Alkaline Protease Production by Solid State Fermentation on Polyurethane Foam

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This paper investigated the process of solid state fermentation (SSF) using PUF (polyurethane foam) as inert solid support to produce alkaline protease. Maximal enzyme activity was 2185 U ml^{-1} at pH 9.0 incubation temperature 32 °C, inoculum amount of ρ = 1.0 %, nutrient solution 3.75 ml g^{-1} PUF, incubation time for 2 h and 15.0 m mol L^{-1} of added $CaCl_2$. Under the same conditions, the yield of alkaline protease produced by SSF using PUF as support is higher than that by submerged fermentation (SMF).

Key words:
Inert support, solid state fermentation, alkaline protease, PUF

Introduction

Alkaline protease is an important additive used to improve the decontamination effect in the detergent industry. Until now, alkaline protease has been produced mostly by submerged fermentation (SMF), a process plagued with problems, including serious pollution, low product concentration and, high production cost. Compared to SMF, solid-state fermentation (SSF) has received more attention recently, as it uses simpler fermentation medium, requires a smaller space, is easier to aerate, and has higher productivity, lower waste water output, lower energy requirement, and less bacterial contamination.[1]

SSF is generally defined as the growth of microorganisms on solid substrates in the absence or near absence of free water.[1,2,3] Until now, the conventional method is mostly applied in industry, and often uses a farm produce as substrate, which acts not only as a support of the microorganism but also as the medium.[4] This type of SSF has a number of disadvantages, such as a large space requirement and incontinuity. Inert material has recently been used in SSF as support of microorganisms. In this type of SSF, microorganisms receive nutriment from the liquid medium absorbed on the inert solid support.[5] As a novel SSF, it has not only advantages of SSF mentioned above, but also some characteristics of SMF. For example, media can be accurately designed like SMF and productivity can be promoted further. Because liquid media are evenly absorbed on inert support and the fermentation environment is homogeneous, like SMF, monitoring of process parameters, scaling-up of strategies become possible. The most important advantage of this type of SSF is the good aeration condition which is difficult for SMF and conventional SSF. The volume of media absorbed on the surface of support is controlled lest there exists free water. Media exist in the form of incontinuous liquid film on the surface of inert support and continuous air surrounds it. The microbe growing in the liquid film can get enough air and do not need any mechanic stirring which is essential for SMF and most types of SSF. Good aeration condition makes the microbe grow better and get higher productivity. Although, application of inert support increases its fermentation cost, the extract cost is reduced because of high concentration of product and easy separation from the support. Totally this type of SSF is feasible in industry and especially in producing high added--value products, such as metabolites and enzymes.[6]

Polystyrene, a commercially-available insulating and packaging material, could be used as the inert solid support for the production of enzymes,[7,8,9] while ion exchange resins,[10] polyurethane foam[6] and computer cards 11 have also been used as inert carriers for SSF. However, production of alkaline protease by inert support absorption SSF has not been yet reported. In this study, we evaluated the potential of *Bacillus Pumilus AS* 1.1625 for producing alkaline protease by SSF on PUF was evaluated. Furthermore, we compared the enzymatic activity of SSF with that for SMF, in order to identify the advantages of SSF was compared. The study was carried out in laboratory scale and its results provided important references to the design and optimization of the continuous reactor.

Materials and methods

Microorganisms and inoculum preparation

The fungal strain *Bacillus Pumilus AS* 1.1625, obtained from Culture Collection Centers, Chinese Academy of Sciences, was used in the present study. The strain was grown and maintained on agar slants

by cultivation at 32 °C. Cultures were preserved at 4 °C for short-term storage. Ten mL of sterile distilled water with 0.1 % Tween-80 was added to fully sporulated one-week old agar cultures. The spores were then scraped using an inoculation needle under strict aseptic conditions. The resulting spore suspension was used as the inoculums. Viable spores in the spore suspension were determined using the plate count (colony count) technique.

Moistening medium

A salt solution (beef extract 5, bean peptone 10, sodium chloride 5 and calcium chloride 1.67 g L^{-1}l) was used as the moistening medium.

Inoculation and incubation

PUF was cut into cubes of 5 mm × 5 mm × 5 mm and dried in the oven until the weigh kept constant. Four grams of PUF pieces was then placed in a 250.0 mL conical flask which had been cleaned and dried. The flask with the PUF was sterilized at 121 °C for 20 min and cooled to room temperature. Each flask was added in about 5~35 mL medium and inoculum under strict aseptic conditions and pressed softly by a glass stick in order to allow the PUF to fully and evenly absorb them. The contents were then incubated in a autonomous incubator of constant temperatue and humidity for a desired length of time.

Experiment of SMF was carried out as the control of SSF on PUF. The same amounts of medium and inoculum were added. Then the contents were incubated at 32 °C for a desired length of time.

Enzyme extraction and assay

From the fermented solid substrate, enzyme extraction was carried out using 93.0 mL distilled water with 0.1 % Tween-80 so, that the final extraction volume was 100.0 mL (93 mL distilled water +with 7 mL moistening medium). First the fermented substrates were properly mixed with distilled water and the flasks were kept on a rotary shaker at n = 150 min^{-1} for 30 min. After this, the solids were separated from the solution by filtering through a nylon cloth sieve. The solution was centrifuged at 12,000 min^{-1} for 20 min at 4 °C in a refrigerated centrifuge. The supernatant was collected and used for enzyme assay.

Alkaline protease was assayed according to Folin. [12] The buffer solution was 0.05 mol L^{-1} borax-sodium hydroxide (pH 10.5). One international unit of alkaline protease activity was defined as the amount of enzyme required to release one microgram of tyrosine from casein at 40 °C and pH 10.5. Enzyme activity was expressed as units mL^{-1} moistening medium (U mL^{-1}).

Total biomass determination

Biomass dry mass was determined as described by Zhu, Y et al.[6] Samples were extracted with 100 mL distilled water in a laboratory blender for 30 s and then filtered through a pre-weighed filter paper. The residue on the paper was washed two times with 100 mL distilled water. The remaining biomass and PUF were subsequently dried overnight at 105 °C. Because of the inert character and the exactly known weight of PUF in every sample, the dry mass of biomass could be estimated by subtracting the weight of PUF and filter paper from the total mass after drying.

Optimization of process parameters for alkaline protease production

Due to application of the liquid medium, optimization of this type of SSF matches that of SMF parameters. The medium described above was taken as a basal medium and the process parameters under study were varied: fermentation temperature (25~38 °C), initial pH of the moistening medium (7~10), inoculum size (ρ = 0.2-~3.0 %, volume of added moistening medium (5-~35 mL), and added casein and calcium chloride were optimized for alkaline protease production.

The procedure, adopted for the optimization of various process parameters influencing alkaline protease production, was to evaluate the effects of individual parameters while keeping all other parameters constant, and to incorporate it at the optimized level in the experiment before optimizing the next parameter. All experiments were carried out in duplicate and the mean values are reported.

Results and discussions

Influence of moistening medium volume on enzyme activity

The results presented in Fig.1 indicate that alkaline protease production increased as the volume of moistening medium increased up to 15.0 mL, where the maximal enzyme activity (1800 U mL^{-1}) was recorded. When the volume of moistening medium was less than 15.0 mL, the microorganism was not able to get enough nutrients, thus resulting in low enzyme activity. On the other hand, when the volume of the moistening medium was more than 15.0 mL, which exceeded the absorption capacity of the PUF, liquid accumulated on the surface of the PUF, thus limiting the transfer of oxygen in the pores of the PUF and hindering normal metabolism.

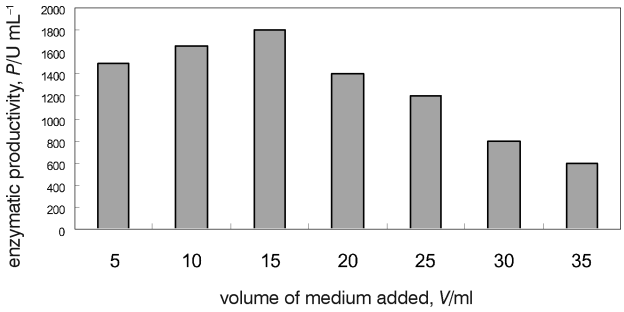

Fig. 1 – *Effects of the volume of medium added on the enzymatic productivity. Fermentation temperature: 30 °C, initial pH 8.0, culture time: 24 h.*

Influence of fermentation temperature on enzyme activity

As shown in Fig. 2, fermentation temperature influenced the yield of alkaline protease for maximal enzyme activity of 1950 U mL^{-1} at 32 °C. Fermentation temperature has a complex effect on the growth of *Bacillus Pumilus AS 1.1625*. At low temperatures, an increase in temperature would enhance the growth of the microorganism, and, therefore improve enzyme activity. At high temperatures, an increase in temperature negatively influences the microorganism by making some important protein denaturalize, and, thus limiting microbial growth. Additionally, high temperature would accelerate the volatilization of water in the medium, thus restraining the growth and enzymatic production of the microbe.

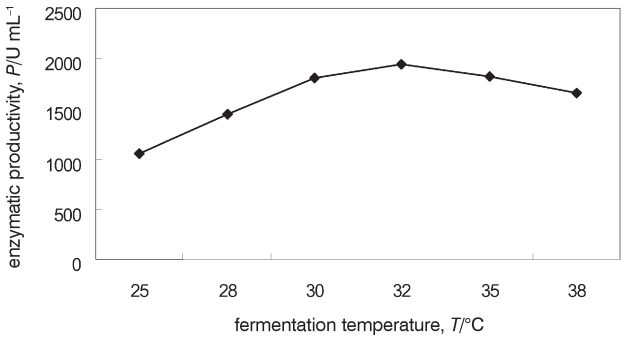

Fig. 2 – *Effects of fermentation temperature on the enzymatic productivity. Culture time: 24 h, initial pH 8.0, volume of medium 15.0 mL*

Influence of inoculum amount on enzyme activity

In this study, the concentration of inoculum was $8.2 \cdot 10^8$. Figure 3 indicates that there was a gradual increase in the synthesis of enzyme when the amount of inoculum was increased up to ρ = 1 %. Further increase of inoculum amount reduced the amount of nutrient solution that the microbe obtained.

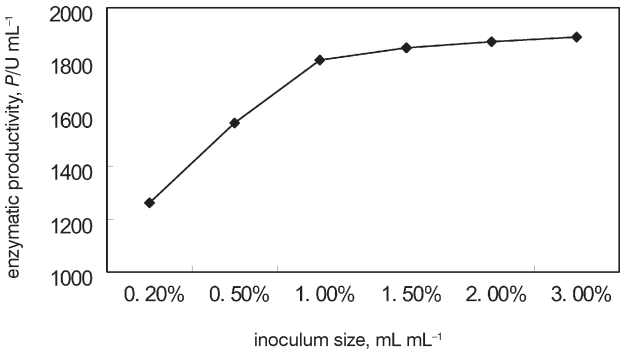

Fig. 3 – *Effects of the inoculum size on the enzymatic productivity. Fermentation temperature: 32 °C, culture time: 24 h, initial pH 8.0.*

Influence of initial pH of moistening medium on enzyme activity

The optimum pH required for maximal enzyme activity by SSF on PUF was evaluated using various initial pH levels (7~10) of the moistening medium. Figure 4 shows that maximal enzyme activity was 1950 U mL^{-1} at pH 9.0, implying that higher or lower pH generally leads to poor growth and results in a decline of enzymatic activity.

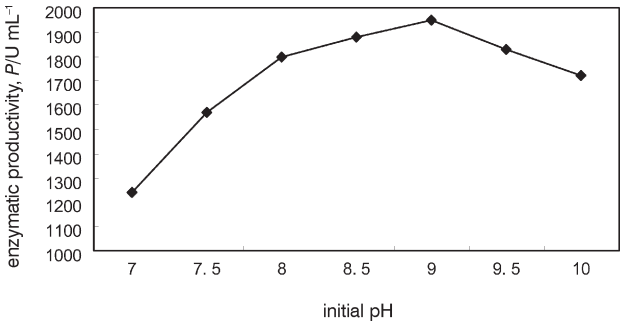

Fig. 4 – *Effects of initial pH on the enzymatic productivity. Fermentation temperature: 32 °C, culture time: 24 h, inoculum size: 1 %*

Influence of added casein on enzyme activity

Different concentrations of casein were also added to the fermentation medium to evaluate the induction of alkaline protease by *Bacillus Pumilus AS* 1.1625. Figure 5 indicates that there was no increase in enzyme activity by casein supplementation at the tested concentrations.

Influence of added Ca^{2+} on enzyme activity

According to some reports,[13,14] Ca^{2+} could improve, both, the activity and stability of alkaline protease. Different concentrations of casein were also added to the fermentation medium to evaluate its influence on enzyme activity. Figure 6 indicates that, when the Ca^{2+} supplement was 15.0 m

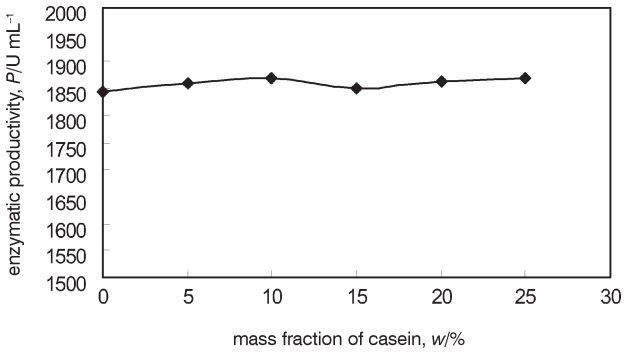

Fig. 5 – *Effect of casein on enzymatic production. Fermentation temperature: 32 °C, culture time: 24 h.*

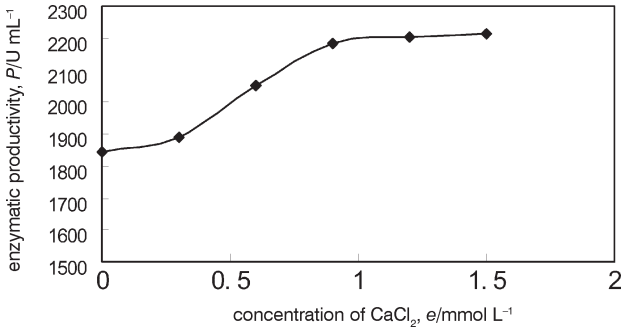

Fig. 6 – *Effect of $CaCl_2$ on enzymatic production. Fermentation temperature: 32 °C, culture time: 24 h.*

mol L^{-1}, an appropriate amount of Ca^{2+} enhanced alkaline protease production up to the maximal enzyme activity of 2185 U mL^{-1}.

Comparison of enzyme activity and biomass between SSF on inert solid support and SMF

Figure 7 indicates that the velocity of alkaline protease production by SSF on PUF was higher than that for SMF. The maximal activity of the former was also higher than that of the latter. Additionally, the enzyme activity by SSF on PUF was more stable than that for SMF, which decreased near the end of fermentation.

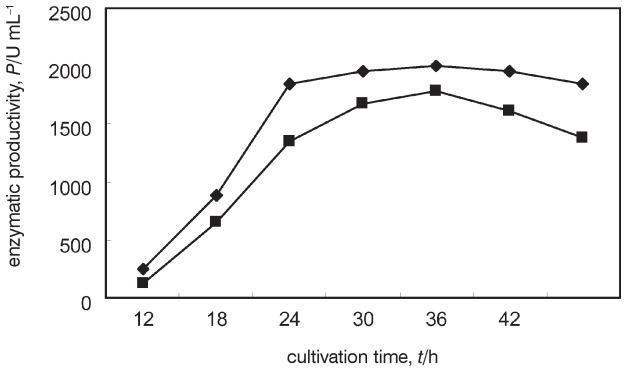

Fig. 7 – *Comparison of the effects of inert carier adsorption SSF and SnF. Fermentation temperature: 32 °C, culture time: 24 h.*

Figure 7 also indicates the change of biomass in SSF on PUF and SMF. Biomass of SSF on PUF has increased during the fermentation process, but the enzymatic activity began to decrease since 36 h. The similar trend was also found in SMF. Biomass of SSF on PUF was higher, than SMF during the total process of cultivation. This accounts for the reason why the enzymatic activity of the former was higher than the latter. Application of inert support provides the microbe better living condition, I.E. better aeration, and thus promotes the propagation of them. More enzymes were produced due to higher concentration of the microbes in the media when other formation parameters are the same. Since 36 h, the biomass still increased, showing that the concentration of the microbes was yet high. However, the number of live cell decreased and thus the enzymatic activity was reduced.

Conclusions

The results presented in this work showed that maximal enzyme activity (2185U mL^{-1}) was observed when SSF was carried out with substrate at pH 9.0 incubation temperature 32 °C, inoculum amount of ρ = 1.0 %, nutrient solution 3.75 mL per 1.0 g PUF , incubation time 24 h and 15.0 mmol L^{-1} of added $CaCl_2$.

Compared to SMF, the most popular method for alkaline protease production, SSF on PUF provides higher protease yield under the same conditions, in concordance with earlier observations,[15] thus forecasting that it may bring in larger economic benefits.

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