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*Full Length Research Paper*

# Biosynthesis of phytases and phosphatases by *Aspergillus niger* 551

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**The purpose of this study was to determine the ability of the *Aspergillus niger* 551 strain to produce phytases and phosphatases in 24 days shake cultures at a pH adjusted to 5.5 and a changing (uncontrolled) pH, in a medium containing 0.5% of glucose and 4% of starch. The fermentation process of the culture performed with no adjustment of the pH showed that the pH markedly decreased (to 2.25) on day five and maintained at the same level also on consecutive days. However, the results show that irrespective of the pH of the medium, the fungi synthesized the enzymes through 2.5 weeks, although maximum activity was observed on different days. It was found that although the biosynthesis of phytases and phosphatases began earlier (before day five) at constant of pH 5.5, significantly higher activities of dephosphorylating enzymes were observed in the cultures with uncontrolled pH. The highest phytases activity (0.77 U/ml) of the fungi was observed on day 14, while phosphatases activity (3.257 $U_{Pho}$/ml) was the highest on day 12. The synthesis of amylases began at the same time in both cultures (before day five), but the production of these enzymes was more efficient at a constant pH. The highest amylolytic activity was found on day 12. The proteolytic enzymes in both processes were synthesized at low levels.**

**Key words:** *Aspergillus niger*, phytase, phosphatase, amylase, protease, starch.

## INTRODUCTION

Ecologically oriented food and feed production introduces new requirements for these products, especially in the regulations determining the standards for phosphorus content in animal feed. As a consequence, steadily increasing interest has been observed in enzyme preparations that can be used for enhancement of animal feed quality. The enzyme preparations added to animal feed contain a wide variety of enzymes, which not only improve digestibility of some feed ingredients, but they are also able to reduce the content of inorganic phosphorus (Choi et al., 2001). For this reason, microbiological production of phytases is of great significance in research. The researchers focus their attention mainly on finding appropriate strains and on their modifications (Pandey et al., 2001; Vats and Banerjee, 2004), optimization of fermentation conditions (Pandey et al., 2001; Gargova et all, 1997; Gargova, 2003; Doo-Sang et al., 1999; Vats and Banerjee, 2002) and purification of the enzymes (Casey and Walsh 2003, 2004). The aim of this present investigation was to study the biosynthesis process of phytases and phosphatases by mycelial fungi of *A. niger* in the long-term shake-flasks experiment using starch as a carbon source.

## MATERIALS AND METHODS

### Microorganism

This study used the mycelial fungi of the strain *A. niger* 551 originating from the collection of microorganisms belonging to the Department of Biotechnology and Food Microbiology at Wroclaw University of Environmental and Life Sciences. The culture was stored on potato dextrose agar (PDA) slants at 4°C.

### Medium

The medium consisted of: starch (40 g/L), glucose (5 g/L), KCl (0.5

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g/L), $MgSO_4 \times 7H_2O$ (0.5 g/l), $FeSO_4 \times 7H_2O$ (0.1 g/L) and $NH_4NO_3$ (5 g/L). The pH of the medium was adjusted to 5.5 and next the medium was sterilized at 121°C for 20 min.

**Fermentation conditions**

Fermentation was performed on a G10 Brunswick rotary shaker at 160 to 170 rpm, using 500 ml flasks containing 100 ml of the medium, at 30°C for 24 days (in two replications for each alternative process). The inoculum was a suspension of about $10^6$ spores per 1 ml obtained by washing the slant with 1% Tween 80 solution.

The pH was adjusted daily to 5.5 using 0.5 M NaOH. The sample collection frequency (5 ml) is shown in the results. The pH was measured and the samples were centrifuged in a sigma 3 to 16 K centrifuge at 5500 rpm and 5°C for 15 min. The activities of phytases, phosphatases, amylases and proteases as well as protein content were determined in the supernatant.

**Analytical methods**

**Phytase assay**

The activity of phytase was determined using 0.1 M, pH 5.11 acetate buffer solution containing 1% phytic acid sodium salt (Sigma) as a substrate. The enzyme reaction was performed in test tubes at 55°C for 30 min. The mixture consisted of 600 µl of the substrate, 200 µl of the enzyme extract and 200 µl 25 mMol of $CaCl_2$. The reaction was disrupted by adding 1 ml of 5% trichloroacetic acid (TCA) solution. The enzyme solution in the control samples was added after TCA. When the enzyme reaction was accomplished, the content of the released phosphates was determined using the method described by Fiske-Subbarowa (Fiske and Subbarow, 1925) and compared with a standard containing 80 µg/ml of phosphate. One unit (U) of phytase activity was defined as the concentration of inorganic phosphate, in µmol, released per min per ml of enzyme preparation (U/ml) and also as a specific activity in conversion to 1 mg of protein (U/mg) under defined reaction conditions.

**Phosphatase assay**

The activity was determined using as a substrate 0.15% of 4-nitrophenol phosphate disodium salt hexahydrate (Fluka) in a 0.2 M acetate buffer, pH 5.11. The enzyme reaction was performed at 37°C for 30 min. Individual sample contained a mixture of 250 µl of the substrate and 250 µl of the enzyme extract. The reaction was disrupted by adding 0.5 ml of 0.05 M NaOH. In the control sample, the enzyme extract was added after NaOH. The quantity of released p-nitrophenol was measured at a wavelength of 405 nm according to blank containing 0.5 ml of 0.2 M acetate buffer and 0.5 ml of 0.05 M NaOH. Phosphatase activity was expressed in µMols of p-nitrophenol produced within 1 min in conversion to 1 ml of the enzyme ($U_{Pho}$/ml), and also as a specific activity in conversion to 1 mg of protein ($U_{Pho}$/mg).

**Protease assay**

Protease activity was determined using a modified Anson method (Mejbaum-Katzenelenbogen, 1969) with 1% of casein solution as a substrate. The enzyme reaction was performed at 30°C for 10 min. The individual sample contained a mixture of 1 ml of the substrate, 0.9 ml of 0.066 M phosphate buffer pH = 6.8 and 0.1 ml of the enzyme extract. The reaction was stopped by adding 2 ml of 6% TCA solution. In the controls, the enzyme extract was added after TCA. After centrifuging (at 5500 rpm for 15 min) the quantity of released tyrosine was determined in the supernatant. Protease activity was expressed in Anson's units (jA/ml). It was the quantity of the enzyme that was able to hydrolyze the substrate under certain conditions (30°C; pH = 6.8; 10 min), at such a rate that the quantity of soluble TCA produced during hydrolysis within 1 min corresponded to 1 mMol of tyrosine measured at OD 670 nm.

**Amylase assay**

Amylolytic activity was determined according to the method described by Fischer and Stein (1960) and expressed in the Fischer-Stein activity units (JFS/ml) as µMols of maltose released within 1 min, in conversion to 1 ml of the enzyme measurement at 530 nm OD in present DNS (Sigma) reagent.

**Protein**

Protein content was determined using Lowry's method (Lowry, 1951).

## RESULTS AND DISSCUSION

The ability of mycelia fungi of *A. niger* 551 strain to synthesize phytases and phosphatases in immersed fermentation processes was investigated using the medium containing starch and glucose as a modified carbon sources. Based on the data from the literature (Gargova et al., 1997; Vats and Banerjee, 2002), the fermentation process was carried out for 24 days. The initial pH of the medium was adjusted to 5.5, because the pH for phytases biosynthesis by mycelial fungi reported by other researchers was ranged from 4.5 to 6.0 (Gargova et al., 1997; Vats and Banerjee, 2002; Casey and Walsh, 2003, 2004; Oh et al., 2004). It is worth noticing, that in literature there was no data found about experiments with fungi conducted at a constant pH, therefore in this study the pH changes were under investigation for the entire length of the process (Vats and Banerjee, 2002; Doo-Sang et al., 1999; O'Donnell et al., 2001). For this reason, this present experiment was carried out on two alternative fermentation processes: one at a pH regulated at 5.5 and in the other, the initial pH was set out at 5.5 but no further adjustments were made throughout the process. It was interesting to find decreasing values of pH (2 to 2.5) on the first days of fermentation and maintained at these low levels for the further process (Figure 1).

Comparison of the pH in the two alternatives processes – adjusted to 5.5 (Figure 2) and changing (Figure 1) shows that both phytases and phophatases biosynthesis began earlier (before day five) in the culture with pH regulated at 5.5. In this culture, the highest phytases production by the fungi obtained between day 14 and 19 of the process, but maximum activity of the strain was observed on day 14 and 17 (0.777 U/ml) and (0.731 U/ml), respectively. Presented data suggest that in this condition the decrease in phytases activity observed after

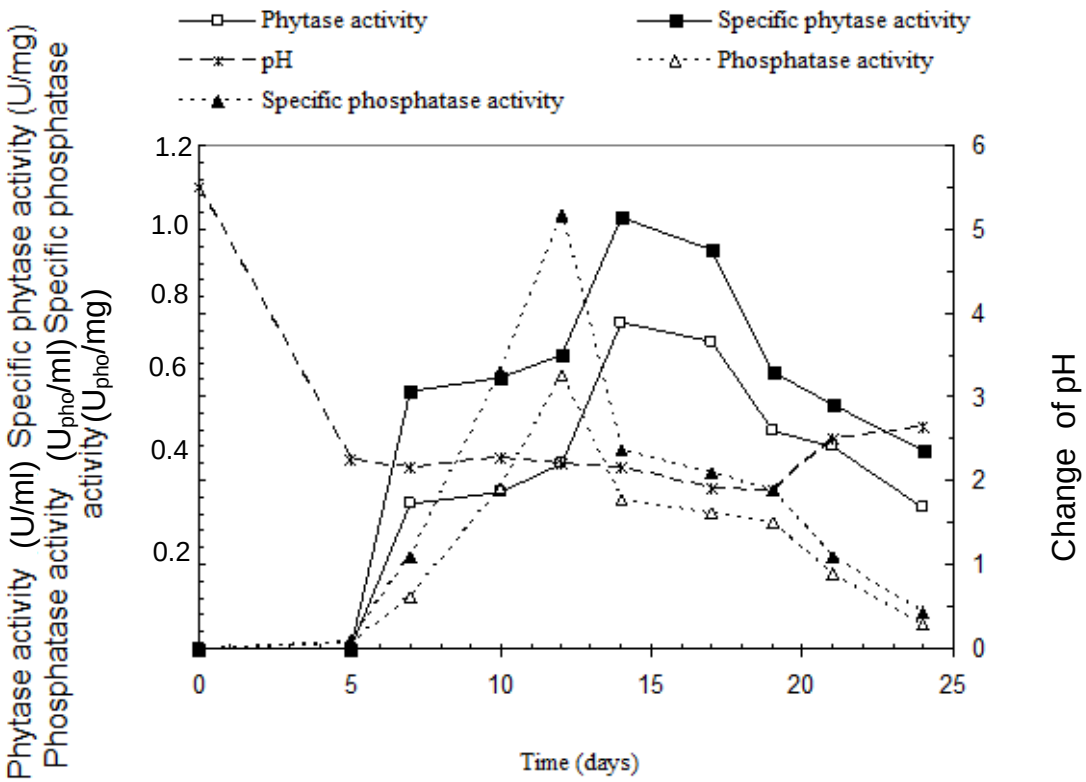


**Figure 1.** Biosynthesis of phytases and phosphatases by *A. niger* 551 strain in submerged fermentation without pH regulation.

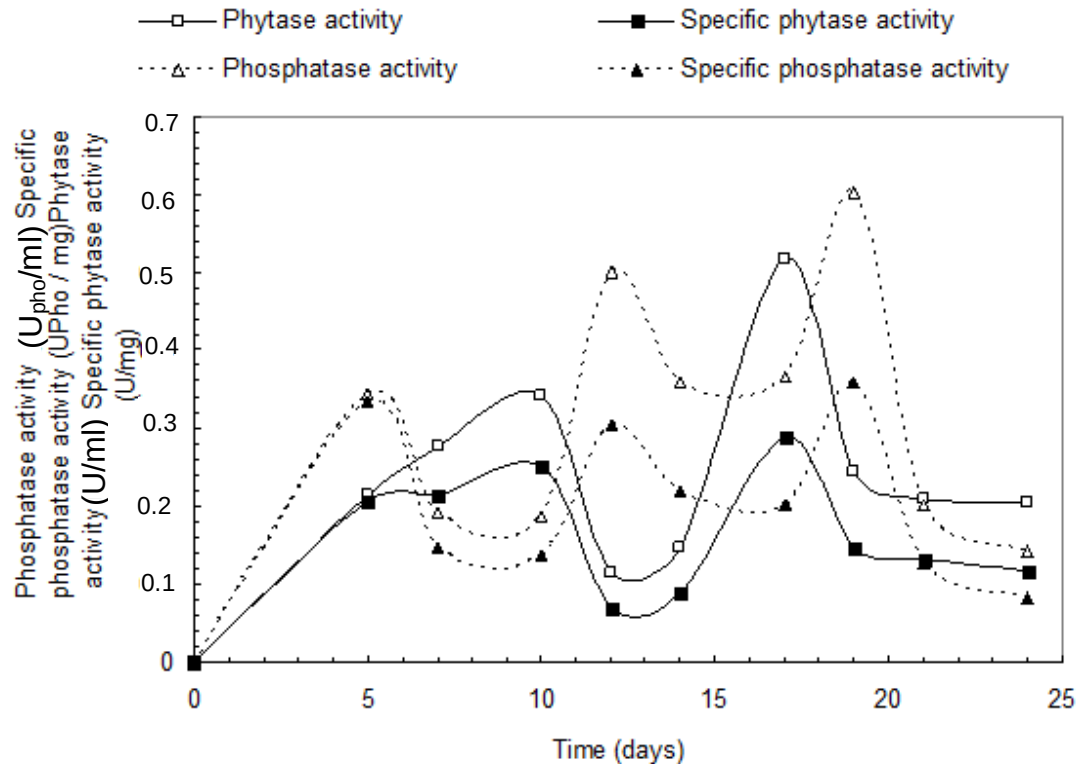


**Figure 2.** Biosynthesis of phytases and phosphatases by 551 strain of *A. niger* in submerged fermentation (Ph = 5.5).

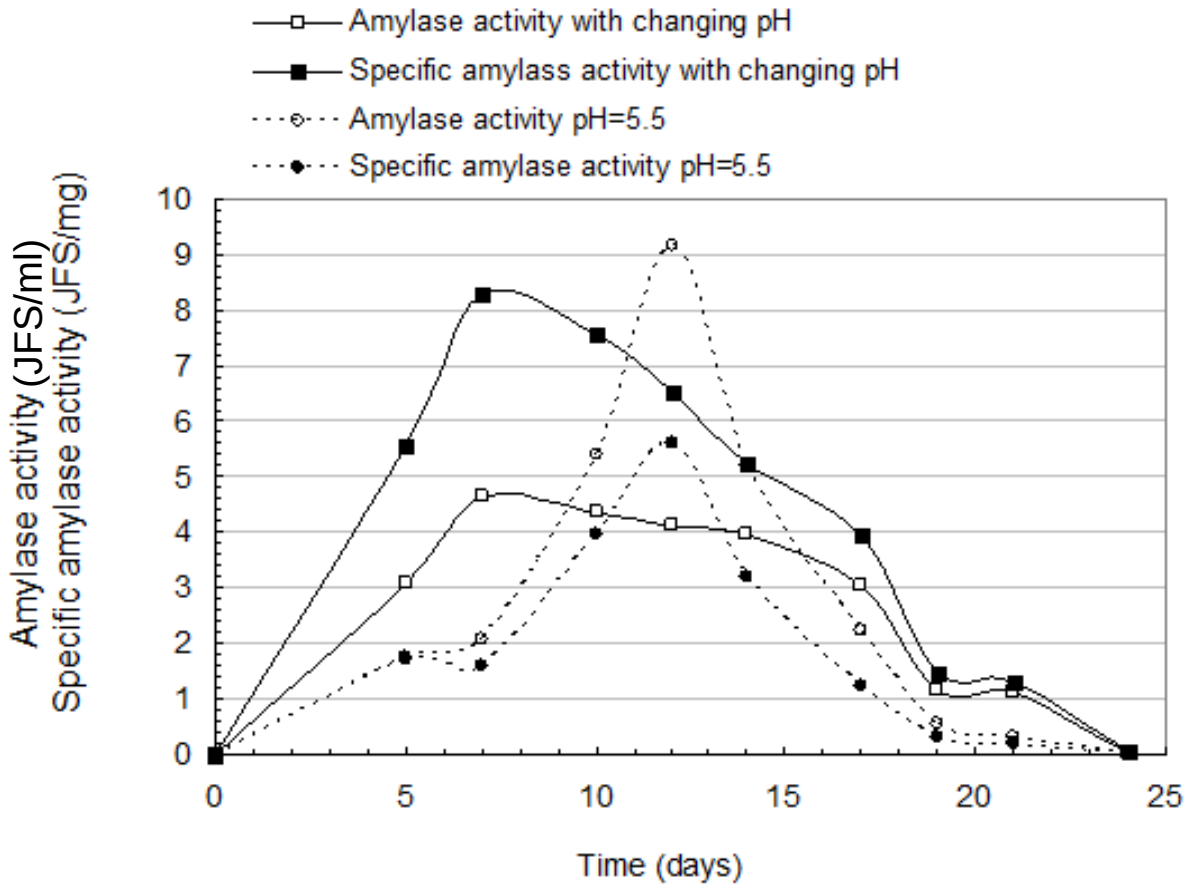


**Figure 3.** Comparison of amylase activity of *A. niger* 551 strain in submerged fermentation with unregulated pH and pH = 5.5

day 14 was due to a slight increase in proteolytic activity obtained on day 19 (Figure 4). The fermentation performed at a constant pH (5.5) exhibited maximum phytases activity (0.518 U/ml) on day 17 (Figure 2). Gargova et al. (1997) studied phytases production by the *Aspergillus* sp. 307 strain and observed the highest activity of the enzymes on day nine. When the strain *A. niger* var. teigham was used in a similar process (3% of glucose and 1% of starch), maximum phytases activity (184 nKat/ml) was also observed on day 17 (Vats and Banerjee, 2002) but the value was several times higher than that obtained in this present investigation. In other conditions, in which saccharose was used as a carbon source, the strain 5990 of *Aspergilus* sp. exhibited the highest phytases production of 13.3 U/ml at pH 2.5 and 14.6 U/ml at pH 5.0 on day eight (Doo-Sang et al., 1999).

Similarly to phytases production, the biosynthesis of phosphatases also started earlier in the process performed at a constant pH (Figure 2), and on consecutive days the activities were even lower as compared to those observed in the process with changing pH values (Figure 1). Maximum phosphatases activity (3.257 $U_{Pho}$/ml) of the *A. niger* 551 strain was observed on day 12 in the culture with changing pH. Gargova and Sariyska (2003) studied the effects of phosphorus concentration and initial pH on the production of acetic phosphatases under similar conditions (4% of starch) and the activities they obtained were slightly higher than those obtained in this present investigation (from 100 to 225 nkat/ml). In other conditions (saccharose used as a carbon source in an eight-days fermentation), the *Aspergilus* sp. 5990 strain reached maximum activity (approx. 55 $\mu Mol_{PO4}$/ml) on day four (Doo-Sang et al., 1999).

The carbon source used in this present study was starch, that can induce the synthesis of amylolytic enzymes, amylase activity was determined in the both processes. However, it is worth noting that, as mentioned earlier, the medium was composed with respect to the efficiency of phytases and phosphatases and not amylases synthesis. The amylases activity observed in this present investigation, ranged from 1.103 to 4.652 JFS/ml in the fermentation with no pH adjustment and from 0.323 to 9.170 JFS/mL in the process in which the pH was adjusted to 5.5. These results are markedly lower than those obtained by Hernandez et al. (2006) when using the cultures of *A. niger* UO-1 in the media containing by-products of meat factories and breweries and the media containing 4% of starch. The activity they

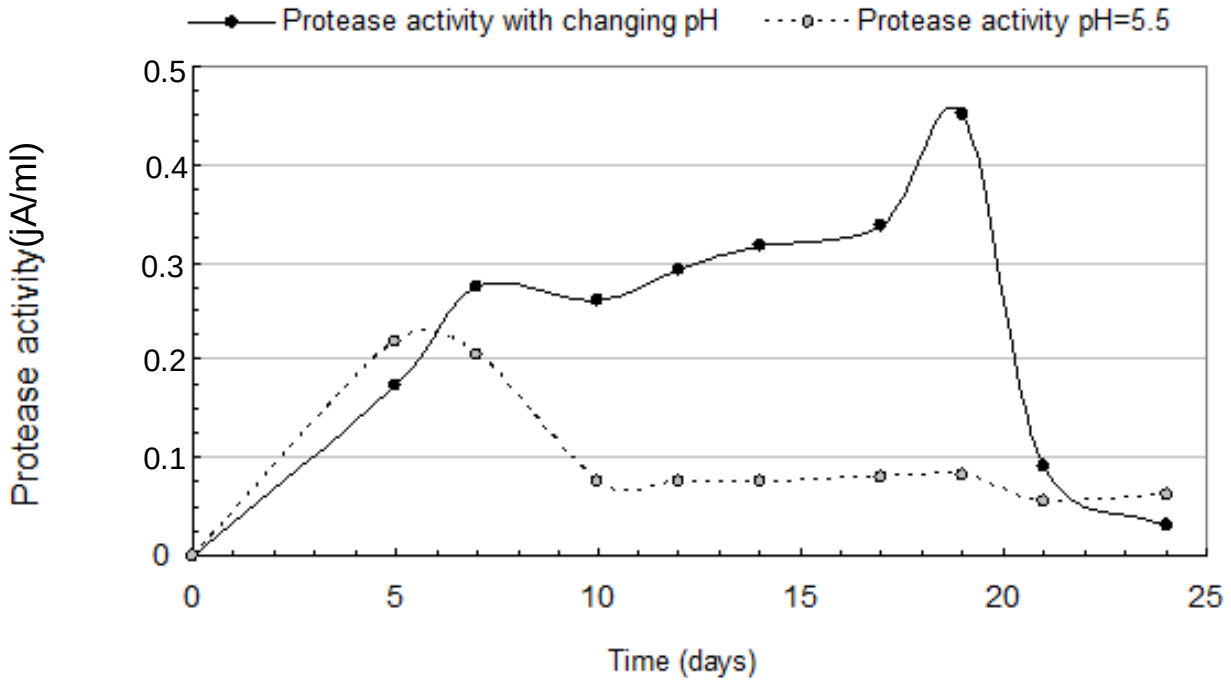


**Figure 4.** Comparison of protease activity of *A. niger* 551 strain in submerged fermentation with unregulated pH and pH = 5.5.

obtained under such conditions ranged from 37 to 70 JFS/ml. Uguru et al. (1997) studied amylase biosynthesis in the media containing 2% of potato or soluble starch and found specific activities of the enzymes within the range of 5 to 16 JFS/mg when using soluble starch in a six-day culture of *A. niger* UO-1. Similar results are obtained in this present study for the culture with un-controlled pH, in which specific amylases activity ranged from 1.340 to 8.292 JFS/mg.

Proteases activity observed in this present study was low, which was a positive phenomenon because the presence of proteolytic enzymes, especially in the processes performed on an industrial scale, may have a degrading impact on the enzymes. For this reason, it is important to minimize proteases production by certain parameters selected for microbiological processes. O'Donnell et al. (2001) studied the pH values in order to find those in which protease production was the lowest. The results of their studies show that maximum protease activity at pH 3.0 was 3.6 jA/ml. The lowest enzymes production ranging from 0 to 0.56 jA/ml was found in the cultures at pH 5.0 and 6.0. These values were twice higher than those obtained in this present study in the processes performed at pH 5.5, which ranged from 0.063 to 0.219 jA/ml. The same authors also performed processes at uncontrolled pH using a recombined strain AB4.1 of *A. niger* in YM medium and obtained markedly higher proteolytic activity (0 to 2.4 jA/ml) than we observed in our studies, in which maximum production of this enzymes (0.452 jA/ml) was observed on day 19 of the process (Figure 3).

To sum up, in the long-term process performed in the medium containing 4% starch and 0.5% glucose, the *A. niger* 551 strain was more efficient in synthesizing both phytases and phosphatases in the cultures with uncontrolled pH, which from technological point of view is more favorable. However, further studies are required to determine optimum fermentation conditions in order to obtain higher activities. According to Doo-Sang et al. (1999), it seems quite likely that the production of depho-sphorylating enzymes in the media containing starch as a carbon source can be improved by higher temperature. Experimentally, it is important to determine the enzymes activity using lower starting pH of the process or performing the process at a lower constant pH.

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