

Enhancement of PVA-Degrading Enzyme Production by the Application of pH Control Strategy

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In batch culture for Poly(vinyl alcohol) (PVA)-degrading enzyme (PVAase) production by a mixed culture, higher pH (pH 7.5) was favorable for PVAase production at the prophase of cultivation, but lower pH (pH 7.0) was favorable at the anaphase. This situation was caused by the fact that the optimum pH for different key enzymes [PVA dehydrogenase (PVADH) and oxidized PVA hydrolase (OPH)] production is various. The activity and average specific production rate of PVADH reached the highest values at constant pH 7.5, whereas those of OPH appeared at pH 7.0. A two-stage pH control strategy was therefore developed and compared for its potential in improving PVAase production. By using this strategy, the maximal PVAase activity reached 2.05 U/ml, which increased by 15.2% and 24.2% over the fermentation at constant pH 7.5 and 7.0.

Keywords: Mixed culture, pH control strategy, PVA-degrading enzyme, PVA dehydrogenase, oxidized PVA hydrolase

Poly(vinyl alcohol) (PVA) is the only carbon–carbon backbone polymer that is biodegradable under both aerobic and anaerobic conditions. It is widely used in the industrial fields. PVA is used for fiber-coating to improve the strength and quality of fiber in textiles and is also used in chemical or manufacturing industries as paper-coating material and adhesives of stamps [22]. Ironically, although PVA has the potential as a biodegradable polymer, it has become one of the major pollutants of present-day industrial wastewater in the textile industry.

The study on biodegradation of PVA has increased significantly with the first discovery of PVA-degrading bacteria *Pseudomonas* O-3 [19]. Since then, researchers

worldwide have focused their attention toward new microbial isolates [10, 24], PVA-degrading enzyme (PVAase) purification [6, 11] and PVA biodegradation mechanism [1, 11]. A pathway for PVA biodegradation in *Pseudomonas* sp. VM15C is the combination of PVA dehydrogenase (PVADH) and oxidized PVA hydrolase (OPH) [17]. PVA is first oxidized by PVADH or secondary alcohol oxidase with formation of β -diketone structure along the polymer chain, and then the β -diketone structure is cleaved by OPH to form methyl ketone, carboxylic acid, and acetic acid, etc. [7, 9, 14].

However, little has been reported about the optimization of fermentation conditions in PVAase production. The relative works were only made in shake flask concerned with the optimization of PVA concentration, carbon and nitrogen sources, and temperature [2, 13]. Recently, researchers discovered that glycine reduced the fermentation time and increased the productivity of recombinant OPH [23]. However, other environmental factors such as pH also play an important role in PVAase production [2]. To our knowledge, few studies have been reported on the effect of pH on the PVAase production in submerged culture.

In this study, the effects of pH on the batch fermentation of PVAase with a mixed culture were studied in detail. Based on kinetic analysis of production rates of PVAase, PVADH, and OPH, an optimal two-stage pH control strategy was developed to improve the activity and productivity of PVAase. The effectiveness of the pH control strategy was verified experimentally.

MATERIALS AND METHODS

PVA Materials

PVA 1799 (average molecular mass: 113 kDa) with a 1,700 polymerization degree and 99.0% saponification degree was purchased from Sichuan Vinyl Factory (Mianyang, China).

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Microorganisms and Culture Conditions

The strains (a mixed culture) used in this study were isolated from PVA-rich soil, which was sampled at Pacific Textile Co. (Jiangsu Province, China). The mixed culture contained *Pseudomonas* sp., *Sphingomonas* sp., *Rhodococcus* sp., *Bacillus* sp., *Brevibacterium* sp., *Flavobacterium* sp., *Micrococcus* sp., *Streptococcus* sp., *Leptothrix* sp., and *Paenibacillus* sp.

The ability of PVA-utilizing of the mixed culture was confirmed [20, 24]. The PVA could not be degraded completely by the single strains isolated in a previous study. However the mixed culture degraded 5 g/l of PVA thoroughly within 36 h. At the same time, the PVAase activity produced by the mixed culture was higher than other single strains. Therefore, the mixed culture was used for the study.

The seeds and fermentation medium composition were as follows (g/l): PVA1799, 5; yeast extract, 2 [the type of "yeast" used for the extracts is *Saccharomyces cerevisiae*. The reagents were purchased from Sangon Biotech (Shanghai) Co., Ltd.]; $K_2HPO_4 \cdot 3H_2O$, 2; KH_2PO_4 , 0.25; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.05; and NaCl, 0.02. The mixed culture was grown in flasks at 30°C and 200 rpm for 36 h.

Batch Fermentation in the 7 l Stirred Fermentor

Fermentations were carried out in a fermentor (KF-7 l; Korea Fermentor Co., Inchon, Korea) with 4 l of fermentation medium and an inoculum size of 10% (v/v). The agitation speed and aeration rate were controlled at 300 rpm and 2 vvm, respectively. pH was controlled according to requirement with addition of 2 M NaOH or 2 M HCl solution. The culture was centrifuged at 15,000 $\times g$ and 4°C for 20 min to obtain the supernatant. For each pH control experiment, about 12 samples (every three hours during the culture time of 0–36 h) were taken and analyzed in duplicate. All experiments were done in duplicate.

pH Control

The online pH electrode connected to the fermentor was used as the equipment for pH control. During the fermentation, the pH control was automatic. When the pH was higher than the set value, 2 mol/l of NaOH solution was fed to the fermentor automatically. The pH electrode was made by US Broadley-James Corporation.

Analytical Methods

Quantitative determination of PVA concentration in the culture broth was carried out by spectrophotometric analysis after addition of boric acid and iodine solutions according to the procedure described by Finley [3].

The assay of PVAase activity was based on the methods described by Mori *et al.* [12]. The reaction mixture containing 1 ml of the crude enzyme solution and 1 ml of the 0.1% PVA solution in 100 mM potassium phosphate buffer (pH 7.0) was incubated at 30°C for 3 h. One unit of PVAase activity was defined as an absorbance decrease of 1×10^{-3} per minute at 690 nm under the specified conditions, and was expressed as the total degradation activity for PVA.

The assay of PVADH was referenced to the method described by Matsumura *et al.* [11]. One unit of the enzyme activity was defined as the amount that reduced 1 μM of 2,6-dichlorophenolindophenol (DCPIP) per minute under the assay conditions.

The assay of OPH was referenced to the method described by Kawagoshi and Fujita [6]. One unit (U) of enzyme activity was

defined as the amount of enzyme that produced 1 μM of acetic acid per minute.

RESULTS AND DISCUSSION

Time Courses of PVAase Fermentation at Different pH Values

To explore the influence of different pH values on PVAase production, the pH was controlled at constant values of 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 in the production of PVAase (Fig. 1). With pH lower than 6.5 or higher than 8.0, little PVAase production was observed (data not shown).

As shown in Fig. 1A, a rapid increase of PVAase activity was obtained in the first 15 h and followed by a

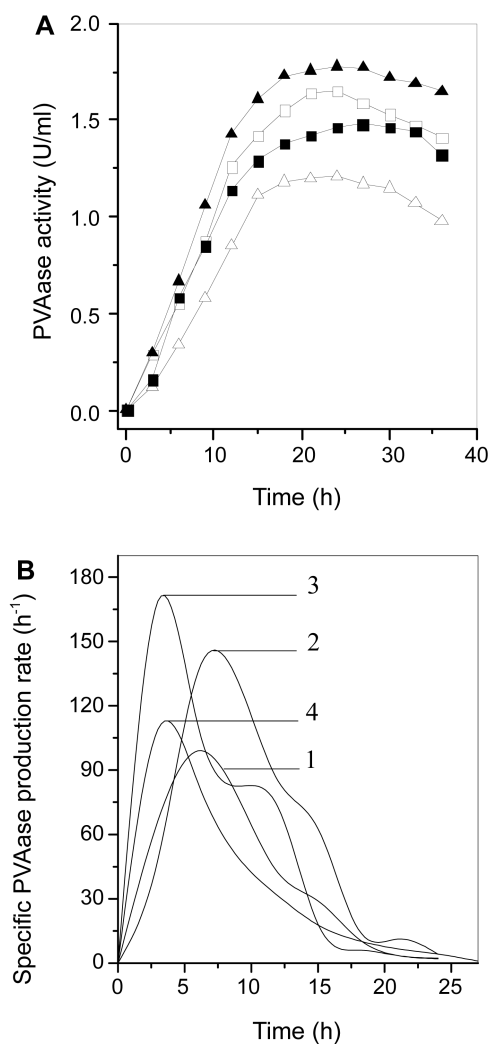


Fig. 1. Time courses of PVAase fermentation (A) and specific PVAase production rate (q_p) (B) during the batch cultivation of a mixed culture at different pH values.

△ and curve 1, pH 6.5; □ and curve 2, pH 7.0; ▲ and curve 3, pH 7.5; ■ and curve 4, pH 8.0.

Table 1. Comparison of parameters in fed-batch fermentations of PVA-degrading enzyme (PVAase) by a mixed culture grown for 36 h at different pH values.

Parameters	pH				
	6.5	7.0	7.5	8.0	7.5–7.0 ^a
Maximum PVAase activity (U/ml)	1.21±0.04	1.65±0.06	1.78±0.05	1.48±0.05	2.05±0.07
Residual PVA concentration (g/l)	1.22±0.03	0.95±0.03	0.81±0.02	1.02±0.04	0.61±0.03
Maximum specific PVAase production rate (U/g/h)	99.0±0.46	135.6±0.52	168.9±0.65	108.9±0.57	161.8±0.69
Average specific PVAase production rate (U/g/h)	32.4±0.21	45.7±0.28	48.3±0.27	29.2±0.25	52.7±0.29
PVAase productivity (U/l/h)	50.4±0.29	68.7±0.31	74.2±0.30	54.8±0.28	85.4±0.35

^aThe pH was switched from 7.5 to 7.0 at 15 h. Data from duplicate fermentations are shown.

slow increasing tendency until a decrease appeared in all pH cases. The maximum PVAase activity (1.78 U/ml) was obtained at pH 7.5 after 24 h of fermentation.

The kinetic parameters of the specific PVAase production rate (q_p), was obtained from the following definitions [8, 18, 21]:

$$q_p = \frac{1}{X} \frac{dP}{dt} = \frac{1}{X} \lim_{\Delta t \rightarrow 0} \frac{\Delta P}{\Delta t}$$

As illustrated in Fig. 1B, the maximum specific PVAase production rate (q_p) was observed at pH 7.5, and it is interesting to find that a relatively high value appeared at pH 7.0 after 15 h.

The comparison of parameters under different pH values is summarized in Tables 1 and 2. Table 1 shows that the average specific PVAase production rate reached the maximum value of 48.3 U/g/h at pH 7.5. However, as illustrated in Table 2, further analysis indicated that it reached the highest value at pH 7.5 in the first 15 h and at pH 7.0 afterwards. This indicates that the suitable pH for PVAase production is different at different fermentation stages.

Effects of Different pH Values on the Key Enzymes Involved in the Biodegradation of PVA

In the PVA biodegradation systems, the carbon-carbon linkage of PVA main-chains is cut first by the action of

PVADH, followed by OPH [16]. A question arises as to whether or not the optimum pHs for PVADH and OPH are different, and subsequently induced the situations that the average specific PVAase production rate was changed at different fermentation stages. The activities of PVADH and OPH were therefore monitored.

As illustrated in Fig. 2, the two enzymes exhibited the highest activities at different pH values. PVADH activity always kept at a higher level and reached the maximum of 47.12 U/ml at pH 7.5 (Fig. 2A), whereas OPH activity reached the highest value of 2.11 U/ml at pH 7.0 and kept a relative higher level during the whole fermentation process (Fig. 2B). The results co-incided with other reports. The optimum pH of PVADH produced by *Pseudomonas* sp. 113P3 was 7.2–7.6 [4], whereas OPH was most active and stable at pH values between 6.0 and 7.0 [15].

The maximum specific production rate of the two enzymes also exhibited different results. The maximum specific production rate of PVADH (Fig. 2C) and OPH (Fig. 2D) was obtained at pH 7.5 and pH 7.0, respectively. The average specific PVADH and OPH production rate reached 1,013.4 U/g/h and 44.2 U/g/h at pH 7.5 and pH 7.0, respectively (Table 2).

In the mixed culture, PVAase activity presented as the total sum activities of PVADH and OPH. They are synergistic effects and should have a certain ratio in the mixture. Only when both of them showed higher activity could a high enough PVAase activity then be achieved. However, it could not be satisfied for both of them under single pH conditions.

Combining this result with that obtained in the analysis of average specific PVAase production rate at different stages (Table 2), it could be concluded that it is important to control different pHs for PVAase production at different fermentation stages.

Therefore, it is favorable to use a two-stage pH control strategy instead of constant pH process to improve PVAase production. An optimal pH-shift strategy was developed as follows: pH was controlled at 7.5 during 0–15 h, and then shifted to 7.0 within 15 min after 15 h.

Table 2. Comparison of average specific production rates of PVAase, PVA dehydrogenase (PVADH), and oxidized PVA hydrolase (OPH) at different pHs.

pH	Average specific production rate (U/g/h)			
	PVAase		PVADH	OPH
	Within 15 h	15 h to 36 h	0 h to 36 h	
6.5	47.5 ± 0.29	6.7 ± 0.09	555.3 ± 1.32	36.1 ± 0.17
7.0	66.4 ± 0.33	10.5 ± 0.08	735.5 ± 1.54	44.2 ± 0.21
7.5	74.8 ± 0.32	6.4 ± 0.12	1013.4 ± 1.86	38.5 ± 0.18
8.0	48.9 ± 0.25	5.7 ± 0.07	692.8 ± 1.57	37.8 ± 0.20

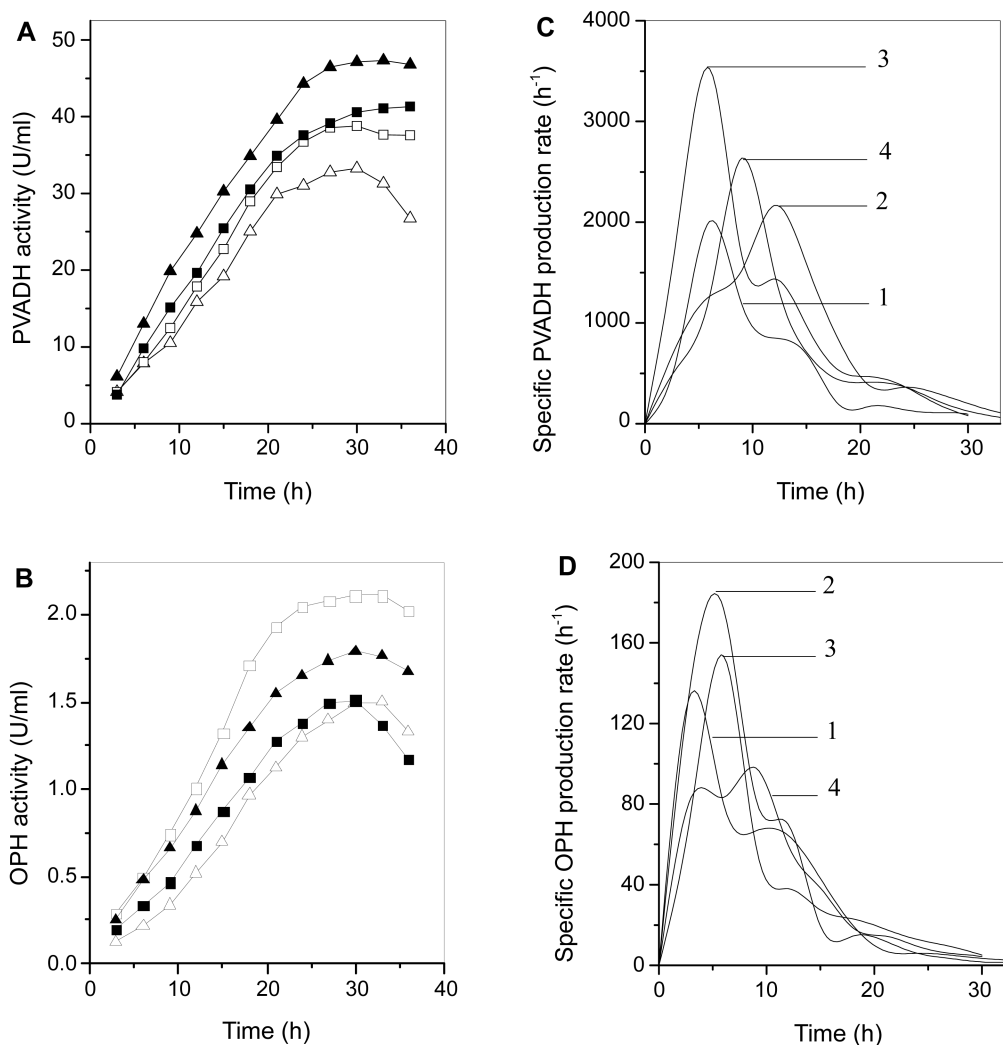


Fig. 2 Time courses of PVADH (A) and OPH (B) and specific production rates of PVADH (C) and OPH (D) during the batch cultivation of a mixed culture at different pH values.

△ and curve 1, pH 6.5; □ and curve 2, pH 7.0; ▲ and curve 3, pH 7.5; ■ and curve 4, pH 8.0.

PVAase Fermentation with Two-Stage pH Control Strategy

With the two-stage pH control strategy, the maximum PVAase activity reached 2.05 U/ml (Fig. 3) and increased by 15.2% and 24.2%, compared with that of constant pH 7.5 and pH 7.0, respectively. Furthermore, the productivity of PVAase of using this strategy was 85.4 U/l/h and 15.1% and 24.3% higher than constant pH 7.5 and pH 7.0 (Table 1), respectively. The result was higher than that of reports by other researchers. Zhang *et al.* [24] reported that the maximal PVAase activity of 0.12 U/ml was produced by *Streptomyces venezuelae* GY1. Moreover, the maximum activity of 1.24 U/ml and 1.36 U/ml produced by mixed bacteria and *Penicillium* sp. WSH02-21 were reported by Ju *et al.* [5] and Qian *et al.* [13]. Therefore, the mixed culture has potential application in degradation of PVA. On

the other hand, PVA was consumed with a relative high rate and the final concentration was 0.61 g/l (Table 1), which were about 35.8% and 24.7% decreases of that of pH 7.0 and pH 7.5.

The microflora was changed with the different fermentation phase. The relative abundances of *Pseudomonas* sp., *Flavobacterium* sp., *Streptococcus* sp., and *Micrococcus* sp. increased with time. *Pseudomonas* sp., *Flavobacterium* sp., and *Micrococcus* sp. were dominant microorganisms in the mixed culture at the end of cultivation, and their relative abundances reached 30.6%, 17.2%, and 19.7%, respectively. The relative abundance of *Bacillus* sp. was 19.5% at the beginning of the degradation, and then decreased with time. *Rhodococcus* sp., *Paenibacillus* sp., and *Sphingomonas* sp. increased firstly until the middle phase of fermentation and then decreased at the end of

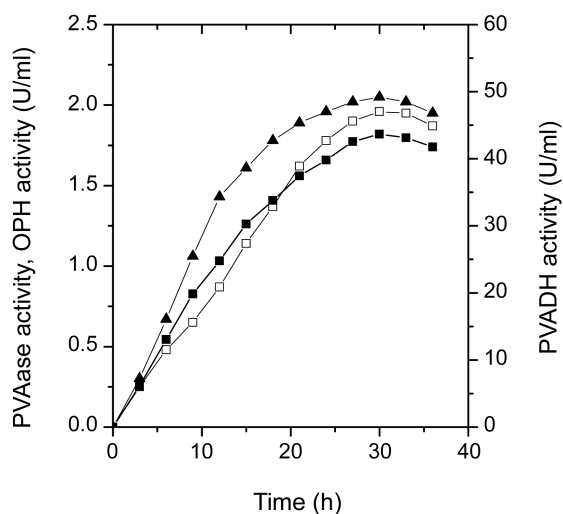


Fig. 3. Time courses of PVAase, PVADH, and OPH activities during the batch cultivation of a mixed culture with two-stage pH control strategy.

▲, PVAase activity; ■, PVADH activity; □, OPH activity.

cultivation [1]. The microflora was mainly changed with the degree of polymerization of PVA during the degradation of PVA. Different microorganisms have specificity in degradation of different degree of polymerization of PVA [1]. The impact of pH on microflora is not very obvious. In our present study, we paid more attention to improving the activity of PVAase. Subsequently, the change of microflora at different pHs was not of much concern to us.

To the best of our knowledge, this is the first time a pH control strategy has been used for the enhancement of PVAase production. Furthermore, the idea of changing the environmental conditions at different culture stages to match the metabolic characteristics of microorganisms could supply an alternative optimization way for other enzymes production in submerged culture.

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