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Phosphodiesterase Production in an Aqueous Two-Phase System by *Nicotiana tabacum* 1507

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Abstract

Studies were conducted on the production of phosphodiesterases by *Nicotiana tabacum* 1507 cell suspension in an aqueous two-phase system formed by adding 4% polyethylene glycol (mol wt 20,000) and 7.5% dextran (mol wt 70,000) to the medium. The time course of growth, biosynthesis, secretion, and partitioning of phosphodiesterases was followed in comparison with *N. tabacum* 1507 cultivation as a free suspension. Partitioning of phosphodiesterases took place mainly in the bottom dextran phase, and a possibility was revealed for obtaining an enzyme preparation with high phosphodiesterase activity.

Index Entries: Aqueous two-phase system; *Nicotiana tabacum* 1507; phosphodiesterases; plant cell suspension culture.

Introduction

Aqueous two-phase systems (ATPSs) are found when two hydrophilic but incompatible substances are simultaneously dissolved in water *(1)*. They are composed of aqueous solutions of either of two water-soluble polymers, usually polyethylene glycol (PEG) and dextran, or of a polymer and a salt, usually PEG and phosphate or sulfate *(1–3)*. ATPSs provided ideal surroundings for the extractive separation of enzymes and proteins *(4–7)* from culture media because of their peculiarities such as high water

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content of both phases (70–80% [v/v]), which means high biocompatibility and low interfacial tension, minimizing product degradation, good resolution, and high yields *(6,8)*. On the other hand, ATPSs have a relatively high capacity, ease of scale-up, low material costs, and the possibility of polymer recycling. Because of these advantages, they are not only a convenient method for easier separation and purification of proteins (enzymes in particular), but they have been used for cultivation of microorganisms and production of enzymes *(9–15)*, as well as for extractive bioconversion *(16)*. Recently their application has been extended to the cultivation of plant cell suspensions *(17,18)* and hairy root cultures *(19)*.

In this article, data are presented for the biosynthesis, secretion, and partitioning of alkaline and acid phosphodiesterases during cultivation of *N. tabacum* 1507 cell suspension in an aqueous two-phase system.

Materials and Methods

Polymers

PEG (mol wt 20,000; Merck) and dextran (mol wt 70,000; Troyan, Bulgaria) were used to obtain the ATPS.

Plant Cell Culture and Culture Medium

The plant cell culture *N. tabacum* 1507 used was cultivated on Linsmayer-Skoog medium *(20)*, supplemented with 3% sucrose and 0.2 mg/L of 2,4-dichlorophenoxyacetic acid. The pH of the medium was adjusted to 5.6–5.8 before autoclaving.

For the formation of the aqueous two-phase cultivation system (ATPCS) marked as PD_5 *(21,22)*, 4% PEG and 7% dextran were added to the medium used for the cultivation of *N. tabacum* 1507 as a free suspension. After preheating the mixture in a water bath to dissolve the polymers, the two-phase medium obtained was autoclaved.

Cultivation of Cell Suspension

The cultivation of *N. tabacum* 1507, both in standard culture medium (used for control cultivation and for inoculum production) and in ATPCS PD_5 was carried out in Erlenmeyer flasks with 1/5 net volume, on a shaker (11.6 rad/s) at 26–28°C in the dark, for 10 d. For inoculation, 20% (v/v) of the cell suspension was used, grown on a standard culture medium for 7 d under the aforementioned conditions.

Analyses

Daily determinations of the following parameters were performed in samples from control and two-phase cultivations.

Cell Growth

The cell suspension growth was monitored by measuring the dry biomass (DB) *(23)*.

Phase Characteristics

The changes in the volume of the biomass and the phase volume during the cultivation were estimated after centrifugation (3000*g*, 5 min) of the cell suspension samples in 10 mL of graduated centrifugal tubes. The results are expressed as a percentage of the total volume.

Determination of Phosphodiesterase

The amount of alkaline (pH 8.0) and acid (pH 5.7) phosphodiesterases in the biomass and in the culture medium as well as their changes during both the control and two-phase cultivations were followed.

The biomass was lyophilized and 10 mg was homogenized using a Modular Homogenizer system (Cole-Parmer) in 1 mL of water. After centrifugation (10 min in an Eppendorf Centrifuge 5415), the supernatant was collected. The two-phase culture medium was centrifuged to separate the phases, from which, the samples were taken for analysis. The samples taken from both phases and the control culture medium were analyzed without preliminary treatment.

Phosphodiesterase activities were measured using *bis p*-nitrophenol phosphate (*bis-p*-NPP), the nonspecific substrate for phosphodiesterases and some nucleases, and 5'-thymidine monophospho-*p*-nitrophenyl ester (*p*-NP.pT), which can distinguish the hydrolytic activity for the 5'-phosphodiester bond *(24)*.

Phosphodiesterase activities in different samples were measured in an incubation mixture containing in a 275-µL total volume 25 µL of the particular substrate in either 150 µL of 0.1 *M* (0.5 *M*) ammonium acetate buffer (pH 5.7) or 0.1 *M* Tris-HCl buffer (pH 8.0). The enzyme reaction was carried out for 20 min at 25°C after the addition of 100 µL of the analyzed solution. The reaction was stopped with 1.5 mL of 0.2 *M* NaOH and the product, *p*-nitrophenyl, was estimated spectrophotometrically (Spectromic 2000, Bausch & Lomb) at 400 nm, using 17,600 as the molar absorption coefficient. To characterize the partitioning of the phosphodiesterases between the phases, the partition coefficient (K_p) was calculated as the ratio of the enzyme activity in the top phase to that in the bottom phase according to the following equation:

$$K_p = (EA \text{ top phase}/EA \text{ bottom phase})$$

And to characterize the effectiveness of ATPCS PD_5 in the phosphodiesterase biosynthesis, secretion, and partitioning, the enzyme yields were calculated in both phases according to the following equation:

$$E_y = EA \times V \text{ phase}$$

in which *EA* is the enzyme activity (U/mL), and *V* is the volume (mL).

The enzyme yield in the biomass was calculated as follows:

$$E_y = EA(b) \times \text{DB}$$

in which *EA*(*b*) is the enzyme activity (U/g of DB), and DB is dry biomass (g). The results represent the averages of the three simultaneous experiments, performed in duplicate.

Results

The comparison between the time courses of *N. tabacum* 1507 growth, cultivated as a free suspension (control) and in ATPCS PD_5 (Fig. 1), showed that the growth maxima were reached on d 7 when cultivated as a free suspension and on d 8 when cultivated in ATPCS PD_5. The rate of growth in the ATPCS PD_5 was lower (13.0 vs 16.5 g/L of DB for the control cultivation (Fig. 1), and the cell biomass remained between the two phases as a layer with a measurable volume designated as a phase interface *(17)*. During cultivation, the changes in the phase characteristics of both cultivation systems (free suspension and ATPCS PD_5) were followed as well (Fig. 2). When *N. tabacum* 1507 was grown as a free suspension, the cell biomass gradually increased to reach a packed volume of up to 80% on d 8 of cultivation. In ATPCS PD_5, the biomass packed volume formed an interface layer that reached a maximum of about 48% on d 8 of cultivation (Fig. 2) as well.

The time courses of the changes in the content of cell phosphodiesterase activities during cultivation, measured with *bis-p*-NPP (Fig. 3) and with the specific substrate for 5'-phosphodiesterases, *p*-NP.pT (Fig. 4), showed that the maxima in the biosynthesis of cell phosphodiesterases were reached on d 5 during the control cultivation and d 4 during the ATPCS PD_5 cultivation. The intracellular content of both the acid and alkaline phosphodiesterases during cultivation in ATPCS PD_5 was lower compared with the control (Figs. 3 and 4).

As shown in Table 1, in ATPCS PD_5 the main part of the extracellular proteins was partitioned in the bottom phase of the system with higher amounts than the control culture medium. The cultivation of *N. tabacum* 1507 in ATPCS PD_5 was favorable for the partitioning of the extracellular phosphodiesterases in the bottom phase (Figs. 5 and 6). However, note that the maximum partitioning of phosphodiesterase activities in the bottom dextran phase was reached 2 d later compared with the maxima of the secreted phosphodiesterases in the culture medium of the control cultivation. The values for the partition coefficients of the phosphodiesterases, measured with *bis-p*-NPP (Table 2), as well as those for 5'-phosphodiesterases, measured with the specific substrate *p*-NP.pT (Table 3), also showed that in ATPCS PD_5 the investigated enzyme activities were almost completely partitioned in the bottom dextran phase and only traces were found in the top PEG phase.

The overall process of biosynthesis secretion and partitioning of phosphodiesterase activities under study during the cultivation of *N. tabacum* 1507 as a free suspension (control) and in ATPCS PD_5 are presented in Figs. 7 and 8.

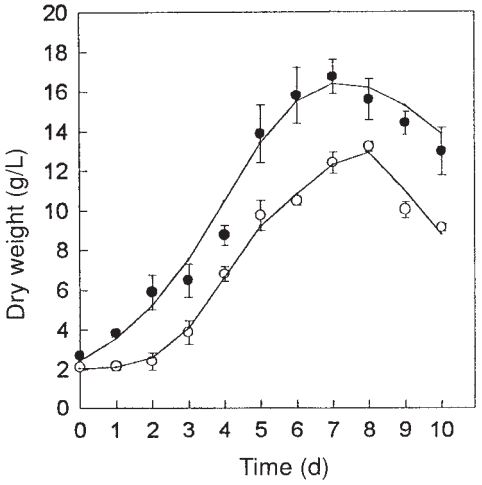

Fig. 1. Time course of growth of *N. tabacum* 1507 cultivated as a free suspension and in ATPCS PD_5. ●, Free suspension; ○, two-phase cultivation.

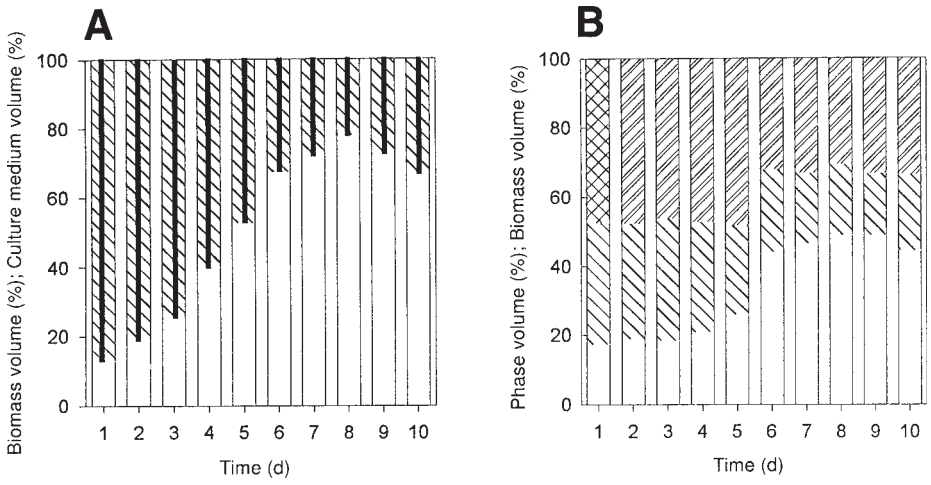

Fig. 2. Changes in phase characteristics of *N. tabacum* 1507 cultivated as a free suspension **(A)** and in ATPCS PD_5 **(B)**. ▭ Biomass; ▨ bottom phase; ▨ top phase; ▨ culture medium.

Discussion

The phosphohydrolytic enzymes (and phosphodiesterases in particular) are of commercial interest for the production of nucleic acid–related compounds with application as food additives, biochemical reagents, and pharmaceuticals *(25,26)*. The plant cell cultures, especially tobacco cell cultures *(26)*, offered a new possibility for their production by plant cell biotechnologies. *N. tabacum* 1507 was selected as a cell culture with high levels of phosphohydrolytic activities *(27)*. In our previous investigations,

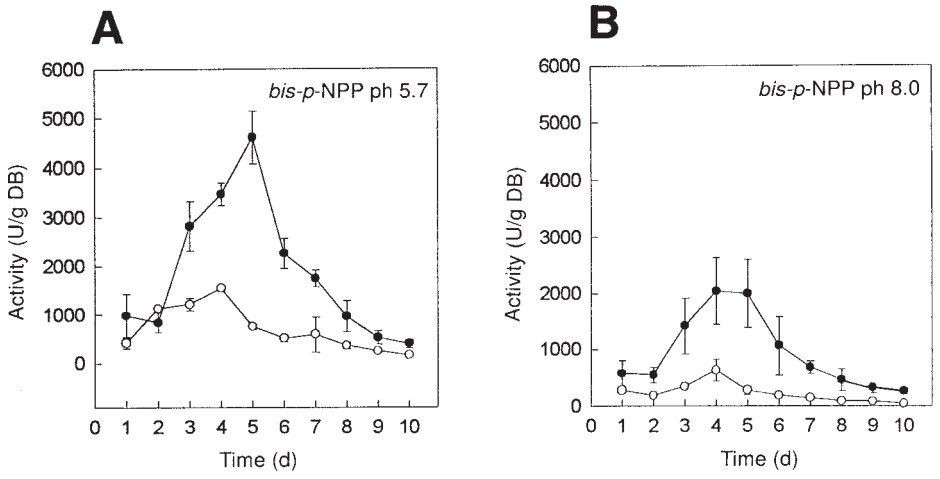

Fig. 3. Changes in the cellular content of acid phosphodiesterases **(A)** and alkaline phosphodiesterases **(B)** (determined by *bis-p*-NPP) during cultivation of *N. tabacum* 1507 as a free suspension and in ATPCS PD_5. ●, Free suspension; ○, two-phase cultivation.

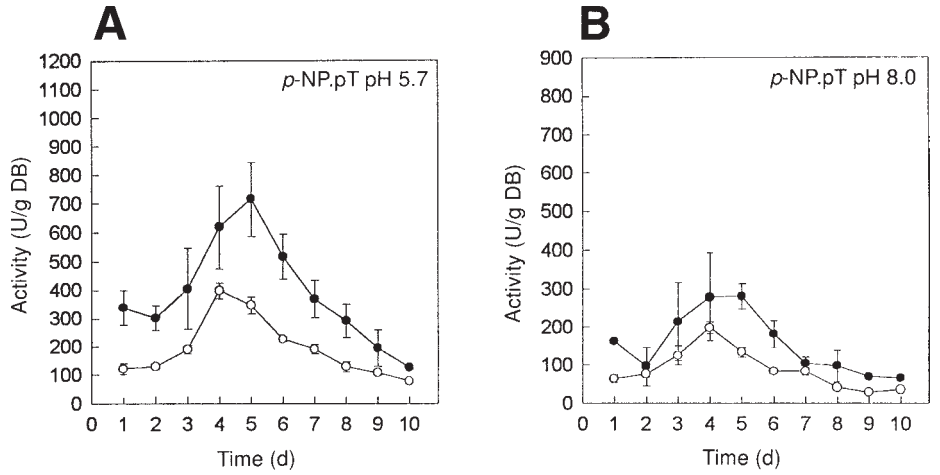

Fig. 4. Changes in the cellular content of acid 5'-phosphodiesterases **(A)** and alkaline 5'-phosphodiesterases **(B)** (determined by *p*-NP.pT) during cultivation of *N. tabacum* 1507 as a free suspension and in ATPCS PD_5. ●, Free suspension; ○, two-phase cultivation.

the cell culture under study was cultivated in different ATPCSs, formed by the addition of PEG and dextran to the culture medium, varying their amounts and molecular masses *(21,22)*. The ATPCS referred to as PD_5 *(22)* proved to be most suitable for the growth and obtaining of enhanced yields of extracellular phosphohydrolases *(3)*.

The plant cell suspension is a physiologically complex system, in which not only the cell biomass but also the culture medium is involved

Table 1
Changes in Extracellular Protein Content
During Cultivation of *N. tabacum* 1507 as a Free Suspension
(control) and in ATPCS PD_5 (bottom and top phases)

Time (d)	Protein (mg/mL)		
	Bottom phase	Top phase	Control
1	0.48	0.25	0.18
2	0.72	0.30	0.37
3	0.90	0.52	0.22
4	0.95	0.40	0.29
5	0.82	0.30	0.17
6	0.81	0.29	0.19
7	0.78	0.18	0.19
8	0.77	0.10	0.18
9	0.62	0.17	0.10
10	0.87	0.15	0.09

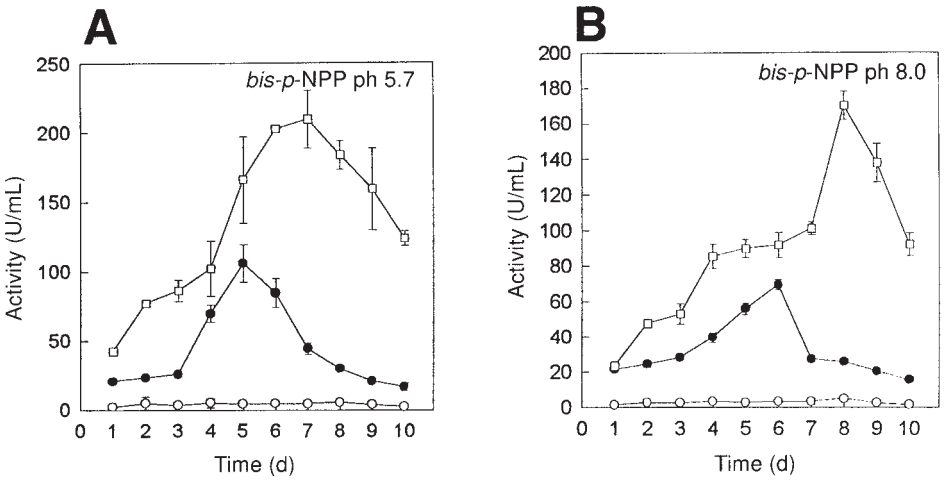

Fig. 5. Time course of the biosynthesis and partitioning of extracellular acid phosphodiesterases **(A)** and alkaline phosphodiesterases **(B)** (determined by *bis-p*-NPP) during cultivation of *N. tabacum* 1507 as a free suspension (control) and in ATPCS PD_5. ●, Control; ○, top phase; □, bottom phase.

(28). In the cell suspension, however, there is always an equilibrium between the content of metabolites in the cell and in the culture medium. The latter is a functional cell compartment in which different metabolites are secreted (including enzymes) and different metabolic performances take place as well. During the formation of an ATPCS, the nature of this functional cell compartment is changed, which leads to changes in the physiologic characteristics of the culture and hence to changes in the overall picture of biosynthesis, secretion, and partitioning of metabolites. Under

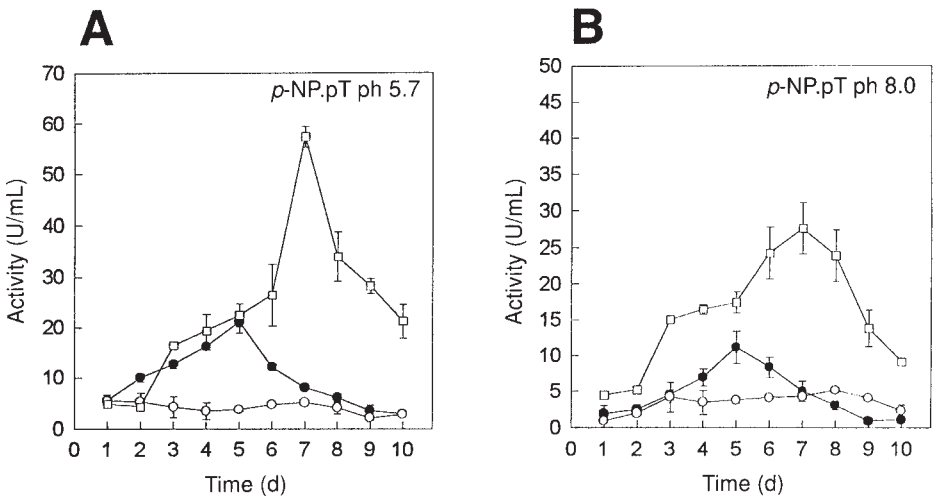

Fig. 6. Time course of the biosynthesis and partitioning of extracellular acid 5'-phosphodiesterases **(A)** and alkaline 5'-phosphodiesterases **(B)** (determined by *p*-NP.pT) during cultivation of *N. tabacum* 1507 as a free suspension (control) and in ATPCS PD_5. ●, Control; ○, top phase; □, bottom phase.

Table 2
Partition Coefficients
of Phosphodiesterases
(determined by *bis*-*p*-NPP)
During Cultivation of *N. tabacum* 1507
in ATPCS PD_5

Time (d)	Partition coefficient	
	pH 5.7	pH 8.0
1	0.05	0.06
2	0.06	0.06
3	0.04	0.05
4	0.05	0.04
5	0.03	0.03
6	0.02	0.04
7	0.02	0.03
8	0.03	0.03
9	0.02	0.02
10	0.02	0.02

these new conditions, a new equilibrium is established between the metabolites in the cells and phases; it is specific for each metabolite and continuously changes in the process of cultivation.

During the cultivation of *N. tabacum* 1507 in ATPCS PD_5, the cell biomass remained between the two phases. Because of that, mass transfer limitations appeared *(16,17)* as much as the contact of the cells with nutrient

Table 3
Partition Coefficients
of 5'-Phosphodiesterases
(determined by *p*-NP.pT)
During Cultivation of *N. tabacum* 1507
in ATPCS PD_5

Time (d)	Partition coefficient	
	pH 5.7	pH 8.0
1	1.13	0.21
2	1.52	0.39
3	0.16	0.28
4	0.27	0.21
5	0.16	0.22
6	0.22	0.17
7	0.09	0.15
8	0.17	0.22
9	0.09	0.29
10	0.14	0.26

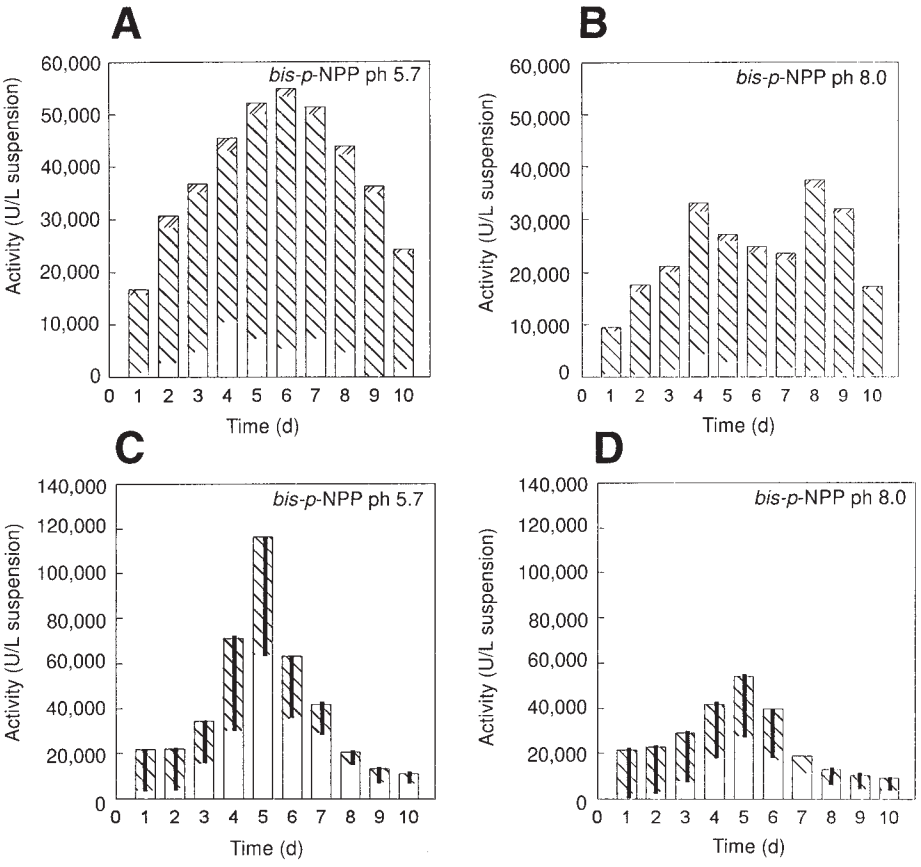

Fig. 7. Overall process of biosynthesis, secretion, and distribution of phosphodiesterases (determined by *bis-p*-NPP) by *N. tabacum* 1507 in ATPCS PD_5 (acid [**A**] and alkaline [**B**]) and as a free suspension (acid [**C**] and alkaline [**D**]). ▭ Biomass; ▨ top phase; ▨ bottom phase; ▨ culture medium.

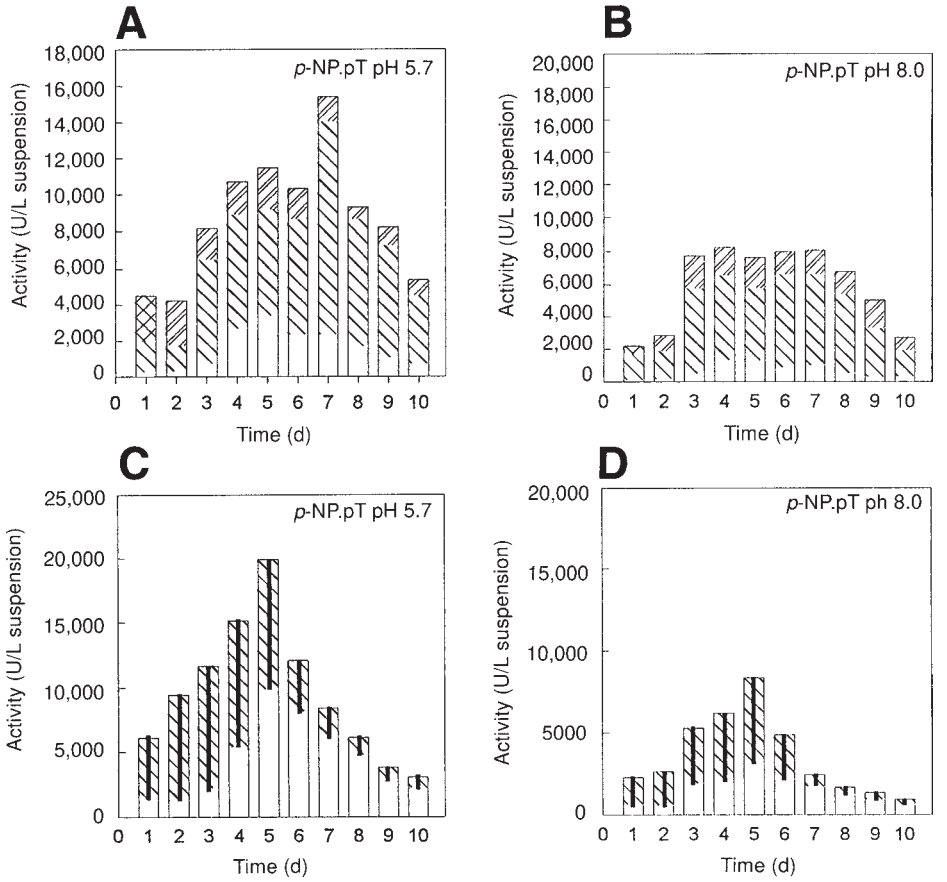

Fig. 8. Overall process of biosynthesis, secretion, and distribution of 5'-phosphodiesterases (determined by *p*-NP.pT) by *N. tabacum* 1507 in ATPCS PD_5. (acid [**A**] and alkaline [**B**]) and as a free suspension (acid [**C**] and alkaline [**D**]). ☐ Biomass; ▨ top phase; ▨ bottom phase; ▨ culture medium.

components in the phases occurred only through agitation, and that could explain the lower rate of growth (Fig. 1). Drastic differences in the biomass packed volume were established during the cultivation of the culture in ATPCS PD_5 and as a free suspension (Fig. 2). The reduced biomass packed volume in ATPCS PD_5 was mainly owing to the osmotic effect, caused by the polymers, forming the PEG and dextran phases. In this system, the cells were located between two layers with considerably higher viscosities.

The intracellular phosphodiesterase activities measured with both substrates (Figs. 3 and 5) were lower during cultivation of *N. tabacum* 1507 in ATPCS PD_5, which could be related both to the changes in the time course of biosynthesis (in ATPCS PD_5 the maximum in the intracellular phosphodiesterase activities was reached on d 4, and on d 5 in the control cultivation, as shown in Figs. 3 and 4), and to the changed time courses of enzyme secretion and partitioning (Figs. 5 and 6).

In the process of partitioning and secretion of the extracellular metabolites between the two phases of ATPCS PD_5, those of protein nature concentrated in the bottom dextran phase; between d 5 and 8 of the cultivation, the amounts of the proteins partitioned in the bottom phase were more than four times higher compared to the control (Table 1). Extracellular phosphodiesterases were partitioned mainly in the bottom phase of ATPCS PD_5 as well (Figs. 5 and 6, Tables 2 and 3), where more than two times higher phosphodiesterase activities were determined. The total yield of phosphodiesterases from a unit fermentation volume (including the intracellular activities and the activities in the bottom phases) was lower in ATPCS PD_5 (Figs. 7 and 8), although in the same two-phase system of cultivation the yield of produced phosphomonoesterases did not change *(18)*. That fact was in favor of the thesis of the specifically altered intensities of biosynthesis and secretion of the different metabolites (phosphodiesterases in particular) under the new conditions in ATPCS PD_5.

In conclusion, during the cultivation of *N. tabacum* 1507 in ATPCS PD_5, the biosynthesized phosphodiesterases were preliminarily extracellular compared with the control cultivation, in which a substantial part of them were intracellular. The total amount of secreted phosphodiesterases in ATPCS PD_5 was equal to or slightly greater than that in the control cultivation, but they were partitioned exclusively in the bottom phase. Therefore, possibilities were revealed for the production of highly active preparations of phosphodiesterases (Tables 2 and 3) in a relatively small volume (Fig. 2) as soon as during cultivation. This finding is very important for the next step of purification of the phosphodiesterases (after cultivation is performed and the bottom dextran phase with high enzyme activities is separated).

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