Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Polymers 56 (2004) 295–300
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Partitioning of pectinase produced by *Polyporus squamosus* in aqueous two-phase system polyethylene glycol 4000/crude dextran at different initial pH values

Mirjana G. Antov*

Faculty of Technology, University of Novi Sad, Blvd. Cara Lazara 1, 21000 Novi Sad, Serbia and Montenegro

Received 24 June 2003; revised 10 October 2003; accepted 5 December 2003

Available online 27 April 2004

Abstract

Partitioning of endo-pectinase and exo-pectinase, produced during the cultivation of *Polyporus squamosus* in an aqueous two-phase system, composed of polyethylene glycol 4000 and crude dextran, at different initial pH values was studied. At all stages cultivation, the biomass produced showed exclusively bottom-sided partition, irrespectively, of the pH. Maximal value of partition coefficient of endo-pectinase, 2.45, was attained on the second day of cultivation at an initial pH 5.0, which was accompanied by a maximal top phase yield of 80.22%. Higher initial pH 7.0 improved the partition coefficient of exo-pectinase about 2.5 times and the top phase yield to 45% on the third day of cultivation in comparison to partition parameters at lower initial pH. *P. squamosus* did not produce endo-pectinase in aqueous two-phase cultivation at initial pH 7.0.

Keywords: Pectinase; *Polyporus squamosus*; Aqueous two-phase system

1. Introduction

Pectic substances are a group of complex acidic polysaccharides that occur in varying amounts in all higher plant tissues and are found in the intracellular spaces. It is not surprising, because of the universal occurrence of pectic polysaccharides in the plant world, that the enzyme systems capable of degrading these structures are not only varied in their mechanism of action, but also widespread in their distribution. Pectinases are produced by plants and micro-organisms, but commercial pectinases for the industrial application are mostly obtained from fungi (Fogarty & Kelly, 1983). One of the major obstacles in downstream processing in these productions is difficult mechanical separation of mycelia from product.

Cultivations of microorganisms in aqueous two-phase systems (ATPSs) for the purpose of production of extracellular enzymes, such as pectinases, are based on the idea of separating cells from enzymes by partitioning them into opposite phases, thus avoiding the conventional techniques of mechanical separation and also allowing process integration (Zijlstra, de Gooijer, & Tramper, 1998). Advantages of ATPSs are also their biocompatibility, due to the high water content in both phases, low process time and energy consumption and relative reliability in scale-up (Andersson & Hah-Hagerdal, 1990) in comparison with other separation and purification procedures. Even more, in some cases an enhanced enzyme production was observed in ATPS when compared to homogeneous cultivation (Antov & Peričin, 2001; Antov, Peričin & Dimić, 2001; Chen & Lee, 1995).

The major industrial applications of pectinases include extraction and clarification of fruit juices and grape musts, citrus fruit juice and wine technology, maceration of vegetables and fruits and extraction of olive oil (Fogarty & Kelly, 1993). The fungus *Polyporus squamosus* is interesting from the viewpoint of simultaneous production of pectinases (Peričin, Kevrešan, Banka, Antov, & Škrinjar, 1992) and biomass for animal nutrition (Peričin, Antov, & Popov, 1998–1999). The pectinase complex from *P. squamosus* does not contain pectin esterase and pectin lyase (Peričin et al., 1992), the former makes it very suitable for application in cloudy citrus juice production and in wine making.

* Tel.: +381-21-350-122; fax: +381-21-450-413.
E-mail address: mantov@uns.ns.ac.yu (M.G. Antov).

0144-8617/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2003.12.008

The aim of this work was to examine partition of pectinases produced by cultivation of *P. squamosus* at different initial pH values in ATPS, composed of polyethylene glycol and crude dextran—a carbohydrate polymer of microbial origin. Partition conditions, concerning concentration of phase-forming polymers and pH, were previously established in model aqueous two-phase system with commercial pectinase preparation, through examination of the influence of the tie-line length, the phase volume ratio and pH, on the partition coefficient and the top phase yield.

2. Materials and methods

2.1. Commercial pectinase preparation

In the partition studies in model system on the influence of phase volume ratio at different tie-line lengths, Vinozym (Novozyme, Denmark) was diluted 100 times in 10 mmol l^{-1} acetate buffer pH 5.0, so that the endo-pectinase and exo-pectinase activities of the basal enzyme solution were 22.73 and 9036.4 U ml^{-1}, respectively.

In order to examine the influence of pH in the presence of phosphate in model ATPS, Vinozym was diluted 100 times in 0.1 mol l^{-1} phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 5.0 and 7.0.

2.2. Preparation of ATPS

The polymers used were polyethylene glycol 4000 (PEG) (MW 3,500–4,500, Merck, Germany) and crude dextran (DEX) (MW $> 500 \times 10^3$). A crude dextran solution of 10% (w/w) contained approximately 0.5% reducing sugars, as determined by the DNS-method (Miller, 1959) with glucose as the standard.

Phase systems for model system experiments were constructed, according to Hotha and Banik (1997), by vortexing mixtures of the required quantities of PEG and DEX in the enzyme solutions, for 5 min at room temperature. The total mass of the two-phase system was 10 g. The two phases were allowed to separate (12 h) before sampling, and then the upper phase was carefully removed with a pipette, leaving a small amount at the interface. The lower phase was then sampled through the interface. Samples of each phase were analysed for enzyme activities.

The tie-line length was defined (Furuya, Yamada, Zhu, Yamaguchi, Iwai, & Arai, 1996) as

$$\text{Tie} - \text{line length} = \lfloor (w_{\text{DEX}}^{\text{TOP}} - w_{\text{DEX}}^{\text{BOT}})^2 + (w_{\text{PEG}}^{\text{TOP}} - w_{\text{PEG}}^{\text{BOT}})^2 \rfloor^{1/2} \quad (1)$$

where w_i^{TOP} and w_i^{BOT} represent the weight percentages of phase-forming component i in the top and bottom phases, respectively.

Weight percentages of polymers of examined systems along the tie-line length 7.44%, with corresponding phase volume ratios (in parenthesis), were: 5% PEG/4% DEX (1.0), 5.5% PEG/3% DEX (2.0), 6% PEG/2% DEX (3.6), and along the tie-line 13.98%: 4.5% PEG/8.4% DEX (0.1), 5% PEG/7.5% DEX (0.6), 5.3% PEG/6.6% DEX (1.0), 6.5% PEG/5% DEX (2.3), 7.2% PEG/4% DEX (3.0), 7.5% PEG/3% DEX (5.6).

2.3. Microorganism

Polyporus squamosus MMOL 87, obtained from the Research Institute NPO Biotechnology, Moscow, Russia, was stored on Saboroaud maltose agar slants at 4 °C. Microorganisms were washed from a slant agar surface into 300 ml shake flasks, containing 100 ml of the basal medium. The culture was incubated at 28 °C for 48 h at 200 rev min^{-1}. The first vegetative generation obtained in this way was used as an inoculum.

2.4. Medium

Basal medium for the inoculum contained (Aguilar & Huitron, 1990) per litre 3 g yeast extract, 2 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 2 g K_2HPO_4 and 5 g pectin (Green Ribbon Pure, Obipektin, Switzerland). The medium contained per litre 4 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4 and 5 g pectin and, in addition, 5% (w/w) PEG and 4% (w/w) DEX. The pH values before sterilisation were adjusted to 5.0 and 7.0. Sterilisation was accomplished at 121 °C for 30 min. Dextran was sterilised separately with the pectin and PEG was sterilised with salts.

2.5. Cultivation of P. squamosus

Erlenmeyer flasks of 300 ml, containing 50 ml of ATPS medium, were inoculated with 5% (v/v) of inoculum, and incubated at 300 rev min^{-1} and 28 °C. Samples were centrifuged for 1 min at 3000 rev min^{-1} in a bench-scale centrifuge in order to separate the phases, and then the bottom phase was centrifuged (10,000 rev min^{-1}, 10 min, Sorvall RC-5B) to separate the biomass. Each phase was suitably diluted and analysed for enzyme activities.

2.6. Biomass

Biomass was dried at 105 °C and the RNA content of the dry matter was determined (Munro & Fleck, 1966). Biomass is expressed as mg of RNA.

2.7. Enzyme assays

Endo-pectinase (endo-p) activity was determined by measuring the decrease of the specific viscosity of the reaction mixture (0.25% pectin Red Ribbon Pure, Obipektin, Switzerland, in 0.1 mol l^{-1} citrate buffer, pH 4.5 as substrate) at 30 °C, according to Peričin et al. (1992). One unit was defined as the amount of enzyme that reduced the initial specific viscosity of the reaction mixture by 20%

in 1 min. In order to avoid the influence of sample viscosities on analytical procedures, suitable dilutions were made such that the initial viscosities of reaction mixture, when basal enzyme solution of commercial pectinases or samples was added, had the same value.

Exo-pectinase (exo-p) activity was measured according to Aguilar and Huitron (1990) by quantifying, using the DNS-method (Miller, 1959), the number of reducing groups, expressed as galacturonic acid, which have been liberated after incubation with 0.9% pectin Red Ribbon Pure, in acetate buffer, pH 5.0, at 45 °C. One unit was defined as the amount of enzyme that catalysed the formation of 1 μmol of galacturonic acid per hour.

The results are the mean value of at least three measurements of activity (the accuracy is considered to be ±5%) on a minimum of three replicas for every partition experimental point.

The partition coefficients for endo-p and exo-p activities in the aqueous two-phase systems were defined as

$$K = \frac{\text{activity}_{\text{top phase}}}{\text{activity}_{\text{bottom phase}}} \quad (2)$$

and yield in top phase as

$$Y(\%) = \frac{100 V_t K}{V_t K + V_b} \quad (3)$$

where V_t and V_b are the volumes of the top and bottom phase, respectively.

2.8. Miscellaneous

Dextran content in phases was determined in a polarimeter (Perkin–Elmer) at 589 nm (Andersson, Johansson, & Hahn-Hagerdal, 1985) and concentration of polyethylene glycol 4000 was measured by method of Skoog (1979).

Samples of cultivation broth from two-phase system were checked for dextranase activity according to the procedure described by Rogalski et al. (1998).

3. Results and discussion

For an efficient separation in aqueous two-phase systems it is important to find both favourable partition coefficient and favourable phase volume ratio (Andersson & Hahn-Hagerdal, 1990). All two-phase systems, with overall composition represented by one of the tie-lines, give phases with identical composition, but different volumes. Some results of examination of the effect of the phase volume ratio on the partition of enzymes and proteins showed its dependence on the length of the particular tie-line (Marcos, Fonseca, Ramalho, & Cabral, 1998). So, partition studies with commercial pectinase preparation were performed by varying phase volume ratio along the two tie-lines, whose lengths were 7.44 and 13.98%.

The change in the phase volume ratio, within the range from 0.1 to 2.0, influenced partition parameters of endo-p activity in the same manner, independently of tie-line length-the partition coefficients were decreased and the top phase yields increased with increasing in V_t/V_b (Fig. 1). But, further increase in the phase volume ratio at the tie-line 7.44% (Fig. 1a) just slightly decreased K, from 1.16 to 1.12, which was, at the same time, followed by increase in the top phase yield of about 10%. In contrast an increase in V_t/V_b over the value 2 at the tie-line 13.98% (Fig. 1b) continued to have a decreasing effect on the partition coefficient of endo-p, and finally caused a decrease in the top phase yield at the highest examined value of the phase volume ratio.

The same behaviour was even more characteristic for the partition of exo-p: at the tie-line 7.44% the increase in the phase volume ratio did not practically influenced the partition coefficient, what was followed by the constant increase in the yield in the top phase (Fig. 2a). In systems with overall composition represented by tie-line 13.98% the influence of the phase volume ratio on the partition of the exo-p (Fig. 2b) showed similar pattern as for the endo-p (Fig. 1b).

These results might indicate that the endo-p and exo-p are showing so-called optimal behaviour in phase diagram region of ATPS, composed of polyethylene glycol and crude dextran, represented by the shorter tie-line, where

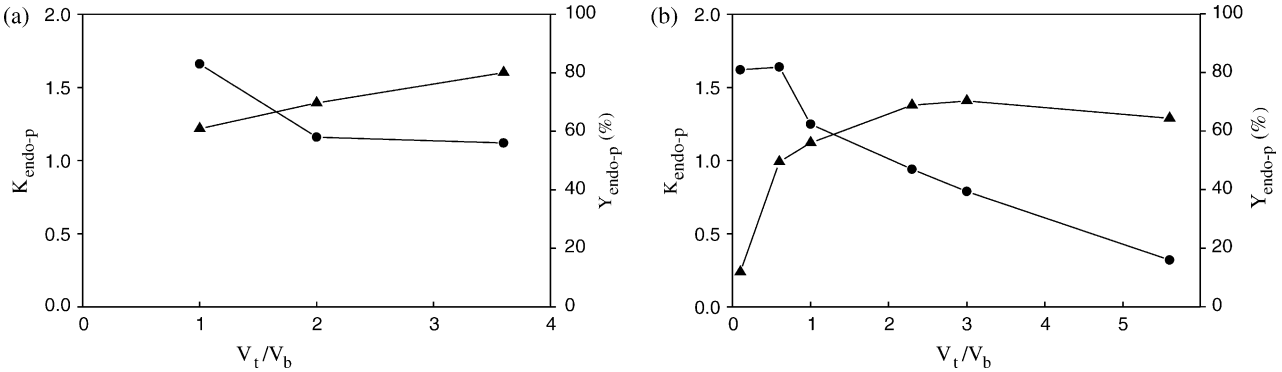

Fig. 1. Partition coefficient (●) and top phase yield (▲) of endo-p activity from commercial enzyme as the function of phase volume ratio at tie-line (a) 7.44% and (b) 13.98% in PEG/DEX aqueous two-phase system.

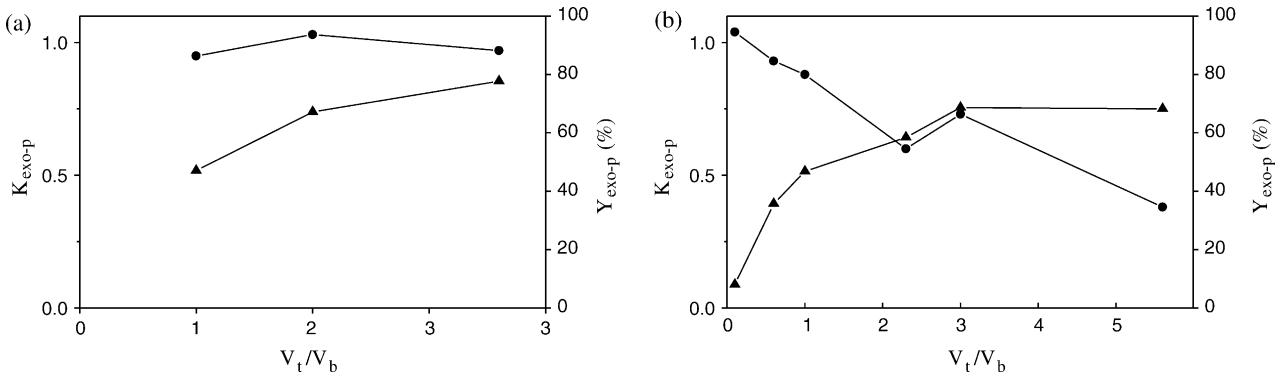

Fig. 2. Partition coefficient (●) and top phase yield (▲) of exo-p activity from commercial enzyme as the function of phase volume ratio at tie-line (a) 7.44% and (b) 13.98% in PEG/DEX aqueous two-phase system.

the differences between phases composition are lower. It means that their partition coefficients have a tendency to change in lesser extent or even to remain constant along the tie-line, which is close to binodal, in polymer/polymer aqueous two-phase systems, similarly to findings of Marcos et al. (1998) for polymer/salt systems.

The important effect of salts on partition behaviour of proteins has been reported in many publications (Antov & Peričin, 2001; Lima, Alegre, & Meirelles, 2002; Marcos et al., 1998) and this is particularly pronounced for the phosphate (Andersson et al., 1985; Antov & Peričin, 2003). As the part of partition studies in model aqueous two-phase systems, we evaluated the effect of two pH values of phosphate buffer in order to find the improved partition conditions for the cultivation in ATPS. According to our previous findings (Antov & Peričin, 2003), the concentration of phosphate was chosen to be 0.1 mol l^{-1} and partition experiments were conducted in the system 5%(w/w) PEG/4%(w/w) DEX, which corresponded to system with the phase volume ratio 1.0 at the tie-line length 7.44%.

The increase in pH of phosphate buffer from 5.0 to 7.0 with commercial pectinase shifted all endo-p activity to the top phase, which resulted in one-sided partition and consequently the maximal top phase yield (Table 1). However, higher values for both the partition coefficient and the top phase yield of exo-p were obtained at pH 5.0, the later as the partial consequence of the lower phase volume ratio at pH 7.0 (data not shown).

Considering previous results, the cultivation of *P. squamosus* was conducted in the system 5%(w/w) PEG/4%(w/w) DEX in the presence of 0.1 mol l^{-1} phosphate at two initial pH values, 5.0 and 7.0, and partitioning of biomass and produced endo-p and exo-p activities was monitored. Presence of dextranase activity was not detected in both aqueous two-phase cultivations.

Table 1
Effect of pH of 0.1 mol l^{-1} phosphate buffer on the partition coefficient and the top phase yield of endo-p and exo-p activities of commercial preparation in 5%(w/w) PEG/4%(w/w) DEX aqueous two-phase system

PH	Endo-p activity		Exo-p activity	
	K	*Y* (%)	*K*	*Y* (%)
5.0	3.51	81.36	1.31	62.00
7.0	∞	100	1.13	45.72

The time course of *P. squamosus* growth showed exclusively the one-sided partition of biomass to the bottom phase, no matter of initial pH of ATPS. The growth curve showed similar trend in both cultivations, but the amount of produced biomass was higher when initial pH was 5.0 (Fig. 3a). It is interesting to note that the higher initial pH in greater extent decreased the amount of biomass when cultivation was conducted in aqueous two-phase systems, i.e. in the presence of polymers, than in simultaneously conducted control homogeneous cultivation, and also, that the amount of biomass generally was higher in two-phase than in control systems, irrespectively, of initial pH. For example, at the second day of cultivation in homogeneous media at initial pH 5.0 and 7.0, biomass amounted 2.18 and 1.86 mg, respectively, and in heterogeneous media, 13.26 and 5.18 mg, respectively.

The changes of pH during the cultivations showed different pattern, but in both cases the slight differences of pH between the phases of the same system were observed (Fig. 3b). Maximal drop of pH in ATPS, from an initial pH 5.0, was on the first day, while in the system at initial pH 7.0 it happened on the third day of cultivation.

The partition coefficient and the top phase yield of endo-p activity produced by *P. squamosus* in ATPS (Table 2) with lower initial pH change during the cultivation, which might be explained by the effects of changeable amount of biomass (Fig. 3a) and metabolites, other than enzymes, on the partition conditions, as has been reported in several papers (Kwon, Kaul, & Matiasson, 1996; Planas, Lefebvre, Tjerneld, & Hahn-Hagerdal, 1997; Zijlstra et al., 1998). The highest K_{endo-p} was achieved on the second day and was followed by the highest obtained top phase yield.

It is very interesting that the endo-p activity was not detected in the ATPS medium when initial pH was 7.0, even though it was present in simultaneously conducted cultivation in heterogeneous medium at the same initial pH

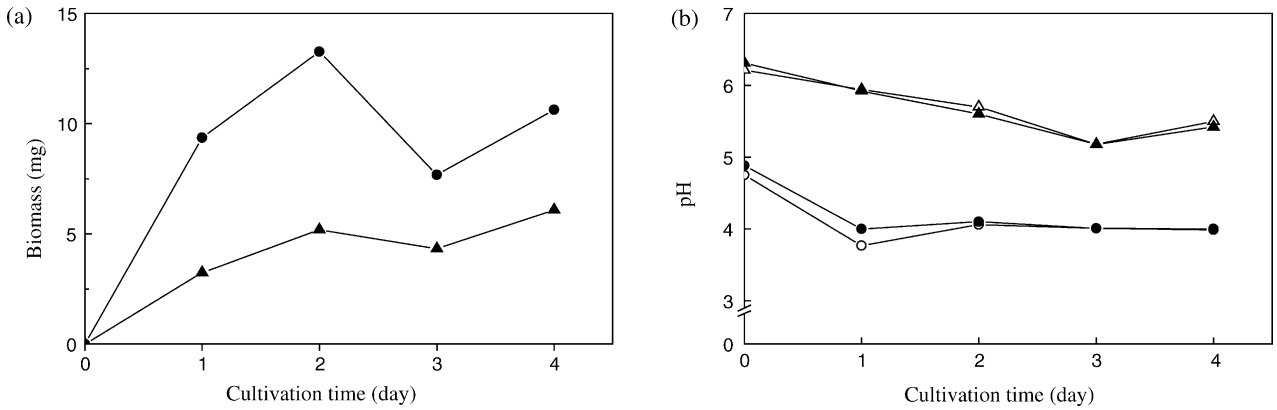

Fig. 3. Time course of (a) growth and (b) pH changes during the cultivation of *Polyporus squamosus* in 5%(w/w) PEG/4%(w/w) DEX aqueous two-phase system in (○) top and (●) bottom phases at initial pH 5.0, and (△) top and (▲) bottom phases at initial pH 7.0.

(data not shown). It may be supposed that the presence of polymers at higher pH did not create suitable environment for microorganism to produce endo-pectinase. Moreover, the lack of endo-p activity in cultivation at initial pH 7.0 may be the cause of significantly lower amount of produced biomass in comparison to cultivation at initial pH 5.0.

Total produced exo-p activity by *P. squamosus* generally was higher at initial pH 5.0 and partition parameters changed a little during the cultivation. The changes in partition parameters for exo-p activity were more characteristic for the cultivation conducted at initial pH 7.0-higher initial pH of ATPS favoured partition of the enzyme to the top phase during the cultivation, which resulted in increased values of the partition coefficient in comparison to those obtained at lower initial pH all along the time course of cultivation (Table 3). The highest value of $K_{exo\text{-}p}$ was attained on the third day of cultivation and it was about 2.5 times higher than the one achieved in cultivation at initial pH 5.0 at the same day. Higher partition coefficient was caused higher value of the yield in the top phase, or, in another words, improvement for about 45% in single extraction step in comparison to cultivation at lower pH, on the third and the fourth day.

The higher initial pH in the presence of phosphate improved both the partition coefficient and the top phase yield of exo-pectinase produced during the cultivation of *P. squamosus* in aqueous two-phase system, but under these conditions endo-p activity was not produced. On the other hand, favourable partition parameters for endo-pectinase were obtained when cultivation was conducted at initial pH 5.0; also the total produced activities of both pectinases were higher. Thus lower pHs are better conditions for cultivation of *P. squamosus* for pectinase production in aqueous two-phase media, composed of polyethylene glycol 4000 and crude dextran, containing pectin as a carbon source.

Table 2
Partition coefficient, top phase yield and total endo-p activity produced by *P. squamosus* during the cultivation in 5%(w/w) PEG/4%(w/w) DEX aqueous two-phase system at initial pH 5.0

Cultivation time (day)	Total (U)	$K_{endo\text{-}p}$	Y (%)
1	2.25	2.20	75.16
2	22.74	2.45	80.22
3	26.80	1.52	70.86
4	22.42	1.17	66.73

Table 3
Partition coefficient, top phase yield and total exo-p activity produced by *P. squamosus* during the cultivations in 5%(w/w) PEG/4%(w/w) aqueous two-phase systems at initial pH 5.0 and 7.0

Cultivation time (day)	Initial pH 5.0			Initial pH 7.0		
	Total (U)	$K_{exo\text{-}p}$	Y (%)	Total (U)	$K_{exo\text{-}p}$	Y (%)
1	963	0.60	45.20	556	0.61	47.78
2	6465	0.60	49.83	1094	1.15	63.30
3	5401	0.59	48.56	1554	1.44	70.59
4	4673	0.50	46.15	1847	1.07	66.90

References

Andersson, E., & Hahn-Hagerdal, B. (1990). Bioconversions in aqueous two-phase systems. *Enzyme and Microbial Technology*, *12*, 242–254.

Andersson, E., Johansson, A. C., & Hahn-Hagerdal, B. (1985). α-Amylase production in aqueous two-phase systems with *Bacillus subtilis*. *Enzyme and Microbial Technology*, *7*, 333–338.

Aguilar, G., & Huitron, C. (1990). Constitutive exo-pectinase produced by *Aspergillus* sp. CH-Y-1043 on different carbon source. *Biotechnology Letters*, *12*, 655–660.

Antov, M., & Peričin, D. (2001). Production of pectinases by *Polyporus squamosus* in aqueous two-phase system. *Enzyme and Microbial Technology*, *28*, 467–472.

Antov, M., & Peričin, D. (2003). Effect of some inorganic salts on the partitioning of pectinase of *Polyporus squamosus* in polyethylene glycol/crude dextran aqueous two-phase system. *World Journal of Microbiology and Biotechnology*, *19*, 151–156.

Antov, M., Peričin, D., & Dimić, G. (2001). Cultivation of *Polyporus squamosus* in aqueous two-phase system containing sugar beet extraction waste. *Journal of Biotechnology*, *91*, 83–87.

Chen, J. P., & Lee, M. S. (1995). Enhanced production of *Serratia marcescens* chitinase in PEG/dextran aqueous two-phase systems. *Enzyme and Microbial Technology*, *17*, 1021–1027.

Fogarty, W., & Kelly, C. (1983). Pectic enzymes. In W. Fogarty (Ed.), *Microbial enzyme and biotechnology* (pp. 131–182). London: Applied Science Publishers.

Furuya, T., Yamada, S., Zhu, J., Yamaguchi, Y., Iwai, Y., & Arai, Y. (1996). Measurement and correlation of liquid–liquid equillibria and partition coefficients of hydrolytic enzymes for DEX T500 + PEG0 + water aqueous two-phase system at 20 °C. *Fluid Phase Equillibria*, *125*, 89–102.

Hotha, S., & Banik, R. M. (1997). Production of alkaline protease by *Bacillus thuringiensis* H 14 in aqueous two-phase systems. *Journal of Chemical Technology and Biotechnology*, *69*, 5–10.

Kwon, Y., Kaul, R., & Matiasson, B. (1996). Extractive lactic acid fermentation in poly(ethyleneimine)-based aqueous two-phase system. *Biotechnology and Bioengineering*, *50*, 280–290.

Lima, A. S., Alegre, R. M., & Meirelles, A. J. A. (2002). Partitioning of pectinolytic enzymes in polyethylene glycol/potassium phosphate aqueous two-phase systems. *Carbohydrate Polymers*, *50*, 63–68.

Marcos, J. C., Fonseca, L. P., Ramalho, M. T., & Carbal, J. M. S. (1998). Variation of penicillin acylase partition coefficient with phase volume ratio in poly(ethylene glycol)-sodium citrate aqueous two-phase systems. *Journal of Chromatography B*, *711*, 295–299.

Miller, G. L. (1959). Use of dinitrosalicilyc acid reagent for determination of reducing sugars. *Analytical Chemistry*, *31*, 426–428.

Munro, H. N., & Fleck, A. (1966). Recent developments in the measurement of nucleic acid in biological materials. *Analyst*, *91*, 78–88.

Peričin, D., Antov, M., & Popov, S. (1998–1999). Simultaneous production of biomass and pectinases by *Polyporus squamosus*. *Acta Periodica Technologica*, *29–30*, 183–189.

Peričin, D., Kevrešan, S., Banka, L., Antov, M., & Škrinjar, M. (1992). Separation of the components of pectinolytic complex produced by *Polyporus squamosus* in submerged culture. *Biotechnology Letters*, *14*, 127–130.

Planas, J., Lefebvre, D., Tjerneld, F., & Hahn-Hagerdal, B. (1997). Analysis of phase composition in aqueous two-phase systems using a two-column chromatographic method: Application to lactic acid production by extractive fermentation. *Biotechnology and Bioengineering*, *54*, 303–311.

Rogalski, J., Szczodrak, J., Glowiak, G., Plszczynska, M., Szczodrak, Z., & Wiater, A. (1998). *Acta Biotechnologica*, *18*, 63–75.

Skoog, B. (1979). Determination of polyethylene glycols 4000 and 6000 in plasma protein preparations. *Vox Sangunis*, *37*, 345–349.

Zijlstra, G. M., de Gooijer, C. D., & Tramper, J. (1998). Extractive bioconversions in aqueous two-phase systems. *Current Opinion in Biotechnology*, *9*, 171–176.