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Clavulanic Acid Production by *Streptomyces clavuligerus* using Solid State Fermentation on Polyurethane Foam

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Clavulanic acid (CA), a metabolite of *Streptomyces clavuligerus*, is a potent β-lactamase inhibitor. In this study, polyurethane foam (PUF) was used as inert solid support to produce clavulanic acid by solid state fermentation (SSF). Maximal CA yield of 263 μg/ml was obtained at pH 6.5, incubation temperature 29°C, 10 ml medium per 3 g PUF, 0.015% added glycerol, 2% added lithium chloride (LiCl), and 2 g/L added ornithine. Under the same conditions, the yield of CA produced by SSF on PUF is apparently higher than that by submerged fermentation (SMF). In addition, CA produced by using this method is of higher purity and easier to be extracted.

Keywords: Inert support; Solid state fermentation; Clavulanic acid; Polyurethane foam

Introduction

Clavulanic acid (CA), a metabolite of *Streptomyces clavuligerus*, is a potent β-lactamase inhibitor [1]. The β-lactamase can hydrolyze the β-lactam ring of penicillin, cephalosporin, and related antibiotics, providing bacteria with antibiotic resistance. The CA binds irreversibly to the serine hydroxyl group at the active center of β-lactamase, producing a stable acylated intermediate that results in the inactivation of the enzyme [2]. The combination of CA with amoxicillin is the best example of the use of a β-lactamase inhibitor.

Until now, CA has been produced mostly by submerged fermentation (SMF), a process plagued with many problems, including serious pollution, low product concentration, and high production cost. Comparing to SMF, solid state fermentation (SSF) has recently received more attention, because it requires simpler fermentation medium and smaller space, is easier to aerate, and has higher productivity, lower waste water output, lower energy requirement, and less bacterial contamination [3].

SSF is generally defined as the growth of microorganisms on solid substrates in the absence or near absence of free water [3,4]. Conventional SSF mostly applied in industry often uses agricultural products as the substrate, which acts not only as a support of the microorganism but also as the medium [5]. It has a number of disadvantages, such as large space requirements and incontinuity. Recently the inert material has been used in SSF as the microorganism support, in which microorganisms receive the nutriment from the liquid medium absorbed on the inert solid support [6]. It possesses the advantages of both SSF and SMF. For example, media can be accurately designed like SMF and

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productivity can be promoted further. Because liquid media are evenly absorbed on inert support and the fermentation environment is homogeneous, process monitoring and scaling-up become possible. The most important advantage of this SSF type is to improve the aeration condition, which was proven to be very difficult for SMF and conventional SSF. During inert support absorption SSF, media exist in the form of incontinuous liquid film on the surface of inert support, and continuous air surrounds it. The microbes 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 achieve higher productivity. Although the application of inert support increases its fermentation cost, the extract cost is reduced because of high concentration of products and simpler separation process. Overall using the inert material in SSF is feasible in industry and especially in producing high value-added products, such as metabolites and enzymes [7].

For the selection of inert materials, polystyrene, which is a commercially-available insulating and packaging material, were used as the inert solid support for the production of enzymes [8-10]; while ion exchange resins [11], polyurethane foam [7,12,13], and vermiculite [14,15] have also been used as inert carriers for SSF. However, production of CA by inert support absorption SSF has not been yet reported. In this study, the potential of producing CA by SSF on polyurethane foam (PUF) was evaluated. Furthermore, CA yields of SSF on PUF and SMF were compared to identify advantages of the former. The study was carried out on a laboratory scale and its results provided important references to the further study on the pilot scale.

Materials and Methods

Microorganisms

Streptomyces clavuligerus CCMCC 4.1611 obtained from the Culture Collection Center at Chinese Academy of Sciences, was used in the present study.

Moistening medium

Agar slant medium contained (in 1 L distilled water) following ingredients: soluble starch 20 g, KNO_3 1 g, $MgSO_4$ 0.5 g, K_2HPO_4 0.5 g, NaCl 0.5 g, $FeSO_4$ 0.01 g, and agar 15 g. Inoculum medium contained (pH 7.0, in 1L distilled water): glycerol 20 g, soybean flour extract 200 ml, and peptone 5 g.

Fermentation medium contained (in 1L distilled water): glycerol 4 g, soybean flour extract 300 ml, peptone 10 g, and KH_2PO_4 0.8 g.

Inoculation and incubation

PUF was cut into cubes of 5 mm × 5 mm × 5 mm and dried in the oven until the weigh was kept constant. The PUF pieces of 4 g was then placed in a 250 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. About 5-35 ml medium was added into each flask with inoculum under strict aseptic conditions, and then the contents were pressed softly by using a glass stick in order to allow the PUF to fully and evenly absorb them. The contents were then incubated in an autonomous incubator at constant temperature 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 29 °C for a desired length of time.

CA extraction and assay

CA extraction was carried out using distilled water. The fermented substrates were properly mixed with distilled water and the flasks were kept on a rotary shaker at 150 rpm for 30 min. After this, the solids were separated from the solution by filtering through a nylon cloth sieve. The solution was centrifuged at 3500 rpm for 40 min at 4°C in a refrigerated centrifuge. The supernatant was collected and used for CA assay.

CA concentration was determined by using a HPLC. A reverse-phase C-18 column (Hichrom) connected to a guard column was used. The mobile phase comprised of 0.1 M KH_2PO_4 and methanol (94:6). The flow rate of the mobile phase was 1 ml/min and temperature and pressure of the column are 25ºC and 7.9 Pa. Prior to injection a 0.8 ml sample, was first reacted with imidazole reagent and incubated for 12 min at 30ºC, then rapidly cooled to 20ºC. Samples were filtered before injection into the column using a Whatman 0.2 PVDF 3 mm disposable syringe filter. The derivatised product was injected to the column and detected with UV absorbance at 311 nm [16,17]. CA standards were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Optimization of process parameters for CA production

Due to application of the liquid medium, optimization of SSF on PUF matches that of SMF parameters. The medium described above was used as a basal medium. To optimize CA production, following process parameters were varied: fermentation time, amount of inoculum liquid, amount of moistening medium, glycerol, lithium chloride (LiCl), and ornithine.

The procedure, adopted for the optimization of various process parameters influencing CA 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 triplicate and the mean values were reported.

Results and Discussion

Influence of fermentation time on CA yield

Figure 1 shows that the yield of CA increased with the fermentation time. The maximum yield of 240 µg/ml was obtained at 48 h and then the yield decreased gradually. It's concluded that the optimum fermentation time was 48 h.

During the progress of the *Streptomyces clavuligerus* fermentation, there were three different periods, growth period of *S. clavuligerus*, production period of CA, and degradation period of CA. The growth period of *S. clavuligerus* happened firstly, followed by production of CA and the degradation period of CA. Sometimes, these three periods might overlay. The degradation of CA reduced the final production, but it was an important protection mechanism which can prevent *S. clavuligerus* from killing itself. Although CA is applied in the clinic together with other antibiotics, it also has slight antibiotic property. CA in high concentration can hurt *S. clavuligerus* itself. *S.*

clavuligerus has several pathways to convert or degrade CA to some levels which will not harm itself. These pathways are probably converting CA to secondary products which have no antibiotic properties or modifying the structure of CA to lose its antibiotic property [18,19].

Researchers have studied some methods, like controlling pH, to reduce the degradation of CA and improve its final yield. Liao used glycerol and soy bean flour as the main nutrition resource to produce CA. The growth period of *S. clavuligerus* and production period of CA can be separated, avoiding the synthesis of enzymes degrading CA. He also found that soy bean absorbed CA and inhibited the decomposition by *S. clavuligerus* [20]. This is one of reasons why we used porous inert support in CA production. The absorbance of CA on the support can inhibit the degradation to some degree.

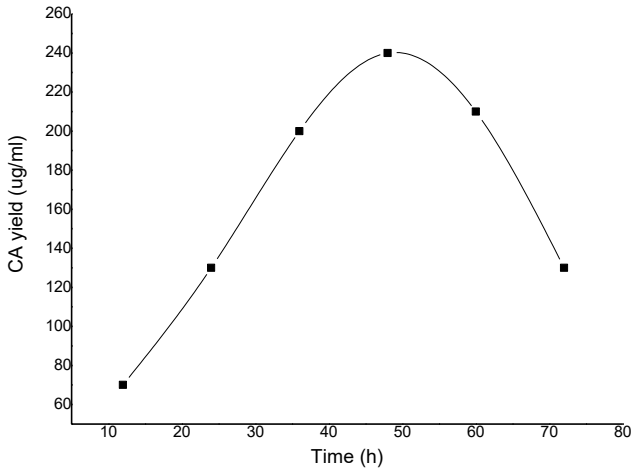

Figure 1 Effect of fermentation time on CA yield
(3g PUF, 10 mL medium, 29°C, 10% inoculum, initial pH 6)

Influence of medium amount on CA yield

The results presented in Figure 2 indicate that CA yield increased as the medium amount increased up to 15 ml, where the maximal CA yield (243 μg/ ml) was recorded. When the medium amount was less than 15 ml, the microorganism was not able to get enough nutrients, thus resulting in a low CA yield. On the other hand, when the volume of the moistening medium was more than 15 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.

Influence of inoculum amount on CA yield

In this study, the concentration of inoculum was 7.9×10^8 cells/mL. Figure 3 indicates that there was a gradual increase in CA yield when the amount of inoculum was increased from 2% to 10 %. Further increasing inoculum amount did not cause significant difference.

PUF used in this study is water-repellent, but it can absorb medium and microbes because of its porosity. Its porosity is not limitless and furthermore there is an optimal absorbed-amount for the microbe. Excessive inoculum lead to over-crowded living

circumstance and reduced the nutrient resource that microbe can obtain. Growth of *S. clavuligerus* and production of CA were thus hindered.

Influence of initial pH of moistening medium on CA yield

The optimum pH required for maximizing CA yield from SSF on PUF was evaluated by varying initial pH levels (4-8) of moistening media. Figure 4 shows that the maximal CA yield was obtained at pH 6.5, implying that higher or lower pH generally lead to poor growth or resulted in the degradation of CA.

CA is not stable after it is produced by fermentation, and could be degraded by adding acid or alkaline. The alkaline conditions tested in this study (pH 8.0) were closer to the optimal stability range found in this study (pH 6.0-7.2) than the acidic ones (pH 4.0). It can be concluded that the alkali-catalyzed degradation of CA was faster than the acidic one. These results were in good agreement with the literature [19]. Results in this study demonstrated that the optimal pH for CA stability was pH 6.5. Therefore, in order to get the higher CA yield, adjusting the initial pH of moistening medium and applying the buffer solution were effective.

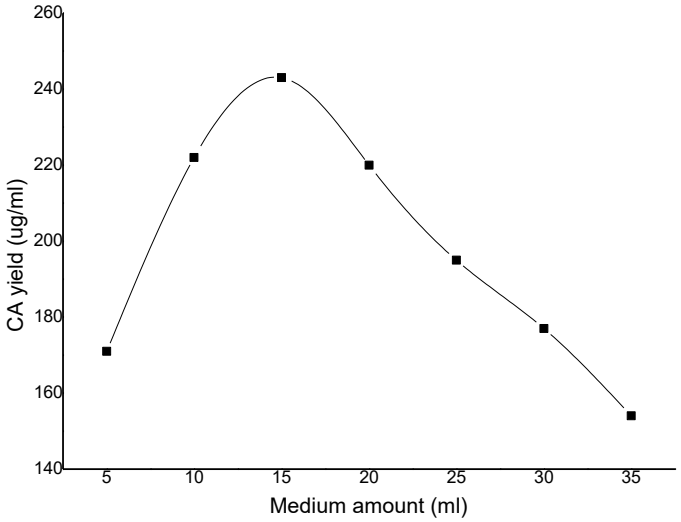

Figure 2 Effect of medium amount on CA yield
(3g PUF, 10 mL medium，29°C, 48 h, initial pH 6)

Influence of glycerol on CA yield

Figure 5 shows that *S. clavuligerus* did not produce CA without glycerol present in the medium. The CA yield increased with the increase of the amount of glycerol, and the maximum yield of 245 μg/ml was obtained when 0.015% glycerol was added. Further increasing glycerol resulted in the decrease of CA yield.

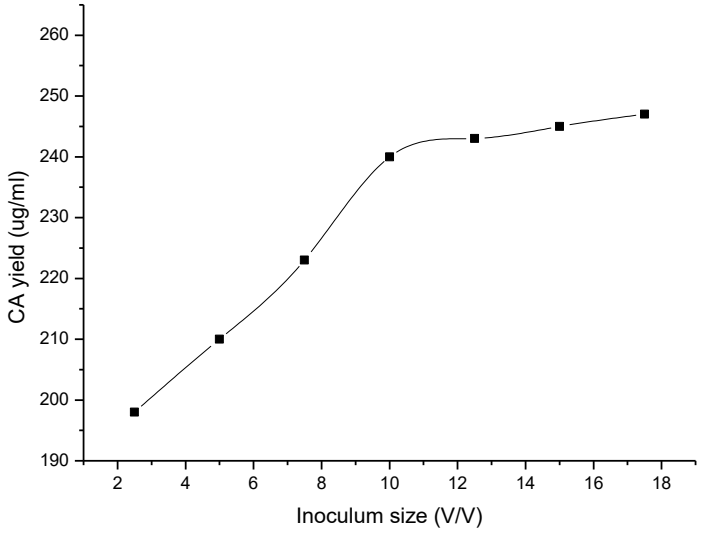

Figure 3 Effect of inoculum size on CA yield
(3g PUF, 10 ml medium, 29 °C, 48 h, initial pH 6)

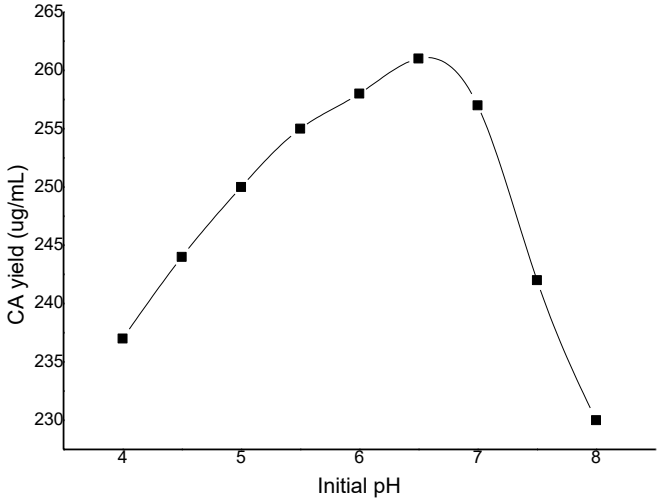

Figure 4 Effect of initial pH on CA yield
(3g PUF, 10 ml medium, 29°C, 48 h)

Glycerol is an essential component in the medium for CA production. It provides carbon skeleton to β-lactamase ring of CA [21]. Adding glycerol in the medium can improve the CA production. On the other hand, *S. clavuligerus* has a tolerance dose for the glycerol. There exists a glycerol conversion system (GTS) which is induced by glycerol. *S. clavuligerus* with the high tolerance to glycerol has active GTS and can effectively convert glycerol into CA. *S. clavuligerus* with low tolerance dose can not convert glycerol effectively, and accumulation of glycerol will be poisonous to the microbe. It can explain why CA yield decreased when glycerol increased to some extent in the medium [22].

Influence of lithium chloride on CA field

Figure 6 shows that lithium chloride improved the CA production. The maximum CA yield was obtained when the concentration of lithium chloride was 2%. These results were in accordance with the literature [23]. Lithium chloride combines with CA into a complex which is not easy to be degraded. This method can be applied in the industry to improve the CA yield in the final products.

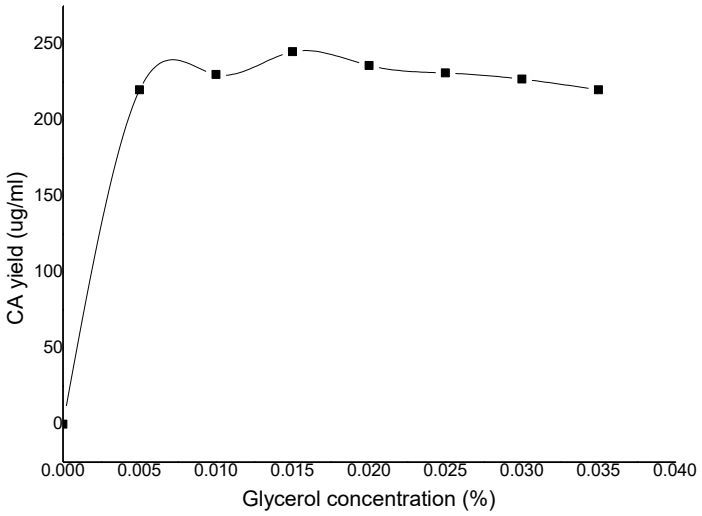

Figure 5 Effect of glycerol on CA yield
(3g PUF, 10 ml medium, 29ºC, 48h, initial pH 6.5)

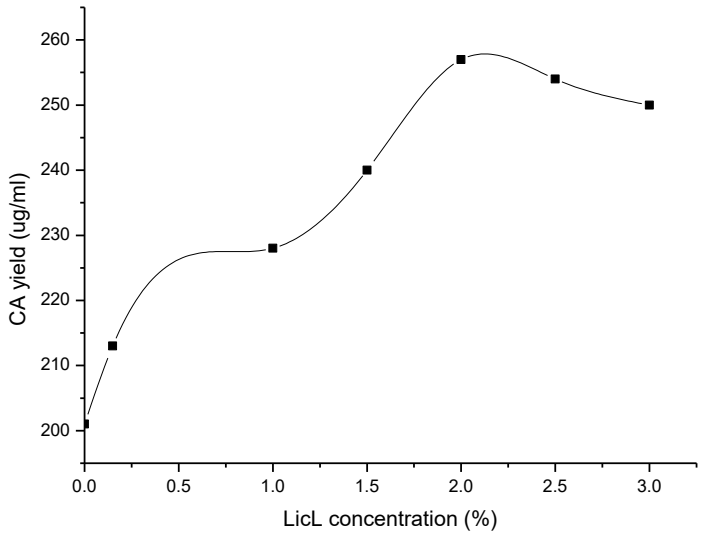

Figure 6 Effect of lithium chloride on CA yield
(3g PUF, 10 ml medium, 29ºC, 48h, initial pH 6.5)

Influence of ornithine on CA yield

Figure 7 shows that ornithine enhanced the biological production of CA. When the concentration of orithinie was 2 g/L, the maximum CA yield of 260 μg/ml was obtained, which was 1.2 times more than that without orithine. Increasing orithine in the medium lead to the decrease of CA yield, but still more than the yield from the process without adding orithine.

Besides CA production pathway, there are other pathways in *S. clavuligerus* fermentation which produce isopenicillin N and deacetylcephalosporin C. The priority of each pathway depends on the sulfur source in the medium. In order to achieve the higher CA yield, the sulfur concentration in the medium have to be controlled. Although the sulfur source is essential for the growth, extra sulfur will inhibit CA synthesis. As a result, existing sulfur-containing amino acids in the medium is not good for CA production. Ornithine has no sulfur atom and is the best precursor for oxazole ring of CA. Results in this study matched other published reports [24-26].

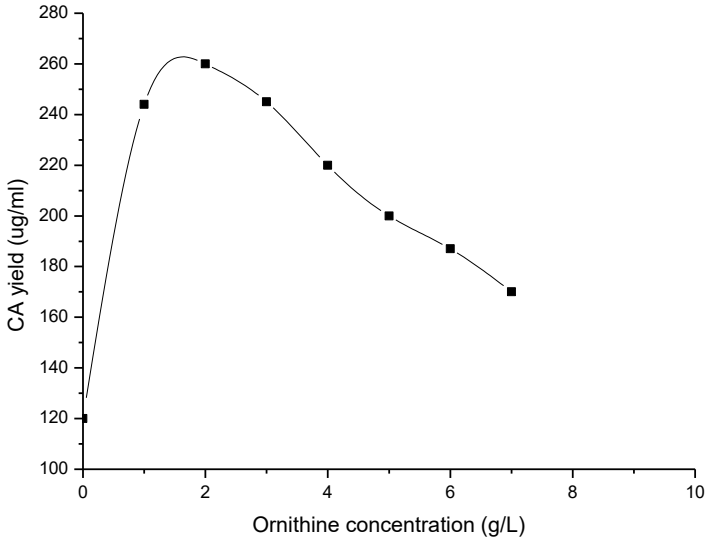

Figure 7Effect of ornithine on CA yield
(3g PUF, 10 ml medium, 29ºC, 48 h, initial pH 6.5)

Table 1 Comparison of SSF on inert support and SMF
(3 g PUF, 10 mL medium , 29ºC, 48 h, initial pH 6.5, 0.015% glycerol, 2% lithium chloride, 2 g/L ornithine)

	CA yield (μg/mL)	Concentration of the microbe (/mL)
SMF	203	1.2×10^9
SSF on inert support	263	60,000

Comparison with CA production in SMF

Table 1 is the comparison of SSF on inert support and SMF under same conditions, including culture temperature, time, inoculum concentration, and addition of glycerol. It can be found that the SSF on inert support improved the CA yield by 29.6%. Besides, concentration of the microbe in SSF on support is 1/20,000, which facilitated the extraction and purification of CA.

CONCLUSIONS

The results presented in this work showed that maximal clavulanic acid yield (263 μg/mL) was observed when solid state fermentation was carried out on polyurethane foam with the substrate at pH 6.5, incubation temperature 29°C, 10 mL medium per 3 g PUF, 0.015% added glycerol, 2% added lithium chloride, and 2 g/L added ornithine. Comparing to SMF that is the most popular method for CA production, SSF on PUF produced CA with higher yield and higher purity under the same conditions. Based on results in this study, inert support absorption SSF could be a novel way to produce CA with higher economic benefits.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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