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Solid state fermentation for cephalosporin production by *Streptomyces clavuligerus* and *Cephalosporium acremonium*

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Summary. Solid state fermentation systems were developed for the production of cephalosporins with *Streptomyces clavuligerus* and *Cephalosporium acremonium*. *S. clavuligerus* NRRL 3585 was grown on moistened barley under optimum solid state fermentation conditions for 7 days; approximately 300 µg cephalosporins per g substrate were extracted from the kernels. *C. acremonium* C-10 produced approximately 950 µg cephalosporin C per g substrate after 10 days of solid state fermentation.
Key words. Solid state fermentation; β-lactam antibiotics; cephalosporins; *Streptomyces clavuligerus*; *Cephalosporium acremonium*.

Introduction

As defined by Cannel and Moo-Young[4], solid state fermentation (SSF) refers to the growth of microorganisms on solid materials without the presence of free liquid. In SSF the moisture necessary for microbial growth exists in an absorbed state or complexed within the solid matrix. An exact definition of SSF with respect to free liquid is not easily established; while the minimum moisture level at which SSF can occur is about 12% (this level being that below which all microbial activities stop), the upper limit for SSF is more a function of absorbancy than moisture content. For example, free water becomes apparent in maple bark at about the 40% level, and in straw at about the 75% level[4]. Because of the low water content and a_w-value of the substrate, solid state fermentation systems provide a selective environment for a large number of filamentous fungi and a few bacteria, such as actinomycetes, which grow in mycelial form.

Solid state fermentations have been used for centuries in the Far East for the preparation of a variety of fermented foods such as miso, soy sauce, tempeh and others as described by Hesseltine[13]. Recently, this technology has been studied for the production of mycotox-

ins[12,13,18,21,29], animal feed[7,24], ethanol[8,9,11], citric acid[2,10], extracellular cellulases, amylases, pectinases[30], proteases[16], lipases[32], as well as for the mass production of spores of filamentous fungi[2,19]. The results of these studies indicate that SSF technology might be efficient for increasing product yields, producing new metabolites, improving nutritive value of animal feeds and alleviating waste disposal problems with the concomitant production of utilizable metabolites. The objective of this study was to determine whether β-lactam antibiotics could be produced from cereals via SSF with *Streptomyces clavuligerus* and *Cephalosporium acremonium*. *S. clavuligerus* produces four β-lactams[14,23]: two of these are cephalosporins [7-(5-amino-5-carboxyvaleramido)-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (= carbamoyldeacetylcephalosporin C) and 7-(5-amino-5-carboxyvaleramido)-7-methoxy-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid (= cephamycin C)] and the others are penicillin N and clavulanic acid. *C. acremonium* is a penicillin N and cephalosporin C producer[27].

Materials and methods

Microorganisms and their maintenance

S. clavuligerus NRRL 3585, and *C. acremonium* C-10, both producers of cephalosporins, were used. For the maintenance of *S. clavuligerus* NRRL 3585, the organism was grown on solid medium containing 0.4% yeast extract, 1.0% malt extract, 0.5% tapioca dextrin and 2.0% agar at pH 7.3, in petri dishes placed in an incubator with forced ventilation at 30 °C (J. M. Piret, personal communication). Sporulation took place after approximately 10 days; the spores were harvested and stored in a 20% (v/v) glycerol solution at −20 °C. *C. acremonium* was maintained on agar slants as previously described[20]. *Escherichia coli* ESS, a mutant strain supersensitive to β-lactam antibiotics, was used as the cephalosporin assay organism. It was grown in nutrient broth for 18 h at 37 °C with shaking at 250 rpm, the mixed 1:1 with glycerol to attain a 50% (v/v) glycerol cell suspension, which was stored at −20 °C.

Sporulation of S. clavuligerus

For the production of spores, *S. clavuligerus* was grown at 25 or 30 °C on the medium described by Brana et al.[3], the yeast extract, malt extract, dextrin agar medium of Shirling and Gottlieb[28] and the medium described above for maintenance (J. M. Piret, personal communication). The media were inoculated using a 60-h seed culture prepared as described by Aharonowitz and Demain[1]. With each medium, six different incubation procedures were adopted: in petri dishes, in 250-ml square bottles and in 2-l flat flasks, at 25 and 30 °C. The spores were harvested after 10–21 days (depending on the medium used), and stored in 20% (v/v) glycerol at −20 °C. The best sporulation was obtained by incubating the organism on the medium described by Piret (personal communication) in petri dishes, and by placing a single layer of plates in an incubator with forced ventilation at 30 °C.

Seed media and culture conditions. One ml of thawed *S. clavuligerus* spore suspension was used to inoculate 50 ml of the seed medium described by Aharonowitz and Demain[1] in 250-ml baffled Erlenmeyer flasks. The seed culture was incubated at 30 °C and 250 rpm for 60 h. Seed cultures of *C. acremonium* were obtained by inoculating seed medium No. 1 of Shen et al.[27] with mycelia from an agar slant and incubating at 25 °C and 250 rpm for 50 h.

Liquid fermentation media and culture conditions. With *S. clavuligerus* liquid fermentations were carried out in glucose-asparagine (GA)-medium[1], Whitney medium[31], GSPG medium[26], Perlman medium[25] and TSB medium (3% trypticase soy broth, 1% corn starch in 100 mM MOPS buffer at pH 7.0) using 500-ml baffled Erlenmeyer flasks and shaking at 250 rpm and 30 °C (J. Zhang, personal communication). 100 ml of medium were inoculated with 2 ml seed culture. At 24-h intervals for 7 days, samples were taken and the production of β-lactams was assayed as described below. With *C. acremonium*, liquid fermentations were done in complex[27] and chemically defined[33] media in 500-ml unbaffled Erlenmeyer flasks at 25 °C and 250 rpm. 100 ml of medium were inoculated with 2 ml seed culture. At 24-h intervals for 7 days, samples were taken and the production of β-lactams was assayed as described below. Fermentations were conducted in duplicate flasks.

Solid state fermentation (SSF). During the fermentations, the pH was measured by mixing 20 g grains with 20 ml Milli-Q water, grinding with pestle and mortar and immersing the sensor in the mashed grains. For growth measurements, the method of Brana et al.[3] was used. Mycelia grown on 20 g grains were scratched off and suspended in 40 ml Milli-Q water. Two ml were then taken and mixed with 2 ml of water, sonicated (Branson Sonifier, 45 s, setting 2 and 50%) and diluted to 10 ml. Growth was measured as Klett units in a Klett-Somerson colorimeter with the red filter. Klett units were converted into dry cell weight (DCW) (for *S. clavuligerus* Klett units/260 = mg DCW/ml; for *C. acremonium*, Klett units/180 = mg DCW/ml). The solid state cultures in 250-ml Erlenmeyer flasks were incubated in a cabinet without forced ventilation at a relative humidity (RH) of approximately 96%, controlled with saturated potassium sulfate solution placed in trays at different points of the cabinet. In this way, it was possible to maintain the water content of the grains at a constant level, their water activity value varied between 0.940 and 0.970 (Hygrometer Model DT; Rotronic AG, Zurich, Switzerland), depending on the amount of liquid used to moisten them. All SSF experiments were conducted in triplicate flasks.

Extraction. A large number of preliminary experiments were done on extraction of cephalosporins from barley after fermentation. Organic solvents (acetonitrile, te-

trahydrofuran, dimethylsulfoxide, dimethylformamide and n-butanol) extracted no antibiotic activity. With distilled water (pH 5.5), a number of extractions were required. At low levels of cephalosporins (ca 60 µg/g), about three extractions were sufficient. However, when cephalosporins were present at 200 µg/g, 5–6 extractions were necessary. Decreasing the pH of the water to 2.5 had no beneficial effect. A comparison of buffers (phosphoric acid, succinic acid, citric acid) at 0.1 and 0.4 M and pH 2.5 and 4.0 revealed that buffers were more useful than water as extractants. We finally chose three extractions with 0.1 M phosphate buffer at pH 3.0 as our standard procedure. For each extraction, 20 g of fermented grain was agitated at 175 rpm (rotary shaker) for 60 min at 25 °C in 60 ml extractant. In this way, 93% of the cephalosporin produced was extracted from the grain.

Antibiotic assay. The production of cephalosporins was assayed by the standard disk-agar plate technique, using as assay organism a β-lactam supersensitive mutant of *Escherichia coli* (strain Ess). Tryptic soy agar (9 ml) containing narrow spectrum β-lactamase (Bacto-penase concentrate, Difco, 1 µl/ml agar) was used to hydrolyze penicillin N and detect cephalosporins only. The amount of enzyme used was sufficient to completely destroy the zone produced by a disk dipped into 100 µg of penicillin N per ml, even when the penicillin N sample included 100 µg/ml of clavulanic acid, a penicillinase inhibitor. In parallel, agar plates containing broad-spectrum β-lactamase (Sigma penicillinase No. P-4524; 10 µg/ml agar) were used to hydrolyze both penicillin N and cephalosporins and to detect clavulanic acid or any non-β-lactam antibiotics produced. The amount of enzyme used was sufficient to destroy the zone produced by a disk dipped into 500 µg cephamycin C per ml, even in the presence of clavulanic acid. Cephamycin C (Merck L-619, 183-006 Y 005) was used as standard.

Results and discussion

Production of cephamycin C and cephalosporin C in liquid media. Figure 1 shows the results of fermentation experiments in liquid media. The complex fermentation medium of Whitney et al.[31] was found to support the best production of cephamycin C by *S. clavuligerus* among several media tested, while the complex fermentation medium of Shen et al.[27] was better than the chemically defined medium in supporting cephalosporin C production by *C. acremonium*. In both Whitney medium[31] and TSB medium (J. Zhang, personal communication), *S. clavuligerus* was able to produce an antibiotic resistant to broad-spectrum β-lactamase, presumably clavulanic acid or holomycin[15].

Solid state fermentations with S. clavuligerus. The following paragraphs describe the development of a solid state fermentation process.

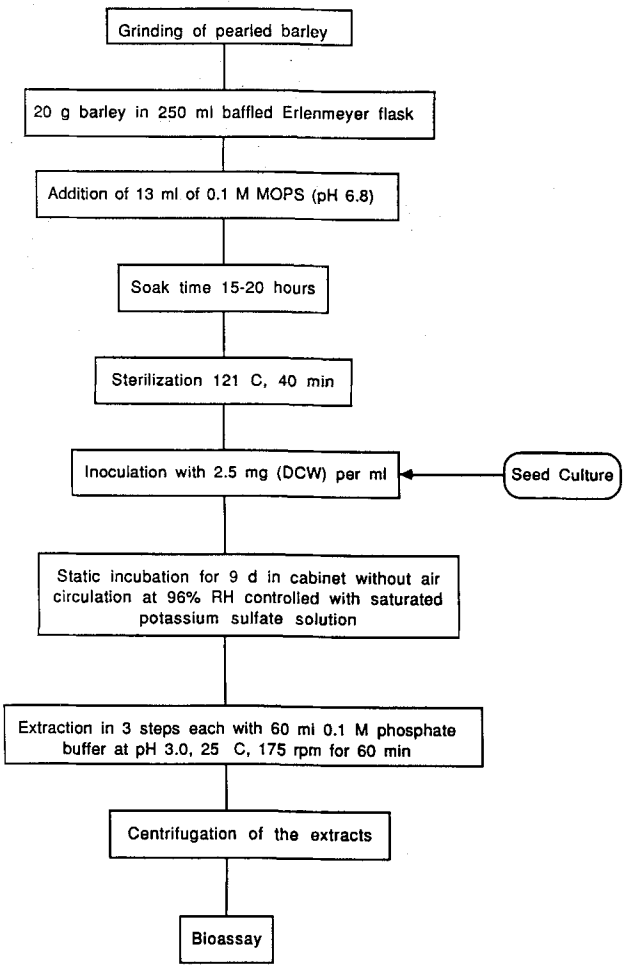

Figure 1. Processing sequence for production of β-lactam antibiotics by *Streptomyces clavuligerus* using the solid state fermentation method.

Substrates

Barley, winter red wheat, glutinous rice and brown rice were compared as substrates. Barley and wheat were ground in a coffee mill (5 pulses each of 0.5 s) while the rice samples were untreated. The 20-g samples of grains were moistened with 13 ml double distilled water which had a pH of 5.5. Flasks were incubated at 30 °C for 4–17 days after inoculation with 0.8 mg DCW seed culture per ml (60 h old).

Growth was poor on brown and glutinous rice but good on barley and wheat. Production on barley was more extensive and faster than on wheat. Of interest was the observation that use of 0.1 M MOPS buffer (pH 6.8) instead of water to moisten the grain eliminated production on wheat but enhanced production on barley. About 17 µg cephalosporin/g barley was produced in 15 days. When Whitney medium was tested as moistening agent, production was virtually eliminated. No clavulanic acid or any non-β-lactam antibiotics were found to be produced in any of the above SSF's.

Moisture content

Ground barley was used to determine the effect of moisture content on production. 5, 10 and 13 ml of 0.1 M MOPS were compared in an 11-day experiment. Growth was observed only at the 10- and 13-ml levels of MOPS. The production with 13 ml MOPS amounted to 181 µg/g barley which was 4.5 times greater than with 10 ml MOPS.

Inoculum level

An experiment was done to examine the effect of inoculum level (0.75, 1.0, 1.5, 2.5 mg DCW/ml) on the ground barley fermentation. A general trend was seen at each sampling time (7, 11 and 15 days), i.e., higher inoculum levels yielded higher cephalosporin production. We thus shifted to 2.5 mg DCW/ml for further work. About 225 µg/g maximum production was observed in this experiment.

Treatment of barley

Pearled and unpearled barley samples were compared in the ground state. Pearled ground barley was the preferred substrate (230 µg/g vs 190 µg/g in 11 days). It was also found that grinding was beneficial for production on barley.

Temperature

A temperature comparison showed that 25 °C was superior to 30 °C. Further studies were done at 25 °C.

Agitation

Static incubation was compared to agitation at 150 rpm in a 10-day experiment. Static incubation was found to be superior. The final procedure adopted for SSF is shown in figure 1.

Time course of production

Figure 2 shows the time course of cephalosporin production in SSF by *S. clavuligerus*. Production amounted to approximately 300 µg cephamycin per g barley.

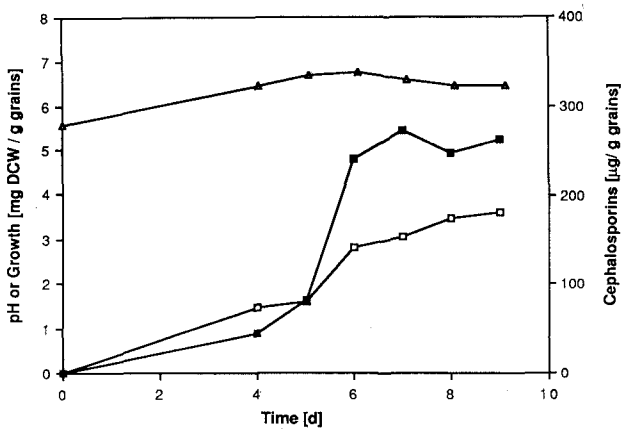

Figure 2. pH, growth and cephalosporin production by *Streptomyces clavuligerus* NRRL 3585 on ground pearled barley under optimum solid state fermentation conditions [20 g barley in a 250-ml Erlenmeyer flask, moistened with 13 ml of 0.1 M MOPS buffer, inoculated with 2.5 mg (DCW) mycelia per ml and incubated statically at 25 °C].

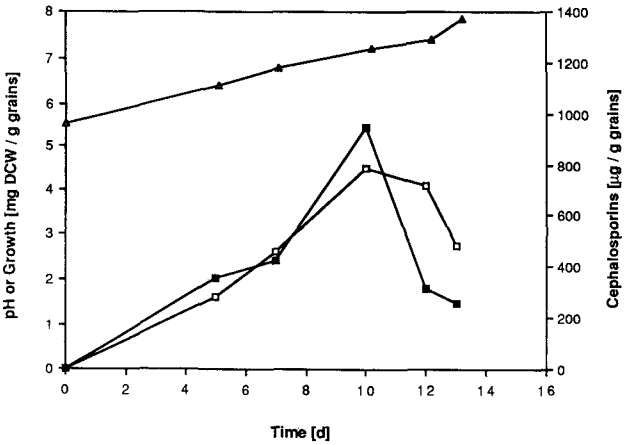

Figure 3. pH, growth and cephalosporin production by *Cephalosporium acremonium* C-10 on ground pearled barley under optimum solid state fermentation conditions [20 g barley in a 250-ml Erlenmeyer flask, moistened with 10 ml chemically defined fermentation medium as described in Zhang et al.[33], inoculated with 1.6 ml packed mycelia and incubated statically at 30 °C].

Solid state fermentations with C. acremonium

Studies with the fungus, *C. acremonium*, indicated that conditions developed for SSF with *S. clavuligerus* were also useful with *C. acremonium*. However, improvement in cephalosporin production by the fungus was observed when the temperature was 25 °C and moistening fluid was changed from 13 ml MOPS to 10 ml of the chemically defined medium of Zhang et al.[33]. Production under these conditions is shown in figure 3. About 950 µg cephalosporins per g barley were produced.

Final comments

The data presented in these experiments show that significant concentrations of cephalosporins (including cephamycins) can be produced in solid state culture on barley. Although we were not surprised that this would occur with fungi, the possibility with actinomycetes was much less clear. It is amazing that nothing is known about the production of medically useful antibiotics in solid state culture on grains and virtually nothing is known about the production of any antibiotics by actinomycetes on grains[22]. On the other hand, there is extensive literature on mycotoxin production by fungi on grains[12,13,22] and many of these are antibiotics[5,6,17]. We suggest that solid state culture might be a cost-effective means of producing expensive antibiotics in developing countries especially those agents used in animal and plant growth and protection.

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