p*-nitrophenol. One unit of enzyme activity was defined as the enzyme that released 1 μmole of *p*-nitrophenol per milliliter per minute under assay conditions.

Shelf Life of Enzymes

Different temperature conditions were tested for storage of partially purified and purified enzymes. The partially purified enzyme was subjected to ammonium sulfate precipitation and the precipitate was stored at 4°C. Fractions containing purified enzymes were stored at −20 C in the buffer in which they were eluted.

Results and Discussion

CIM has emerged as one of the important support medium for purification of large macromolecules like proteins, polysaccharides, and oligosaccharides. The major advantage of this support is that the mechanical stability is not affected by large pore sizes. No diffusional limitations are reported and hence scale up can be expected to be linear [14]. Negligible pressure drops, even with high flow rates, are an added advantage. The simplicity of assembling the column and productivity achieved in terms of time and capacity, without compromising the resolution, make CIM monoliths a better support for scale up of purification. The purification of β-glucosidases usually requires multiple chromatographies. Initial precipitation by ammonium sulfate followed by a minimum of two chromatographic steps has been reported [22, 23]. Affinity chromatography has also been coupled as a last step [24]. Use of hydroxyapatite is also reported as many β-glucosidases bind to hydroxyapatite [9, 22]. The yield values range from 1.7% with a purification fold of 144 [23] to 46% with a purification factor of 16 [25]. However, no comment was made in these studies about presence of other β-glucosidases and the efficiency of the procedure in terms of resolving these. In our previous study [9], a yield of 17% with a purification fold of 30 for BGL I and a yield of 7.5% with purification fold of 19.4 for BGL II was reported. In this study, we demonstrate purification of these two closely related β-glucosidases using CIM monolith columns.

Step I—CIM-DEAE Chromatography

The wet biomass of 24 g was obtained from 1.8 l of culture. This was subjected to ultrasonication and the cell extract, obtained after centrifugation, was used for chromatographic purification. Initial trials were done using 0.34 ml CIM-DEAE disks.

Optimization of Binding and Elution Conditions

Among the 50 and 25 mM phosphate buffers of pH 7.2, the 25 mM buffer facilitated stronger binding of both BGL I and BGL II, and the contaminating proteins exhibited lesser binding and were eluted first. With 50 mM phosphate buffer, the proteins of interest were eluted at 0.1 M NaCl along with contaminant proteins. The difference in the binding strength with a change in the molarity of the buffer may be due to the fact that the ions of the buffer mask the protein binding sites to some extent so that both

BGL I and BGL II get eluted at lower salt strengths. As the column is of ion-exchange chemistry, this kind of competition by the buffer ions for binding on the matrix is not unexpected.

Scale-up Experiments

A total of 32 mg of protein was loaded on the CIM-DEAE tube equilibrated with 25 mM phosphate buffer, pH 7.2. The elution was done with an increasing step gradient of NaCl (0.1, 0.2, 0.3, and 1 M) in 25 mM phosphate buffer, and the results are shown in Fig. 1a. Enzyme activities of eluted fractions were checked with both *p*NPG and cellobiose. The fractions eluted with 0.2 M NaCl showed activity on both *p*NPG and cellobiose. Representative samples were run from each group on SDS-PAGE, and the results are shown in Fig. 1b. As seen, two protein bands of 97 and 90 kDa, corresponding to BGL I and BGL II respectively, were detected (Fig. 1b, lanes 3 and 4) in fractions eluted with 0.2 M NaCl. Very few contaminating proteins were observed. These results are very different from our earlier results wherein DEAE-sepharose chromatography was used and resulted in elution of large number of other contaminating proteins [9] which had to be subsequently removed.

When lesser concentration of protein was injected, the contaminant proteins which were seen in the non-retained fractions at high enzyme loadings, co-eluted with β-glucosidases in 0.1 and 0.2 M NaCl elutions. This indicated that at lower protein loadings, proteins with comparatively less binding strength could adsorb to the DEAE ligands and get eluted with the enzymes of our interest. Thus, the protein concentrating loaded on to the column is also an important factor during purification.

Step II—CIM-EDA Chromatography

Binding and Elution

The 0.2 M NaCl eluted fractions from the CIM-DEAE chromatography step were pooled (30 ml) and concentrated to 2 ml using Amicon ultra-15 centrifugal filtration unit (10 kDa cut off). As shown in Table 1, 84% yield was obtained at this step with a purification fold of 13.5. An effective concentration of the protein was also achieved and a high specific

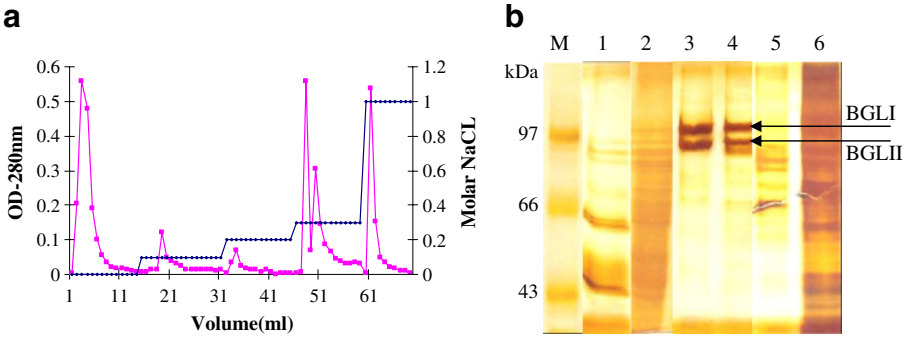

Fig. 1 **a** Chromatogram of 50 ml crude extract (containing a total protein of 32 mg) loaded on CIM-DEAE 8 ml tube. Flow rate, 10 ml/min, buffers: binding, 25 mM phosphate buffer, pH 7.2. Elution was performed with 25 mM phosphate buffer, pH 7.2 with NaCl step gradient (0.1, 0.2, 0.3, 1 M). **b** SDS-PAGE 8% resolving gel stained using silver nitrate for CIM-DEAE elutions. *M* Marker, *lane 1* non retained, *lane 2* 0.1 M NaCl, *lanes 3, 4* 0.2 M NaCl, *lane 5* 0.3 M NaCl, *lane 6* 1 M NaCl

activity of nearly 4,000 IU/ml was obtained. This is by far the highest activity reported after the first chromatographic/ultrafiltration step.

The concentrated protein was loaded on the CIM-EDA 0.34 ml disk after equilibrating the disk with 25 mM MOPS buffer, pH 6.5. The elution was done with the same buffer using a continuous gradient (0–1 M) of NaCl. The two enzymes were separated from each other as shown in Fig. 2a (peaks 2 and 3). Selected fractions were run on the gel which showed homogeneity of BGL I (Fig. 2b, lanes 3, 4) and BGL II (Fig. 2b, lanes 5, 6). The bands with molecular masses of ~97 and ~90 kDa correspond to the monomeric forms of BGL I and BGL II [9]. Addition of mercaptoethanol did not change the band pattern, indicating that subunits are not bound by disulfide bridges. As observed in peak 2 (Fig. 2a), BGL I appeared as a sharper peak than BGL II, and this was confirmed by the electrophoresis data. The peak fractions (of peak 2 and 3) were also run on native PAGE (Fig. 3a, lanes 1 and 2) and showed distinct bands when stained with Coomassie R-250. On zymogram analysis with *p*NPG, both lanes confirmed the presence of β-glucosidase activity (Fig. 3b, lanes 1 and 2).

Shallowing of the NaCl gradient by increasing the gradient time from 10 to 15 min did not show any improvement in the chromatogram pattern over the results of Fig. 2a. So optimization of chromatographic conditions for CIM-EDA anion exchange chromatography was done using different initial salt concentrations and different elution conditions. The chromatograms obtained under different conditions indicated that at 5% initial salt concentration, additional protein peak (s) appeared before elution of BGL I. This was removed by increasing the initial salt concentration to 14%. The conditions of elutions were very critical for the complete separation of the two peaks. Considering the constraints of gradients on separation, the conditions used were initial 14% of salt followed by elution with 20% NaCl till BGL I peak reached baseline and 40% NaCl till BGL II reached the baseline. Although under these conditions, the two enzymes appeared to be well separated, the electrophoretic data indicated co-elution in many fractions (data not shown). Hence, this method of separation was not considered for determining the final yields of the two enzymes. The yields reported (Table 1) are the pure fractions of single enzymes excluding the fractions where both the enzymes co-eluted.

A summary of purification steps with yields obtained at each stage is given in Table 1. While BGL I is reported [9] to be more active on *p*NPG and BGL II on cellobiose, the purification summary is reported using *p*NPG as substrate. As seen, a total 84% recovery was seen after the first chromatographic step, although this step was followed by an ultrafiltration step as well, where some loss may have occurred. After the second chromatographic step, a total yield of 33.7% was obtained. This may not be entirely due to the system being inefficient as this yeast produces multiple β-glucosidases and the initial activity in the cell-free extract is likely to be due to these "minor" enzymes. Three

Table 1 Summary of Purification of BGL I and BGL II as monitored with pNPG as substrate

Steps	Vol (ml)	Protein (mg/ml)	Total protein (mg)	Activity (IU/ml)	Total activity (IU)	Specific activity (IU/mg)	Yield (%)	Purification fold
Cell-free extract	50	0.65	32.5	190.5	9,525.0	293.0	100	1.0
CIM-DEAE (0.2 M NaCl)	2	1.01	2.02	4,009.5	8,019.0	3,969.8	84.19	13.5
CIM-EDA								
BGL I (peak 2)	1	0.22	0.22	2,019.6	2,019.6	9,180.0		31.3
BGL II (peak 3)	3	0.17	0.51	398.7	1,196.1	2,345.3		8.0

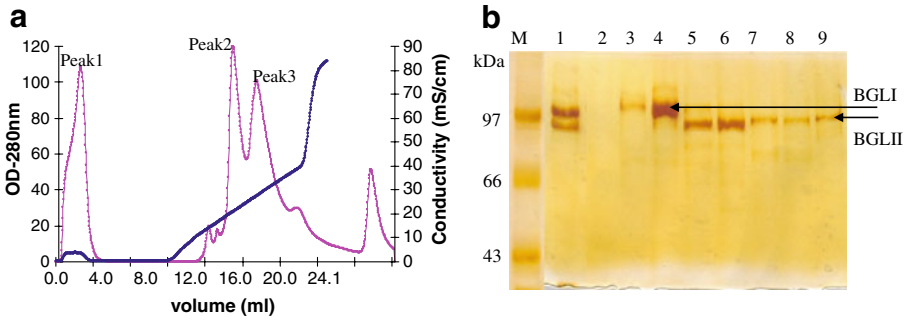

Fig. 2 **a** Chromatogram of 0.2 M NaCl eluted fractions from CIM-DEAE loaded on CIM-EDA column. Flow rate, 3 ml/min. Buffers: binding, 25 mM MOPS, pH 6.5. Elution was performed with binding buffer+ 1 M NaCl continuous gradient (0–1 M in 10 min). **b** SDS-PAGE analysis of CIM-EDA fractions: 8% resolving gel was used and the gel was stained using silver nitrate. *M* Marker, *lane 1* load, *lane 2* blank, *lanes 3*, *4* samples from *peak 2*, *lanes 5–9* samples from *peak 3*

additional β-glucosidases (and β-glucosidase-like enzymes) have also been identified in this yeast by expression in *Escherichia coli* [26–28]. All the chromatographic steps were reproducible with consistent separation pattern and purity as observed by SDS-PAGE. The activity of the pure enzymes was retained for 1 week without any significant loss at 4 C. However, when purified enzymes were stored at −20 C, only the enzyme fractions having concentration of protein (>0.2 mg/ml) retained activity. This is expected as proteins are known to denature in dilute solutions. The fractions containing both the enzymes (CIM-DEAE elutions) retained their activity for more than a year at 4 C in ammonium sulfate precipitated form.

Conclusion

CIM monolith columns were used for purification of two closely related β-glucosidases from *P. etchellsii.* In the first step, the two β-glucosidases were effectively separated from cell-free extract proteins. The process was linearly scaled up to 8 ml column. Factors such as buffer ion concentration and total protein loading affected this separation process. An

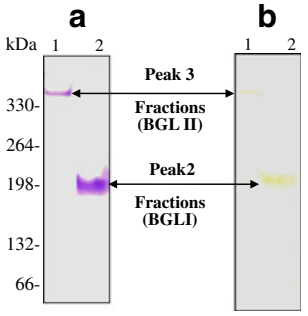

Fig. 3 **a** Native PAGE 6% resolving gel stained with Coomassie brilliant blue showing two bands for two proteins corresponding to peaks 2 and 3 from CIM-EDA chromatography. **b** Zymogram on native PAGE 6%. The gel was stained with *p*NPG showing two *yellow bands* for BGL I and BGL II corresponding to two peaks from CIM-EDA chromatography

overall recovery of 84% was obtained. The two enzymes were further resolved in the next chromatographic step on CIM-EDA within a narrow range of salt gradient with an overall yield of 33%. Some cellobiose hydrolyzing activities were lost during the initial steps. The procedure is amenable to linear scale up and gives a promise for separation and achieving high yield of this category of enzymes.

Acknowledgements This work was supported by a funding from Department of Science and Technology, (No.SR/SO/BB-21/2006 dated 10-08-2007) Government of India. We thank Dr. Ales Podgornik (BIA separations, Slovenia) for giving required technical details regarding CIM monoliths and Prof. Nadir T. Mrabet (Nancy University, School of Medicine, France) for his help in designing the optimization experiments on CIM-EDA chromatogram using different gradients.

References

1. Tomme, P., Warren, R. A. J., & Gilkes, N. R. (1995). *Advances in Microbial Physiology, 37*, 1–81.
2. Bisaria, V. S., & Mishra, S. (1989). *CRC Critical Reviews in Biotechnology, 5*, 61–103.
3. Sahoo, D. K., Mishra, S., & Bisaria, V. S. (1986). *Microbiology, 132*, 2761–2766.
4. Gueguen, Y., Chemardin, P., Janbon, G., Arnaud, A., & Galzy, P. (1996). *Journal of Agricultural and Food Chemistry, 44*, 2336–2340.
5. Fujita, Y., Ito, J., Ueda, M., Fukuda, H., & Kondo, A. (2004). *Applied and Environmental Microbiology, 70*, 1207–1212.
6. Den Haan, R., Rose, S. H., Lynd, L. R., & Van Zyl, W. H. (2007). *Metabolic Engineering, 9*, 87–94.
7. Klibanov, A. M. (2001). *Nature, 409*, 241–246.
8. Varghese, J. N., Hrmova, M., & Fincher, G. B. (1999). *Structure, 7*, 179–190.
9. Wallecha, A., & Mishra, S. (2003). *Biochimica et Biophysica Acta, 1649*, 74–84.
10. Baranwal R., Mishra S., GenBank Accession No.EU914813.
11. Asif M. (2010), Ph.D. Thesis, Indian Institute of Technology Delhi, India.
12. Wallecha A. (2000), Ph.D. thesis, Indian Institute of Technology Delhi, India.
13. Mishra A.K., Baranwal R., Asif M., Schneider K., Keller U., Chand S., Mishra S. and Roderich D. (2010), Biotech. Bioengg. submitted 8th Jan 2010, under review.
14. Tennikova, T. B., & Reusch, J. (2005). *Journal of Chromatography A, 1065*, 13–17.
15. Strancar A., Podgornik A., Barut M. and Necina R. (2002), Advance in Biochemical Engineering, Biotechnology 76, 49–85.
16. Isobea, K., & Kawakami, Y. (2005). *Journal of Chromatography A, 1065*, 129–134.
17. Burke, D. (2000). Methods in Yeast genetics: A Cold Springer Harbor Laboratory Course Manual, 2 ed., CSHL Press, NY.
18. Laemmli, U. K. (1970). *Nature, 227*, 680–685.
19. Nesterenko, M. V., Tilley, M., & Upton, S. J. (1994). *Journal of Biochemical and Biophysical Methods, 28*, 239–242.
20. Pandey, M., & Mishra, S. (1995). *Journal of Fermentation and Bioengineering, 80*, 446–453.
21. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
22. Daniels, B. L., Coyle, J. P., Chiao, Y. B., Glew, R. H., & Labow, R. S. (1981). *The Journal of Biological Chemistry, 256*, 13004–13013.
23. Yan, Q., Zhou, W., Li, X., Feng, M., & Zhou, P. (2008). *Bioscience, Biotechnology, and Biochemistry, 72*, 352–359.
24. Saha, B. C., & Bothast, R. J. (1996). *Applied and Environmental Microbiology, 62*, 3165–3170.
25. Bhat, K. M., Gaikwad, J. S., & Maheshwari, R. (1993). *Journal of General Microbiology, 139*, 2825–2832.
26. Pandey, M., & Mishra, S. (1997). *Gene, 190*, 45–51.
27. Bhatia, Y., Mishra, S., & Bisaria, V. S. (2005). *Applied Microbiology and Biotechnology, 66*, 527–535.
28. Roy, P., Mishra, S., & Chaudhuri, T. K. (2005). *Biochemical and Biophysical Research Communications, 336*, 299–308.