

## Elimination of 4-chlorophenol from Water Solutions Using Commercial Peroxidases

A. Bódalo\*, J. L. Gómez, E. Gómez, J. Bastida, and M. F. Máximo.

Chemical Engineering Department, University of Murcia,  
Campus de Espinardo, 30071 Murcia, Spain.

E-mail: abodalo@um.es; carrasco@um.es; egomez@um.es;  
jbastida@um.es; fmaximo@um.es.

Original scientific paper  
Received: September 11, 2006  
Accepted: March 1, 2007

A comparative study of the two most widely used commercial peroxidases (E.C. 1.11.1.7) for removing 4-chlorophenol from aqueous industrial effluents is presented. Both the peroxidases tested, horseradish peroxidase (HRP) and soybean peroxidase (SBP), showed maximal removal efficiency in a neutral pH medium although they maintained more than 70 % of their activity in a pH range of between 6.0 and 8.0. The influence of temperature on the elimination levels was negligible between  $T = 25$  and  $40$  °C for both enzymes. To minimize the treatment period and enzyme dose, the effect of adding different amounts of a protective agent (polyethylene glycol, PEG) was explored. The final choice of the peroxidase source will depend on the convenience of using such protective agents, SBP being the most suitable when the addition of PEG is not possible or desirable.

*Key words:*

Horseradish peroxidase, soybean peroxidase, 4-chlorophenol, polyethylene glycol, removal efficiency

### Introduction

Chlorophenols are one of the most prominent groups of pollutants in various industrial effluents, such as those generated by high-temperature coal conversion, petroleum refining, and the manufacture of plastics, resins, textile, iron, steel and paper. They may be considered refractory compounds as regards traditional microbiological treatment and conventional physicochemical treatments are not economically viable. Due to their toxicity and suspected carcinogenicity, several methods for removing chlorophenols are profusely being investigated.<sup>1–6</sup>

The catalytic elimination of phenolic compounds from wastewaters using horseradish peroxidase (HRP) and hydrogen peroxide has been the focus of extensive research since the initial work of Klibanov in 1980.<sup>7</sup> Many papers have been published since then and these investigations have showed that HRP is effective in the enzymatic removal of toxic aromatics. However, the prohibitive costs of extracting and purifying HRP, as well as its susceptibility to enzyme inactivation by various side reactions of the treatment process, limit its use in industrial situations.<sup>8</sup>

Some studies have demonstrated that enzyme inactivation can be reduced by the use of chemical additives,<sup>9–11</sup> while the problem of enzyme cost can

be minimized by using a less expensive source of enzyme. In 1991 the seed coats of soybean were identified as a rich source of soybean peroxidase<sup>12</sup> (SBP) and, since these are the by-product of the soybean food industry, SBP has the potential of being a cost effective alternative to HRP for wastewater treatment.<sup>13</sup>

Therefore, the main objective of this study was to compare the removal efficiency of the pollutant 4-chlorophenol from water solutions using as catalyst the two most widely used commercial peroxidases, HRP and SBP. For this purpose, the optimal conditions (temperature and pH) of both experimental systems were explored, as well as the effect of enzyme dose and the possibility of adding enzyme protectors and their optimal concentration. The time necessary to complete chlorophenol elimination may also influence the experimental conditions chosen.

Although a similar study for the elimination of phenol, present in other industrial effluents, has been previously carried out and published by the authors,<sup>14</sup> the different behaviour of phenol and 4-chlorophenol, when treated in identical conditions with peroxidase for their elimination, justifies the present study. Furthermore, while the phenolic compounds are considered as a single pollutant by the environmental legislation (named as total phenols), different elimination strategies have to be designed for each particular phenolic species.

\*Corresponding author

## Materials and methods

**Chemicals:** Soybean peroxidase (lyophilized powder, 54 U mg<sup>-1</sup> of solid,  $RZ \geq 0.5$ ), horseradish peroxidase Type I (lyophilized powder, 148 U mg<sup>-1</sup> of solid,  $RZ \approx 1$ ), 4-chlorophenol (99 %), catalase (E.C. 1.11.1.6) from bovine liver (lyophilized powder, 2200 U mg<sup>-1</sup> of solid), polyethylene glycol (average molar mass  $\bar{M} = 3350$  g mol<sup>-1</sup>) were purchased from Sigma. One unit of peroxidase will form  $m = 1.0$  mg of purpurogallin from pyrogallol in 20 s, at pH 6.0 and 20 °C. One unit of catalase will decompose 1  $\mu$ mol of H<sub>2</sub>O<sub>2</sub> per min at pH 7.0 and 25 °C, while the H<sub>2</sub>O<sub>2</sub> concentration falls from 10.3 to 9.2 mmol l<sup>-1</sup>. The analytical chemicals, 4-aminoantipyrine (AAP) and potassium ferricyanide, were also supplied by Sigma. Hydrogen peroxide solution ( $w = 35$  %) was provided by Aldrich Chemie. All other chemicals used were of analytical grade and were used without further purification.

**Experimental procedure:** Experiments were conducted in a  $V = 30$  ml total volume batch reactor thermostated at 30 °C (except when optimum temperature was studied). All chemicals added to the reactor were dissolved in phosphate buffer 0.1 mol l<sup>-1</sup>, pH 7.0 (except when optimum pH was studied). First 4-chlorophenol, PEG (when used) and buffer solutions were placed in the reactor. When the desired temperature had been reached, the enzyme solution and finally hydrogen peroxide solution were added. 4-chlorophenol and hydrogen peroxide concentrations were kept constant (2.0 mmol l<sup>-1</sup> for both substrates), while enzyme concentration, PEG mass concentration, pH value and temperature were varied. The reaction course was followed by taking 1 ml samples and analysing its 4-chlorophenol concentration until its value remained constant.

**Sample processing:** Samples from the reactor were immediately poured over 1 ml of catalase solution (2200 U ml<sup>-1</sup>) to stop the reaction by breaking down the hydrogen peroxide. To 1 ml of the this solution, 0.2 ml of coagulant (AlK(SO<sub>4</sub>)<sub>2</sub> 40 g l<sup>-1</sup>) were added and centrifuged for 30 min at 10 000 g, giving a colourless supernatant. In consequence, its 4-chlorophenol content could be analysed using a colorimetric method.

**Analytical method:** The concentrations of 4-chlorophenol were measured by a colorimetric method<sup>15</sup>. Solutions of potassium ferricyanide (83.4 mmol l<sup>-1</sup> in 0.25 mol l<sup>-1</sup> sodium bicarbonate solution) and 4-aminoantipyrine (20.8 mmol l<sup>-1</sup> in 0.25 mol l<sup>-1</sup> sodium bicarbonate solution) were prepared. In a spectrophotometer cuvette (3 ml) 2.4 ml of the diluted sample (chlorophenol concentration up to  $c = 0.2$  mmol l<sup>-1</sup>) were placed together with 0.3 ml of ferricyanide solution and 0.3 ml of AAP solution.

After approximately 10 min for the colour to develop fully, the absorbance was measured at 505 nm against a blank (2.4 ml of water, ferricyanide solution, and AAP solution). Absorbance values were transformed to chlorophenol concentrations in the sample by using a calibration curve ( $[4\text{-chlorophenol}] = 0.1023 \cdot \text{Abs}_{505}$ ,  $r = 0.9998$ ).

## Results and discussion

### Optimal pH and temperature for 4-chlorophenol removal

For the 4-chlorophenol removal efficiency of both biocatalysts to be compared, SBP and HRP should be allowed to act under their optimal operational conditions. Therefore, two experimental series were carried out, first varying pH ( $T = 30$  °C) and then temperature (pH 7). All the experiments were performed with a constant reactor volume (30 ml) and 4-chlorophenol and hydrogen peroxide concentrations ( $c = 2$  mmol l<sup>-1</sup>). The initial H<sub>2</sub>O<sub>2</sub> to 4-chlorophenol molar ratio ( $r = 1 : 1$ ) was chosen according to the work of Nicell et al. (1992). Different concentrations of both enzymes were used (450 U l<sup>-1</sup> SBP and 75 U l<sup>-1</sup> HRP) because, from the outset, the higher affinity of HRP for 4-chlorophenol was observed. Moreover, PEG was added to the reaction media ( $\gamma = 0.05$  g l<sup>-1</sup>) in order to prevent the rapid enzyme inactivation observed in preliminary studies. Samples were taken at different times and the 4-chlorophenol concentrations were determined. The reactions were allowed to progress until 4-chlorophenol elimination was constant (approximately 400 min for SBP and 150 min for HRP).

The results obtained in these experiments are summarised in Tables 1 (for pH) and 2 (for temperature). The removal efficiency was defined as the maximum efficiency (%) of 4-chlorophenol re-

Table 1 – Effect of pH on the removal efficiency of 4-chlorophenol by SBP and HRP at 30 °C. Reaction time is 400 min for SBP and 150 min for HRP.

pH	SBP		HRP	
	removal efficiency	relative removal efficiency	removal efficiency	relative removal efficiency
6.0	65	70	81	94
6.5	79	85	82	95
7.0	93	100	86	100
7.5	85	91	85	99
8.0	82	88	83	97

moved from the solution under the experimental conditions. As regards pH, maximal 4-chlorophenol elimination was attained at neutral pH and both enzymes tolerated a slightly basic medium better than a slightly acid medium. SBP seemed to be more sensitive to an acid environment than HRP. It is very difficult to compare these results with those published previously mainly because of the great discrepancies reported. Thus, the optimum pH for 4-chlorophenol removal by SBP could be 8<sup>16</sup> or 9<sup>17</sup>, while for HRP, several optimum pH values and ranges have been reported: 5.5<sup>7</sup>, 9<sup>18–19</sup>, 3.9 – 9<sup>20</sup>, 5 – 8<sup>10</sup>, 6 – 9<sup>21</sup> and 6 – 8<sup>11</sup>. However, in spite of these discrepancies, it is important to note that both enzymes showed an appreciable percentage of activity over a wide range of pH, meaning that they can be used for wastewater treatment without prior adjustment of pH.

As regards the temperature, little change was observed in the efficiency of elimination achieved when it was varied between 25 and 40 °C, for both enzymes (see Table 2). This is another advantage because, unlike with most enzymatic systems, it is not necessary to strictly control this parameter. The optimum temperature for 4-chlorophenol elimination by SBP has not been quantity reported while only few researchers have studied the influence of temperature on 4-chlorophenol removal by HRP.<sup>19,21–22</sup> Their finding that the removal efficiency increased when reaction temperature was lowered was attributed to the lower solubility of the polymer at low temperatures. This effect was not significant at temperatures between 30 °C and 60 °C. In this paper we did not use very low or high temperatures because our main interest was to study the elimination of 4-chlorophenol from wastewater with no previous treatment, such as cooling or heating.

To compare the two enzymes in the same conditions, a pH of 7 and a temperature of 30 °C were used in the subsequent experiments.

Table 2 – Effect of temperature on the removal efficiency of 4-chlorophenol by SBP and HRP at pH 7.0. Reaction time is 400 min for SBP and 150 min for HRP

Temperature, T/°C	SBP		HRP	
	removal efficiency	relative removal efficiency	removal efficiency	relative removal efficiency
25	92	99	85	99
30	93	100	86	100
35	93	100	86	100
40	91	98	85	99

### Influence of peroxidase concentration

In order to compare the 4-chlorophenol removal efficiency of SBP and HRP, a series of experiments were performed using the same pollutant and H<sub>2</sub>O<sub>2</sub> concentrations ( $c = 2 \text{ mmol l}^{-1}$ ) and different enzyme doses (450 U l<sup>-1</sup>, 900 U l<sup>-1</sup> and 1800 U l<sup>-1</sup>). The results obtained are shown in Fig. 1, where removal efficiency was plotted against reaction time for both biocatalysts and different enzyme concentrations.

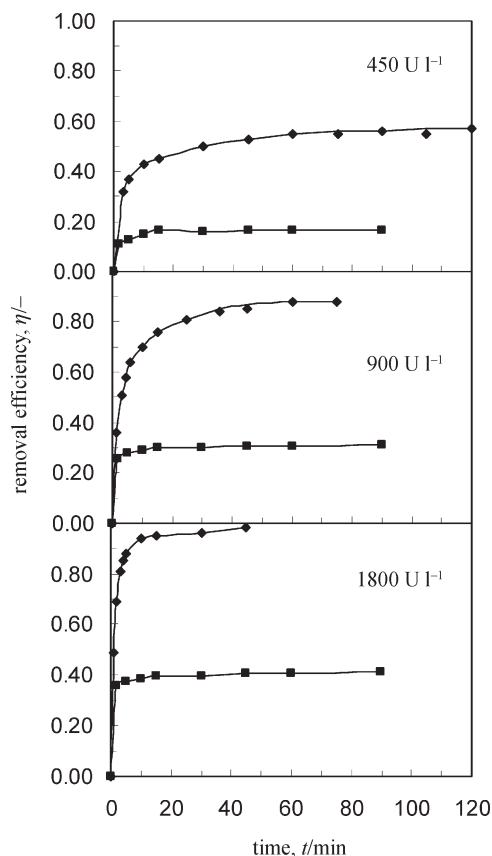


Fig. 1 – Time course of the removal efficiency of 4-chlorophenol using three different enzyme doses in the absence of PEG. (■) HRP and (◆) SBP

It can be observed that the removal efficiency increased with increasing biocatalyst concentrations although the behaviour of both peroxidases was quite different. Thus, within the experimental range used in the present study, the HRP-catalysed elimination of 4-chlorophenol was a very fast reaction, reaching maximal removal in less than 10 min, after which no further 4-chlorophenol was eliminated. This, together with the low removal efficiency, suggest enzyme inactivation either by the phenoxy radicals or by the phenolic polymers produced in the enzymatic reaction, implying that large amounts of HRP are needed.<sup>10–11</sup> On the other hand, the SBP-catalysed elimination system was a slower re-

action, which needed longer reaction periods to reach the maximal removal efficiency, although these times were shortened as the SBP concentration increased. Moreover, it is important to notice that when the same enzyme dose was used, the amount of 4-chlorophenol removed from the solution was significantly higher for SBP than for HRP, and 100 % elimination could be achieved after 50 min by adding 1800 U l<sup>-1</sup> of SBP. In the above experimental conditions, seven million units of SBP were necessary to completely remove 1 kg of the pollutant.

To summarise, SBP was seen to be slower than HRP for the removal of 4-chlorophenol but was less sensitive to the inactivation effect produced by the phenoxy radicals and/or the phenolic polymers. These results cannot be properly checked with the results of other authors because no previous studies comparing the behaviour of both peroxidases have been found. Although we found several papers on enzymatic 4-chlorophenol removal, the results varied greatly. For example, it has been described that 95 % of 1 mmol l<sup>-1</sup> 4-chlorophenol solution could be polymerized, in three hours, with 200 U l<sup>-1</sup> or 350 U l<sup>-1</sup> of SBP,<sup>16–17</sup> in other words, 1.6 million and 2.9 million units of SBP, respectively, and a three hours treatment, were necessary to almost completely remove (95 %) 1 kg of 4-chlorophenol. As regards HRP the bibliography reflects that higher units of enzyme were necessary to achieve more than 90 % removal of  $c = 1$  mmol l<sup>-1</sup> 4-chlorophenol solutions:<sup>10,20–21</sup> 1020 U l<sup>-1</sup>, 1400 U l<sup>-1</sup> or 2000 U l<sup>-1</sup>. If all the above results are resumed, we may affirm that they agree with the experimental observations obtained in the present paper.

### Influence of PEG mass concentration

It has previously been reported that some additives may be useful for preventing peroxidase inactivation<sup>11</sup>. In our case, PEG with an average molar mass of  $M = 3350$  g mol<sup>-1</sup> was selected based on the results of *Nakamoto* and *Machida*,<sup>9</sup> who determined that PEG of molar mass in excess of  $M = 1000$  g mol<sup>-1</sup> were most effective in protecting HRP. On the other hand, it has been demonstrated that PEGs with molar mass of  $M = 1000$  g mol<sup>-1</sup> and below were ineffective in protecting SBP.<sup>23</sup>

In order to study the protective effect of PEG on the 4-chlorophenol removal efficiency of SBP, experiments were conducted by using different PEG doses (0.05 g l<sup>-1</sup> and 0.25 g l<sup>-1</sup>) and enzyme concentrations (175 U l<sup>-1</sup>, 450 U l<sup>-1</sup> and 900 U l<sup>-1</sup>). Fig. 1 shows that the addition of 1800 U l<sup>-1</sup> of SBP to the reaction mixture, provoked the total elimination of 2 mmol l<sup>-1</sup> 4-chlorophenol in only 50 min in

the absence of PEG. Therefore, in order to investigate the influence of PEG on the 4-chlorophenol removal, lower enzyme concentrations were used. The results obtained are illustrated in Fig. 2, where time data for up to 2 h are presented, although 4-chlorophenol elimination with the lowest SBP concentrations continued after this time. It can be seen that the addition of PEG enhanced the removal efficiency in all cases, reaching 100 % elimination in only 30 min, when 900 U l<sup>-1</sup> of SBP and 0.25 g l<sup>-1</sup> of PEG were used. In other words, the total amount necessary to eliminate 1 kg of 4-chlorophenol was reduced to 3.5 million units of SBP, at the same time obtaining a substantial reduction in treatment time.

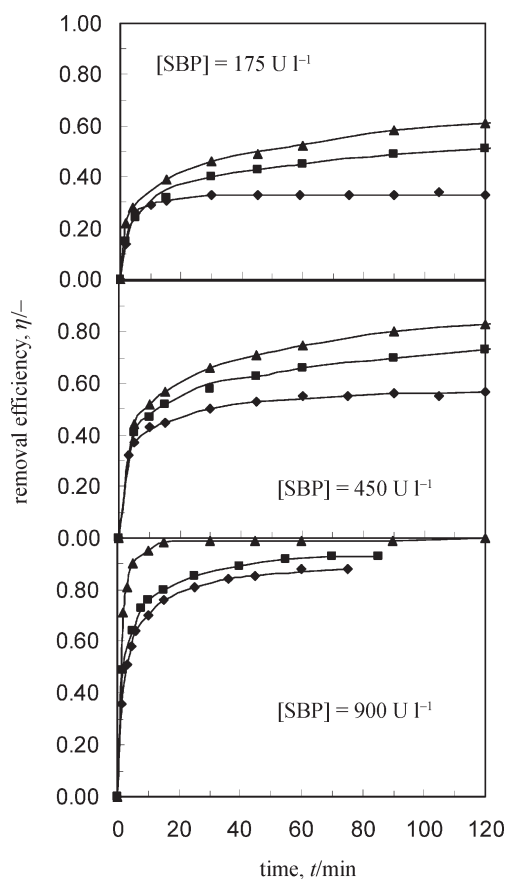


Fig. 2 – Effect of adding different concentrations of PEG on the removal efficiency of 4-chlorophenol using SBP as catalyst. (◆) 0.0 g l<sup>-1</sup>; (■) 0.05 g l<sup>-1</sup>; and (▲) 0.25 g l<sup>-1</sup>

In order to compare the behaviour of SBP and HRP, a similar series of experiments was planned with the latter. However, in all cases, 100 % removal was reached during the first stages of the reaction when HRP was used as biocatalyst and PEG was added to the reaction mixture. Therefore, a new series of experiments was planned using three different PEG doses (0.025 g l<sup>-1</sup>, 0.05 g l<sup>-1</sup> and 0.25 g l<sup>-1</sup>) and four very low HRP concentrations (25 U l<sup>-1</sup>,

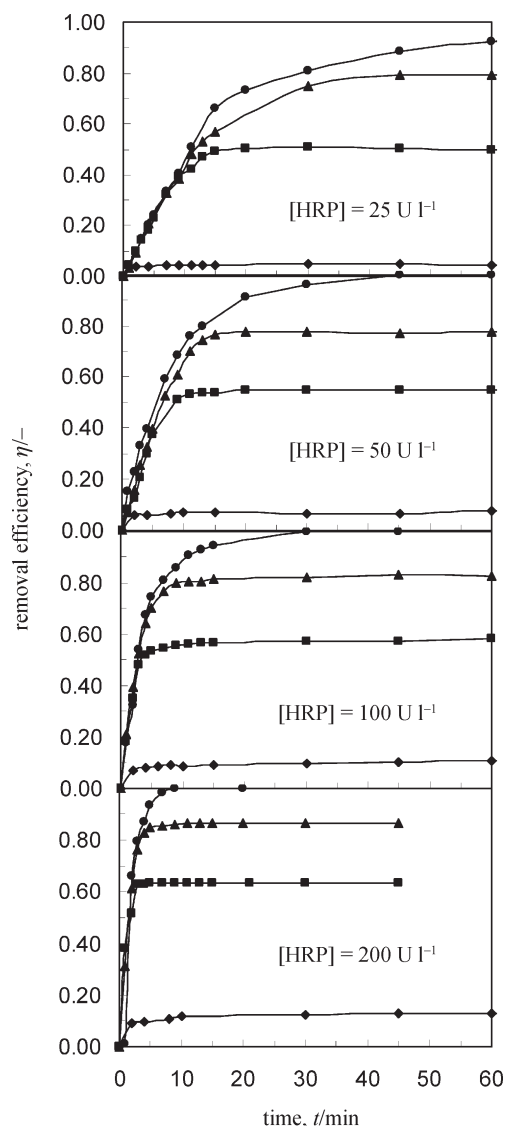


Fig. 3 – Effect of adding different concentrations of PEG on the removal efficiency of 4-chlorophenol using HRP as catalyst. (◆) 0.0 g l<sup>-1</sup>; (■) 0.025 g l<sup>-1</sup>; (▲) 0.05 g l<sup>-1</sup> and (●) 0.25 g l<sup>-1</sup>

50 U l<sup>-1</sup>, 100 U l<sup>-1</sup> and 200 U l<sup>-1</sup>). Fig. 3 shows the significant effect of PEG on the removal efficiency of HRP. The presence of 0.25 g l<sup>-1</sup> of PEG allowed us to reach 100 % elimination in 120 min if 25 U l<sup>-1</sup> of HRP were used, that is, 97 000 units were enough to completely remove 1 kg of pollutant. If an even shorter treatment time is required, the same elimination percentage can be reached in 45 min by doubling the HRP concentration. The reaction time could be substantially reduced (less than 10 min) by increasing the enzyme concentration to 200 U l<sup>-1</sup>.

In the light of the above, it is concluded that PEG was a beneficial additive in the 4-chlorophenol removal process independently of the enzyme used as biocatalyst, although this effect was more pronounced when HRP was used. To compare these results with those previously published, a

compilation of the findings described in several papers had to be made. Thus, it has been described that the addition of 0.3 g l<sup>-1</sup> of PEG to a reaction medium containing 1 mmol l<sup>-1</sup> 4-chlorophenol slightly reduces the amount of SBP necessary (from 0.2 U l<sup>-1</sup> to 0.15 U l<sup>-1</sup>) to obtain 95 % removal efficiency in three hours.<sup>16</sup> These data agree with the results obtained in the present study, where a slight effect of PEG on 4-chlorophenol removal by SBP was observed. On the other hand, some authors have described a noticeable effect of the additive on the 4-chlorophenol removal efficiency attained with HRP. The addition of different PEG mass concentrations, ranging from 0.3 g l<sup>-1</sup> to 5 g l<sup>-1</sup>, led to the elimination of more than 90 % of the 4-chlorophenol (1 mmol l<sup>-1</sup> solution) using only 30 U l<sup>-1</sup> or 15 U l<sup>-1</sup>. The reaction times necessary to achieve such elimination levels ranged from 1 to 16 h, depending on the enzyme concentration.<sup>10–11</sup>

## Conclusions

Based on the experiments conducted with SBP and HRP in the same operational conditions, it can be concluded that both enzymes are suitable for eliminating 4-chlorophenol from wastewater because pH and temperature need only be loosely controlled at around neutral pH and ambient temperature.

The action of both peroxidases on 4-chlorophenol removal is quite different, HRP being slightly more active at the outset but very susceptible to deactivation. On the other hand, SBP is slower in its action but is quite resistant to deactivation, the amount of 4-chlorophenol removed from the solution being significantly higher with SBP than with HRP when the same enzyme concentration was used.

When complete elimination of the pollutant is necessary, two different strategies can be adopted. If the addition of PEG is not desirable, the best choice is the soybean enzyme. Seven million units of this enzyme will eliminate 1 kg of 4-chlorophenol from the effluent in less than 1 hour. When the addition of PEG is not a difficulty, HRP will be the best choice. By using fewer than 200 000 units of HRP, 1 kg of the pollutant can be removed in 45 min.

## ACKNOWLEDGEMENTS

The present study was supported by the grant 00505/PI/04 from Fundación Séneca. Comunidad Autónoma de la Región de Murcia.

**List of symbols**

$c$	– concentration, mmol l <sup>-1</sup>
$M$	– molar mass, g mol <sup>-1</sup>
$\bar{M}$	– average molar mass, g mol <sup>-1</sup>
$r$	– molar ratio
$T$	– temperature, °C
$t$	– time, min, h
$V$	– volume, ml, l
$w$	– mass fraction, %
$\gamma$	– mass concentration, g l <sup>-1</sup>
$\eta$	– efficiency, %, –

**REFERENCES**

1. Spigno, G., Zilli, M., Nicoletta, C., *Biochem. Eng. J.* **19** (2004) 267.
2. Cea, M., Seama, J. C., Jara, A. A., Mora, M. L., Diez, M. C., *J. Colloid. Interf. Sci.* **292** (2005) 171.
3. Enterazi, M. H., Mostafai, M., Sarafraz-Yazdi, A., *Ultrason. Sonochem.* **13** (2006) 37.
4. Hu, Y., Xu, J. J., Yuan, C. W., Lin, J., Yin, Z. D., *Chinese Sci. Bull.* **50** (2005) 1979.
5. Nordin, K., Unell, M., Jansson, J. K., *Appl. Environ. Microb.* **71** (2005) 6538.
6. Wu, F. C., Tseng, R. L., Juang, R. S., *Sep. Purif. Technol.* **47** (2005) 10.
7. Klibanov, A. M., Alberti, B. N., Morris, E. D., Felshin, L. M., *J. Appl. Biochem.* **2** (1980) 414.
8. Bassi, A., Gen, Z., Gijzen, M., *Eng. Life Sci.* **4** (2004) 125.
9. Nakamoto, S., Machida, N., *Water Res.* **26** (1992) 49.
10. Wu, J., Bewtra, J. K., Biswas, N., Taylor, K. E., *Proceedings of the 48<sup>th</sup> Purdue Industrial Waste Conference*, 1993, pp 421–431.
11. Wu, J., Taylor, K. E., Biswas, N., Bewtra, J. K., *Water Res.* **31** (1997) 2699.
12. Gillikin, J. W., Graham, J. S., *Plant Physiol.* **96** (1991) 214.
13. Kraus, J. J., Munir, I. Z., McEldoon, J. P., Clark, D. S., Dordick, J. S., *Appl. Biochem. Biotechnol.* **80** (1999) 221.
14. Bódalo, A., Gómez, J. L., Gómez, E., Bastida, J., Máximo, M. F., *Chemosphere* **63** (2006) 626.
15. *Standard Methods for the Examination of Water and Wastewater* 19<sup>th</sup> Edition. Eaton AD, Clesceri LS, Greenberg AE, Eds. American Public Health Association, Washington, 1995, Part 5530.
16. Caza, N., Bewtra, J. K., Biswas, N., Taylor, K. E., *Water Res.* **33** (1999) 3012.
17. Wright, H., Nicell, J. A., *Bioresource Technol.* **70** (1999) 69.
18. Dec, J., Bollag, J. M., *Arch. Environ. Con. Tox.* **19** (1990) 543.
19. Song, H. Y., Liu, J. Z., Xiong, Y. H., Weng, L. P., Ji, L. N., *J. Mol. Catal. B-Enzym.* **22** (2003) 37.
20. Nicell, J. A., Bewtra, J. K., Biswas, N., Taylor, K. E., *Water Res.* **27** (1993) 1629.
21. Nicell, J. A., Bewtra, J. K., Taylor, K. E., Biswas, N., St Pierre, C. C., *Water Sci. Technol.* **25** (1992) 157.
22. Masuda, M., Sakurai, A., Sakakibara, M., *Enzyme Microb. Tech.* **28** (2001) 295.
23. Kinsley, C., Nicell, J. A., *Bioresource Technol.* **73** (2000) 139.