Appl Biochem Biotechnol (2008) 147:23–32
DOI 10.1007/s12010-007-8081-3

Lignin Peroxidase from *Streptomyces viridosporus* T7A: Enzyme Concentration Using Ultrafiltration

Leda M. F. Gottschalk · Elba P. S. Bon · Ronaldo Nobrega

Received: 18 May 2007 / Accepted: 10 October 2007 /
Published online: 20 March 2008

Abstract It is well known that lignin degradation is a key step in the natural process of biomass decay whereby oxidative enzymes such as laccases and high redox potential ligninolytic peroxidases and oxidases play a central role. More recently, the importance of these enzymes has increased because of their prospective industrial use for the degradation of the biomass lignin to increase the accessibility of the cellulose and hemicellulose moieties to be used as renewable material for the production of fuels and chemicals. These biocatalysts also present potential application on environmental biocatalysis for the degradation of xenobiotics and recalcitrant pollutants. However, the cost for these enzymes production, separation, and concentration must be low to permit its industrial use. This work studied the concentration of lignin peroxidase (LiP), produced by *Streptomyces viridosporus* T7A, by ultrafiltration, in a laboratory-stirred cell, loaded with polysulfone (PS) or cellulose acetate (CA) membranes with molecular weight cutoffs (MWCO) of 10, 20, and 50 KDa. Experiments were carried out at 25 °C and pH 7.0 in accordance to the enzyme stability profile. The best process conditions and enzyme yield were obtained using a PS membrane with 10 KDa MWCO, whereby it was observed a tenfold LiP activity increase, reaching 1,000 U/L and 90% enzyme activity upholding.

Keywords *Streptomyces viridosporus* · Lignin peroxidase · Ultrafiltration · Enzyme concentration · Enzyme stability

L. M. F. Gottschalk (✉) · E. P. S. Bon
Departamento de Bioquímica, IQ, CT, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21949-900, Brazil
e-mail: ledagott@gmail.com

R. Nobrega
Programa de Engenharia Química, COPPE, CT, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

Introduction

There has been a growing interest on the use of lignin peroxidase (LiP), manganese peroxidase, and laccase because of their ability to degrade both lignin and highly toxic phenolic compounds [1]. Regarding the use of biomass as a renewable material for the production of fuels and chemicals, the research on LiPs is of paramount importance because of their potential use for the degradation of the biomass lignin. It is well known that lignocellulose is a complex composite designed to resist degradation and that the enzymatic attack is effective for lignin removal. In biomass-free lignin, cellulose and hemicellulose moieties would be more prone to the attack by cellulases and hemicellulase enzymes. The production of ethanol from plant structural polysaccharides has become essential to improve energy availability, decrease air pollution, and diminish atmospheric CO_2 accumulation [2]. Regarding environmental pollution, the human activity has introduced a great variety of xenobiotic and recalcitrant chemicals into the environment, on a large scale, so that it is necessary to develop innovative bio-based technologies for its degradation [3]. Although the catalytic profile of peroxidase is potentially useful for the pretreatment of lignocellulosic materials [4] and in environmental pollution control [5], its application in industrial scale requires the production of a stable and low-cost biocatalyst.

Since its first characterization [6], several aspects of *Streptomyces viridosporus* T7A LiP production and characterization have been studied [7–10]. There is not a consensus regarding LiP molecular mass as different data have been reported according to the enzyme purification procedure. Although a molecular weight of 17,800 was estimated for the enzyme by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [6], different values were observed for LiP isoenzymes when they were purified by preparative PAGE and electroelution using unboiled (75,800) and boiled (67,100 and 37,100) samples. In addition, the Fergusson plot for the native polyacrylamide electrophoresis indicated a molecular weight of 134,000 [11]. The effect of the temperature and pH on the enzyme stability has been investigated. The enzyme showed to be stable in the pH range 3.0 to 8.2 in temperatures below 40 °C [10, 12]. Sensitivity toward temperature was observed upon the enzyme incubation at 50 °C for 30min at pH 5.5 or 7.0, which resulted on 25 and 38% activity loss, respectively [10, 12].

S. viridosporus LiP has been concentrated by ultrafiltration (UF) for further studies on the enzyme purification and the determination of its chemical and biochemical properties [10, 11, 13, 14]. However, there is no report on the UF operational conditions and on the enzyme activity recovery.

In enzymes, downstream processing activity loss is often observed. As the degree of product loss is critical to the economics of the separation process, the choice of the technique is guided by the preservation of the enzyme structure that is associated to their biological function [15]. One such technique, membrane technology, has been largely used because of its benefits such as: separation can be carried out under mild conditions and in a continuous mode, energy consumption is generally low, up-scaling is easy, membrane proprieties are variable and can be adjusted, no additives are required, and it can be easily combined with other separation processes [16]. Within this context, UF is a cost-effective method that allows both concentration and primary purification of enzymes under low temperature and pressure conditions. UF is also easy to scale-up in comparison to chromatography and electrophoresis. However, UF may present concentration polarization and fouling, which reduce the permeate flux below the theoretical capacity and modify membrane selectivity. This flux decline has a negative influence on the economics of a

given membrane operation and should be prevented [16]. The disadvantages of UF strongly depend on operation conditions and membrane characteristics, such as feed properties, membrane molecular weight cutoff (MWCO), transmembrane pressure (TMP) and cross-flow rate [17].

Our research group has studied *Streptomyces viridosporus* T7A LiP aiming the enzyme chemical characterization and the reduction in the enzyme production cost [7–9, 14]. In this study, LiP concentration by UF was investigated focusing on the effect of UF membrane MWCO and operation conditions. The recovery of fouled membranes using cleaning agents was also accessed.

Materials and Methods

Microorganism and Enzyme Production

S. viridosporus T7A (ATCC 39115) stock spores was maintained at −20 °C in a 20% (w/v) glycerol aqueous solution [18] after cell growth at 37 °C for 6–8 days on a medium composed of malt extract (3 g/L), yeast extract (3 g/L), peptone (5 g/L), glucose (10 g/L), and agar-agar (20 g/L). LiP was produced in batch submerged fermentations at 37 °C (bioreactor model Applikon, 3 L working volume) using an optimized growth medium (6.5 g/L yeast extract, 1 g/L corn oil, 5 g/L calcium carbonate, 0.20 g/L $MgSO_4.7H_2O$; 0.20 g/L NaCl; 0.05 g/L $CaCl_2$, and trace metal stock solution). The use of adequate aeration/agitation conditions (1.0 vvm and 400 rpm) allowed optimization of enzyme production and productivity [19]. Peak enzyme production was observed within 32 h of fermentation. At this time, the culture was filtered in Whatman no. 1 paper. This supernatant was used for UF experiments.

Lignin Peroxidase Activity

The LiP activity was determined by the enzymatic oxidation of 2,4-dichlorophenol (2,4-DCP) in the presence of H_2O_2 and 4-aminoantypirene (4-AAP) [20]. The 1.0-mL reaction mixture contained 164 μM 4-AAP (Sigma), 3 mM 2,4-DCP (Sigma), 4 mM hydrogen peroxide, and 200 μL of the culture supernatant in 50mM of potassium phosphate, pH 7.0. The reaction was initiated by the addition of hydrogen peroxide and the increase in absorbance at 510 nm was monitored for 1 min at room temperature. One unit of LiP activity corresponded to the increase in 1U of absorbance per minute under initial reaction rates. Extracellular LiP concentration was expressed as units of enzyme per liter of culture. LiP activity was measured in the feed before starting the UF process, in the concentrate and in the permeate to calculate the percentages of enzyme in each fraction and the losses of the process. Experiments were performed in duplicate, and standard error was lower than 10% of the mean.

Effect of pH and Temperature on LiP Stability

LiP stability was investigated by incubating the enzyme at 20, 25, 30, 40, and 50 °C for different time intervals at pH 7.0 and 8.0 (fermentation pH at the end of the enzyme production) to allow the design of adequate conditions for UF. After the incubation, the enzyme was placed in an ice bath, and the residual activity was measured under standard conditions. Experiments were performed in duplicate, and standard error was lower than 10% of the mean.

Ultrafiltration Unit and Operating Conditions

Membrane cutoff is not a clear-cut decision as it may not be a direct relationship between molecular mass and its response to the MWCO used in the UF process; the macromolecules may suffer structural deformation depending on the operational conditions and display a peculiar separation profile [21]. Thus, in this work, polysulfone (PS) membranes were used with MWCO of 10, 20, and 50 kDa (PS10, PS20, and PS50, respectively) and a cellulose acetate (CA) membrane with MWCO of 20 kDa (CA20), purchased from Dow Denmark. For the choices of membrane cutoff, the inconsistency on the reported LiP molecular mass was also taken into account. Ultrafiltration runs were performed in a laboratory-stirred cell (initial process volume of 50 mL and minimum working volume of 4 mL) showing an effective membrane area of 15.8 cm^2. Membranes were prewashed to eliminate residual solvent or impurities resulting from the manufacturing process. UF experiments were carried out in room temperature using microfiltered water under pressure of 1 to 3.0 bar. The measurement of the permeate flux indicated the initial hydraulic permeability before each run, taking into account the linear relationship between pressure and permeate flux [16]. Pressure was supplied by a nitrogen gas line. To reduce losses because of LiP adsorption on membrane surfaces and pore walls, all membranes were brought into contact with an enzyme solution during 1h before the experiments. For the UF experiments, 50 mL of LiP solution were transferred to the laboratory-stirred cell (200 rpm) and concentrated as already described. Permeate flux (J_{perm}) was obtained by measuring their volumes during each run. Feed and concentrate samples were taken in the beginning, during the UF process, and at the end of the runs. After the UF experiments, membranes were cleaned with microfiltered water for 30 min, using a pressure of 2 bar and 200 rpm. As water cleaning did not restore the initial permeate flux, a protocol to clean the membranes was further developed. All experiments were performed in duplicate, and standard error was lower than 10% of the mean.

UF Process Parameters

The slope of the corresponding flux–pressure curve corresponds to the hydraulic permeability coefficient (Lp), as shown by the Eq. 1:

$$\text{Hydraulic permeability coefficient(Lp)} = \frac{J_{perm}}{\Delta P} \tag{1}$$

The percentage of LiP in concentrate and in permeate was calculated as:

$$\text{LiP activity in concentrate (\%)} = \frac{\text{Total concentrate activity}}{(\text{Total concentrate activity} + \text{Total permeate activity})} \times 100 \tag{2}$$

$$\text{LiP activity in permeate (\%)} = \frac{\text{Total permeate activity}}{(\text{Total concentrate activity} + \text{Total permeate activity})} \times 100 \tag{3}$$

The LiP activity loss during the UF process was calculated as:

$$\text{LiP activity loss(\%)} = 100 - \frac{(\text{Total permeate activity} + \text{Total concentrate activity})}{\text{Total activity of feed solution}} \times 100 \tag{4}$$

The retention of LiP by the membrane was calculated as follows:

$$\text{Enzyme rejection } (\%) = 1 - \frac{\text{Permeate LiP activity}}{\text{Concentrate LiP activity}} \times 100 \quad (5)$$

The enzyme activity yield during membrane concentration of the feed was determined as the ratio of the final activity of the concentrate to the number of units at the beginning of the operation:

$$\text{Yield } (\%) = \frac{\text{Total concentrate activity}}{\text{Total activity of feed solution}} \times 100 \quad (6)$$

Results and Discussion

Enzyme Preparation and Stability Toward pH and Temperature

The supernatants of fermentations with peak enzyme concentrations in the range of 100 to 180 U/L [19] were used to study the enzyme stability toward incubation time, pH, and temperature. Data presented in Fig. 1a and b indicated for LiP a higher overall stability at pH 8.0 in comparison to pH 7.0, although a gradual enzyme inactivation, in both pH values, was observed in response to the temperature increase and incubation time. Upon incubation at pH 7.0 in the temperature range of 20 to 30 °C, for 4 h, a maximum activity loss of 25% was observed. Under comparative conditions, a more pronounced activity decrease was observed in pH 8.0. The results shows that the activity loss at pH 7.0 was less severe, within 76% of residual activity observed after incubation for 1 h at 50 °C in accordance to previous reports [10]. As at pH 7.0, the enzyme showed to be quite stable at 25 °C (maximum activity loss of 10% upon four hours of incubation), this pH and temperature were chosen for the subsequent UF experiments.

Membrane Selection for LiP Ultrafiltration

The first set of experiments, carried out to compare the performance of membranes with MWCO of 10, 20, and 50 kDa, were carried out using a pressure of 2 bar and 200 rpm agitation, to expose the systems to the same concentration polarization and fouling effects. Before and after the UF experiments, the membrane hydraulic permeability coefficient (Lp) was measured to evaluate the permeability loss.

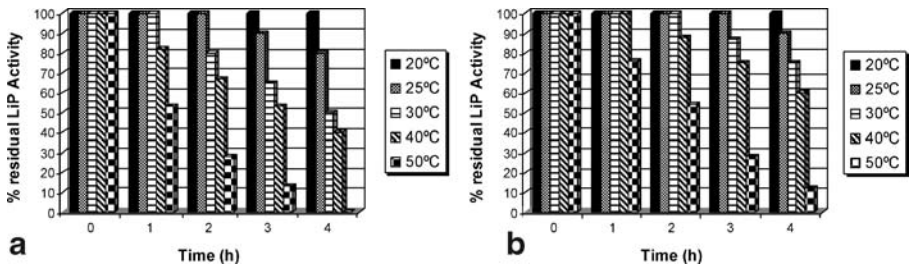

Fig. 1 Comparison of the effect of temperature on residual LiP activity on pH 8.0 and 7.0 (**a** and **b**, respectively)

The PS50 membrane showed to be the most permeable, as expected, with a permeability of 39.8 L/m^2 h bar, followed by the PS20 membrane that presented a permeability of 16.0 L/m^2 h bar. Surprisingly, the PS10 membrane permeability, of 20.7 L/m^2 h bar, was higher than that showed by the membrane PS20. This characteristic might be related to its global porosity and porous size distribution and density. LiP activity was measured in the feed before starting the UF and in the concentrate and in the permeate, at the end of the experiments. Results are summarized in Table 1. In UF experiments carried out with PS50, 83% of total enzyme activity was measured in permeate, in disagreement to previous reports that indicated for the enzyme a molecular mass around 50,000 [11]. The use of PS20 resulted on a better enzyme yield in the concentrate; nevertheless, 27% of total enzyme activity was still measured in permeate. The best results for LiP concentration were obtained using the PS10 membrane, which showed 90% retention of total enzyme activity in the concentrate, a rejection of 99% and a yield of 74%. This result was in agreement to the literature that reported a Lip molecular mass of 17,800 [6] and of 13,500 [14] using SDS-PAGE. In all UF experiments, LiP was concentrated around tenfold.

In the second set of experiments, UF were performed using a CA membrane with MWCO of 20 kDa. The pressure of 1 bar was used because this membrane showed a high initial permeability (37.7 L/m^2 h bar). Table 2 compares LiP UF results for PS10 and CA20 under the same process conditions (1 bar and 200 rpm). The use of PS10 resulted on 96% LiP activity retention on the concentrate (processing time of 340 min). As for results using the CA20 membrane, only 45% of LiP activity was measured in the concentrate (processing time of 65 min). As the CA10 membrane was not available at the time of the experiments, it was not possible to confirm the hypothesis that this membrane would conciliate the selectivity of the PS10 membrane with faster filtration characteristics of the hydrophilic CA membranes. The total PS10 permeability loss of 72% was considerably higher than that observed for CA20, of 12%. Our results are in agreement to previous reports where hydrophobic membrane like PS interacts more strongly with enzymes, being more prone to fouling, in comparison to hydrophilic polymers like CA membranes [22]. Besides, no LiP activity loss was observed for CA20 (0%), whereas 14% loss was measured for PS10,

Table 1 Comparison of $Lp_{initial}$, Lp loss, LiP activity in permeate and concentrate, rejection, yield, LiP activity loss, and UF processing time using membranes PS10, PS20, and PS50 for LiP concentration in a laboratory-stirred cell, with volume of 50 mL, pressure of 2 bar, and agitation of 200 rpm (permeate volume = 46 mL and concentrate volume = 4 mL).

Membrane	PS50	PS20	PS10
$Lp_{initial}$ (L/m^2 h bar)	39.8	16.0	20.7
Lp loss (%)	24	38	65
LiP activity in feed (UI/mL)	100	100	100
LiP activity in concentrate (UI/mL)	187	653	921
LiP activity in permeate (UI/mL)	79	20	9
Total feed LiP activity (UI)	5	5	5
Total concentrate LiP activity (UI)	0.75	2.61	3.68
Total permeate LiP activity (UI)	3.63	0.92	0.41
LiP activity in concentrate (%)	17	73	*90*
LiP activity in permeate (%)	83	27	10
Rejection (%)	58	97	*99*
Yield (%)	15	52	*74*
LiP activity loss (%)	12	29	18
UF process time (min)	30	75	105

Table 2 Comparison of $Lp_{initial}$, Lp loss, LiP activity in permeate and concentrate, rejection, yield, LiP activity loss, and UF processing time using membranes PS10 and CA20 for LiP concentration in a laboratory-stirred cell, with volume of 50 mL, pressure of 1 bar, and agitation of 200 rpm (permeate volume = 42 mL and concentrate volume = 8 mL).

Membrane	PS10	CA20
$Lp_{initial}$ (L/m^2 h bar)	24.1	37.7
Lp loss (%)	72	12
LiP activity in feed (UI/mL)	153	126
LiP activity in concentrate (UI/mL)	786	525
LiP activity in permeate (UI/mL)	6	120
Total feed LiP activity (UI)	7.65	6.3
Total concentrate LiP activity (UI)	6.29	4.2
Total permeate LiP activity (UI)	0.25	5.04
LiP activity in concentrate (%)	*96*	45
LiP activity in permeate (%)	4	55
Rejection (%)	*99*	77
Yield (%)	*82*	67
LiP activity loss (%)	14	*0*
UF Process Time (min)	340	65

indicating absence of LiP adsorption on the CA membrane. The rejection of LiP using PS10 was 99%, and the yield was 82%, being both values higher than that observed for CA20 that showed 77% rejection and 67% of yield.

Operational Conditions for LiP Ultrafiltration

According to the literature, TMP has a negative effect on the protein concentration because of membrane-fouling enhancement associated to TMP increase [17]. To evaluate the occurrence of pressure-related fouling, UF experiments were carried out using a PS10

Table 3 Comparison of $Lp_{initial}$, J_{perm} loss, LiP activity in permeate and concentrate, rejection, yield, LiP activity loss, and UF processing time using membrane PS10 for LiP concentration in a laboratory-stirred cell, with volume of 50 mL, pressure of 1, 2, and 3 bar, and agitation of 200 rpm.

Pressure	1 bar	2 bar	3 bar
$Lp_{initial}$ (L/m^2 h bar)	24.1	15.9	12.7
J_{perm} loss (%)	42	50	63
LiP activity in feed (UI/mL)	153	130	172
LiP activity in concentrate (UI/mL)	786	1,200	1,020
LiP activity in permeate (UI/mL)	6	10	14
Concentrate volume (mL)	8	4	6
Total feed LiP activity (UI)	7.65	6.5	8.6
Total concentrate LiP activity (UI)	6.29	4.8	6.12
Total permeate LiP activity (UI)	0.25	0.46	0.62
LiP activity in concentrate (%)	96	91	91
LiP activity in permeate (%)	4	9	9
Rejection (%)	99	99	98
Yield (%)	*82*	74	71
LiP activity loss (%)	*14*	21	35
UF Process Time (min)	340	250	*180*

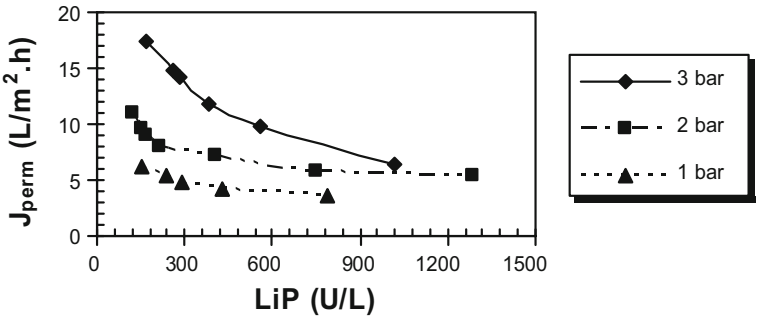

Fig. 2 Comparison of permeate flux values of PS10 membrane during LiP ultrafiltration using TMP of 1, 2, and 3 bar in relation to LiP activity

membrane under TMP of 1, 2, and 3 bar. New membranes with similar initial hydraulic permeability (around 15 L/m^2 h bar) were used in each run. A discrete permeate flux decrease was observed in all cases because of concentration polarization and fouling effects. Table 3 shows that higher initial permeate fluxes were observed in response to TMP increase, although the flux decreased sharply, resulting in a superior permeate flux loss (42, 50, and 63% for the TMP of 1, 2, and 3 bar, respectively). As the enzyme might be susceptible to shear stress and pressure, long processing times could lead to significant loss of enzyme activity [15]. In accordance to the literature, the use of 1bar resulted in the lowest LiP activity loss (14%) and the best yield (82%) even with the highest processing time (340 min). The increased in TMP to 2 and 3 bar resulted on an increase in activity loss to 21 and 35%, respectively. In addition, according to data presented in Fig. 2, a more pronounced decrease in the permeate flux was observed in response to the TMP increase. It is also shown that the increase in LiP initial concentration caused a decrease in permeate fluxes likely related to membrane polarization and fouling.

Membrane Cleaning and Reutilization

Maintenance of UF membrane performance requires the cleaning of fouled membranes [23]. Standard procedures involve the circulation of acid, caustic, and surfactant solutions through the system in a cyclic fashion [24]. In this study, fouled membranes were at first treated with H_2SO_4 6.0 N. This procedure was not adequate as it caused a further decrease in the membrane permeability, likely related to protein precipitation on the membrane surface. A subsequent treatment with NaOH 0.5 N restored to some extent the initial permeability. In a new set of experiments, NaOH 0.5 N was solely used resulting on the

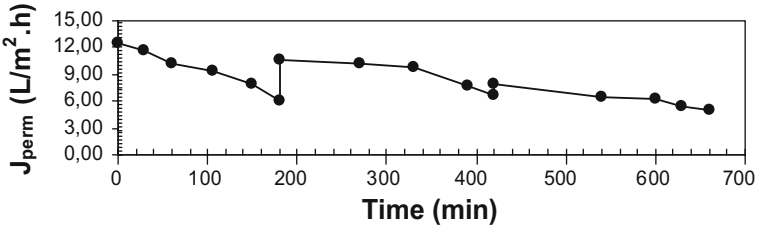

Fig. 3 Flux variation with time for PS10 membrane and flux restoration after cleaning procedure at 180 and 420 h

recovery of 93% of the initial membrane permeability. Afterward, the fouled PS10 membrane was cleaned upon circulation of 10 mL of NaOH 0.5 N (pH bellow 13 in agreement with manufacturer advice) for 20 min at 1 bar and 400 rpm. The cleaning efficiency of this procedure was evaluated, using a PS10 virgin membrane, in three LiP concentration experiments intercalated by a cleaning cycle. Although it was observed a flux decline after the first cleaning cycle, the flux of the subsequent experiments involving UF and cleaning runs showed to be quite stable (Fig. 3). Cleaning with the anionic surfactant SDS increased initial permeability of virgin membranes likely because of the modification of the membrane surface [24].

Final Remarks

In accordance to the enzyme stability data towards pH and temperature the UF experiments for LiP concentration were performed at pH 7.0 and controlled temperature of 25 °C. The best results were obtained using a PS10 membrane that showed 96% of enzyme activity retention in the concentrate. The CA20 membrane showed a lower decrease in total permeability (Lp) in comparison to the PS10 membrane suggesting a relative higher LiP adsorption and pore blocking.

Although the increase in the PS10 TMP from 1 to 3 bar was beneficial to the initial permeate flux, it was deleterious to the enzyme stability, decreasing the process yield. Considering the recovery of fouled membranes, the sole use of NaOH showed to be quite efficient for the PS10 membrane allowing its successful reuse in subsequent experiments. The LiP UF experiments carried out under optimized conditions allowed a tenfold LiP activity increase, reaching 1,000 U/L along with 90% enzyme activity upholding. This concentrate has a potential biotechnological application for the removal of lignin from lignocellulose materials widening its use prospects.

Acknowledgments This work was supported by the Brazilian Research Council/CNPq.

References

1. Minussi, R. C., Pastore, G. M., & Durán, N. (2007). *Bioresource Technology*, *98*, 158–164.
2. Prasad, S., Singh, A., & Joshi, H. C. (2007). *Resources Conservation & Recycling*, *50*, 1–39.
3. Ferreira-Leitao, V. S., Carvalho, M. E. A., & Bon, E. P. S. (2007). *Dyes and Pigments*, *74*, 230–236.
4. Hernandezperez, G., Goma, G., & Rols, J. L. (1997). *Biotechnology Letters*, *19*, 285–289.
5. Gilbert, M., Morosoli, R., Shareck, F., & Kluepfel, D. (1995). *CRC Critical Reviews in Biotechnology*, *15*(1), 13–39.
6. Ramachandra, M., Crawford, D. L., & Hertel, G. (1988). *Applied and Environmental Microbiology*, *54*, 3057–3063.
7. Macedo, J. M. B., Gottschalk, L. M. F., & Bon, E. P. S. (1999). *Applied Biochemistry and Biotechnology*, *77-79*, 735–744.
8. Gottschalk, L. M. F., Macedo, J. M. B., & Bon, E. P. S. (1999). *Applied Biochemistry and Biotechnology*, *77-79*, 771–778.
9. Macedo, J. M. B., Gottschalk, L. M. F., & Bon, E. P. S. (1999). *Brazilian Journal of Chemical Engineering*, *16*, 163–169.
10. Lodha, S. J., Korus, R. A., & Crawford, D. L. (1991). *Applied Biochemistry and Biotechnology*, *28*(29), 411–420.
11. Magnuson, T. S., Roberts, M. A., Crawford, D. L., & Hertel, G. (1991). *Applied Biochemistry and Biotechnology*, *28*(29), 433–442.
12. Zerbini, J. E. (1994). M.S. thesis, Rio de Janeiro Federal University, Rio de Janeiro, Brasil.
13. Spiker, J. K., Crawford, D. L., & Thiel, E. C. (1992). *Applied Environment and Microbiology*, *37*, 518–523.

14. Bon, E. P. S., Nascimento, H. J., Macedo, J. M. B., & Silva Jr, J. G. (1999). *Biotechnology Techniques*, *13*(5), 289–283.
15. Krstic, D. M., Antov, M. G., Pericin, D. M., Höflinger, W., & Tekic, M. N. (2007). *Biochemical Engineering Journal*, *33*, 10–15.
16. Mulder, M. (1991). Basic principles of membrane technology. The Netherlands: Kluwer.
17. Li, Z., Youravong, W., & H-Kittikun, A. (2006). *Bioresource Technology*, *97*, 2364–2370.
18. Hopwood, D. A., Bibb, M. B., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M. et al. (1985). Genetic manipulation of *Streptomyces*: A laboratory manual pp. 3–5. Norwich: The John Innes Foundation.
19. Gottschalk, L. M. F., Nobrega, R., & Bon, E. P. S. (2003). *Applied Biochemistry and Biotechnology*, *105*, 799–807.
20. Pasti, M. B., Hagen, S. R., Korus, R. A., & Crawford, D. L. (1991). *Applied Microbiology and Biotechnology*, *34*, 661–667.
21. Nobrega, R., & Balmann, H. (1989). *Journal of Membrane Science*, *45*, 17–36.
22. Salgin, S., Takaç, S., & Özdamar, T. H. (2006). *Journal of Colloid and Interface Science*, *299*, 806–814.
23. Kim, K. J., Sun, P., Chen, V., Wiley, D. E., & Fane, A. G. (1993). *Journal of Membrane Science*, *80*, 241–249.
24. Lee, H., Amy, G., Cho, J., Yoon, Y., Moon, S. H., & Kim, I. S. (2001). *Water Research*, *35*(14), 3301–3308.