ENHANCEMENT OF SOLID STATE FERMENTATION FOR PRODUCTION OF PENICILLIN G ON SUGAR BEET PULP

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In this study, two local strains of *Penicillium chrysogenum* named EGEK458 and EGEK469 were selected for enhancement of Penicillin G (PenG) production under solid state fermentation (SSF) conditions. These two strains were selected among seven strains according to their fermentation yields for PenG production during previous tests under submerged fermentation conditions. Sugar beet pulp, an agro-industrial residue of the sugar industry, was used as an inert support for the first time in PenG production under SSF. In order to enhance the production of PenG, two points of moisture level and three concentration values of nutrients (impregnated in solid support), which are the key parameters in production of PenG, were compared. As the yields from solid and submerged fermentation were compared, 570U/g of PenG – almost 15 times higher quantities of its production vs. submerged conditions – were obtained under SSF conditions in 50 hours by the strain EGEK458. The conditions for the enhanced production of PenG were 65% moisture content with a four-fold concentrated nutrients impregnated solid support.

Keywords: Penicillin G; Solid state fermentation; Sugar beet pulp; Inert support

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INTRODUCTION

Penicillin and cephalosporins are two major commercialised β-lactam antibiotics. Industrial production of these antibiotics by fermentation over the past 50 years is one of the outstanding examples of biotechnology. The total world market for β-lactam antibiotics is estimated to be about 15 billion US$ penicillin dosage from sales at 5 billion. The β-lactam antibiotics now account for over 65% of the world antibiotics market (Elander 2003).

The commercial fermentation mode for penicillin is a fed batch process carried out in stainless steel tank reactors in which carbon and precursors are fed to the system throughout the cycle. About 65 % of the carbon is metabolized for cellular maintenance, 20-25 % for growth, and 10-12 % for penicillin production (Van Nistelrooij et al. 1998). Sugar and precursor are fed continuously, and the sugar is also used to help regulate the pH of the fermentation to between 6.4 and 6.8 during the active penicillin production phase. Corn steep liquor and cottonseed or soybean meal, ammonia, and ammonium sulphate represent major nitrogen sources. The essential precursor substances are phenyl-acetic acid (for PenicillinG) or phenoxyacetic acid (for penicilin V) that are either fed or batched. Major fermentation producers are now estimated to record harvest titers of 40-50 g/L for penicillin (Elander 2003).

Solid state (substrate) fermentation (SSF) is characterized by a fermentation process on a solid support that has a low moisture content (lower limit ~12 %) and occurs in a non-septic and natural state (Nigam and Singh 1994). Selection of a proper susbtrate is another key aspect of SSF. In SSF, solid material is non-soluble and acts both as a physical support and a source of nutrients (Pandey 2003). SSF has been used successfully for the production of enzymes and secondary metabolites.

The mycelial morphology associated microorganisms predominantly used for secondary metabolite production are well suited to growth on a solid support. In SmF, the morphology and the secretion of these metabolites into the growth media can increase viscosity and cause reduced oxygen transfer. Therefore SSF technology can be exploited as an alternative, allowing better circulation (Elibol and Mavituna 1997). Wild type bacteria and fungi tend to perform better under SSF conditions than do genetically modified microorganisms, reducing energy and cost requirements (Barrios-González et al. 1988). Many of these metabolites are still produced by submerged liquid fermentation (SmF), even though production by this method has been shown to be less efficient than SSF (Robinson et al. 2001).

Production of penicillin by SSF technology is high in yield in a short time period as compared to submerged fermentation (Balakrishnan and Pandey 1996). The methodology of SSF employing inert solid supports, which has been used for the production of several compounds, could also be used for penicillin synthesis. In 1989, penicillin was produced by non-sterile SSF, and the substrate used was bagasse impregnated with culture medium. In this system the yield and productivity were found to be superior to that of SmF.

The factors that have been studied and which affect penicillin production can be divided into environmental and genetic factors. It has been established that those parameters that do not directly control idiophase, such as initial moisture contents and concentrated media, are important to obtain a high yield of penicillin. The production of antibiotics is controlled by the proportion of the SSF-support and other two components in the solid medium e.g. nutrients and water (Pandey et al. 2001).

Recent studies have explored different SSF systems, particularly the use of inert supports impregnated with liquid media. It was shown that some physical and nutritional parameters are also important, such as the initial moisture content, the concentration of the medium, the contents of the support, the particle size, and the packing density during use of SSF (Barrios-González et al. 1993; Dominguez et al. 2001).

It was aimed in this study to investigate the suitability of a byproduct of the sugar industry (agro-industrial residue) as an inert support for the production of PenG initially under solid state conditions. Then, enhancement of the PenG yields under SSF was carried out considering two parameters that define the culture conditions, namely the initial moisture content of the fermentation medium and the concentration of nutrients used to impregnate the solid support. Both parameters were indicated as key parameters for the production of PenG.

EXPERIMENTAL

Microrganisms

A total of seven strains of *Penicillium chrysogenum*, one of which was obtained from the NRRL culture collection centre (NRRL824) and others were our natural isolates, had been previously screened for their PenG production on petri plates (unpublished results). They were submitted to submerged fermentation in order to select the strains with higher titers to use for solid state fermentation experiments.

These strains were deposited in lyophilized form in the microfungi culture collection of *Bioengineering Department Ege University,* İzmir, (Turkey). *Micrococcus luteus* ATCC 9341 was employed as a test organism to check PenG activities from the fermented media extracts.

Sporulation of Fungi and Activation of Test Microorganism

Fungal isolates were inoculated on Potato Dextrose Agar (PDA, Sigma P-2182) and incubated at 28°C for 7 days for sporulation. For determination of the PenG activities of extracts of fermented media, each test microorganism was cultured at Mueller Hinton Broth (Merck 1.10293) at 37 °C for 18 hours before the bioassay test.

Submerged Fermentation (SmF)

Complex penicillin media (CPM) was prepared according to Laich et al. (1999) and sterilized in an autoclave. It consisted of (g/L), corn steep liquor (CSL; Sigma-Aldrich Co., C4648) 20, lactose 55, $MgSO_4.7H_2O$ 3, $CaCO_3$ 10, KH_2PO_4 7, and phenylacetate (PAA) 4. All chemicals except CSL were obtained from Merck Co. The initial pH was adjusted to 6.8. Fermentation was carried in 250 ml flasks containing 100 ml of medium at 28 °C for eight days. Experiments were run in triplicate.

Solid State Fermentation (SSF) and Enhancement of PenG on SSF

SSF was carried in 250 mL flasks. Sugar beet pulp (SBP) was washed twice with hot water and then distilled water, and dried at 70 °C before use as an inert support. It was used after impregnated and moistened with a two-fold concentration of CPM. Later three-fold and four-fold concentrated CPM were examined in enhancement studies. The composition of CPM was prepared according to the desired concentration, and distilled water was added to achieve the desired moisture level. Each of the flasks contained 7.5 grams of SBP. Incubation was carried at 28 °C for seven days. Experiments were performed in triplicate.

For enhancement of PenG production under solid state fermentation conditions, two levels of initial moisture content (IMC), 60 and 65 %, were first studied with our two isolates (EGEK458 and EGEK469). The maximum water retention capacity of the inert support was visualized as 65 %. Then three values of CPM levels (two-, three-, and four-fold concentrations) were used to impregnate solid support of the SSF medium with 65 % IMC. In order to achieve the desired IMC level of the SSF medium, the volume of added water was adjusted. The percentages corresponding to 2, 3, and 4-fold concentration of CPM in water [CPM (g)/distilled water (ml)] were 11.3, 15.3, and 18.7 %, respectively.

Inoculation of Fermentation Media

Inoculation was done by spores of mold cultures, which were previously sporulated on slope PDA in flasks. After the incubation period, CPM solution (that contained 0.1 % Tween 20 solution) was added to sporulated flasks, spores were scraped with a glass stick, and this spore suspension was transferred to another sterile flask. Spores were counted by Thoma slide. Each medium was immediately inoculated with a spore suspension at a 5 % ratio (v/v), which included 10^7 spores per ml for submerged and per gram of solid state fermentation medium.

Extraction of PenG from SSF Medium

PenG was extracted daily from SSF samples with 0.1 M, pH5.5 phosphate buffer at 1:10 ratio (w:v) with a rotary shaker for half an hour. Then extracts were transferred in Eppendorf tubes and centrifuged at 6000 rpm for ten minutes. These clear extracts were used for the determination of PenG activities.

Bioassay Test – Quantitation of PenG

Sixty microliters of the fermented media (from SmF) or extract (from SSF) were used to fill wells (8 mm) in the agar medium seeded with *M. luteus* for the bioassay. Tryptic Soy Agar (Oxoid CM 131) was used as the test medium. The inhibiton zone diameter was linearly related to the logarithm of penicillin concentration in a calibation curve (0-50 U/mL). The diameter of the inhibition zone of the fermentation samples was interpolated in the curve. Production was expressed in units of penicillin per gram of dry fermented sugar beet pulp. Experiments were performed in duplicate. Results for SmF were expressed directly as U/ml, since the suspended mixture was used and no extraction was carried out from the fermentation media. Experiments were performed in duplicate.

RESULTS AND DISCUSSION

SmF Results

The results obtained from SmF experiments for seven strains belonging to *P. chrysogenum* species are represented in Fig. 1. The highest titers of PenG were obtained at between the 48 and 96 hours of fermentation. Among the strains, EGEK458 produced the highest PenG as 45 U/mL at 48 hours. This was followed by EGEK469, which produced 34 U/mL PenG at 72 hours. These two strains were selected for the further SSF experiments. The reference strain NRRL824 produced later and lower titers of PenG than our isolates under these experimental conditions.

SSF Results

PenG production and enhancement studies were carried out on SSF. First, two levels of initial moisture content (IMC) were studied by 2-fold CPM impregnated SBP, and the results are represented in Fig 2. By increasing the IMC from 60 to 65 %, increased titers of PenG were obtained for both strains. The highest titers were 220 U/g at 96 h and 230 U/g at 72 h for the strains EGEK458 and EGEK469 from the media with 65

% IMC, respectively. These results were 4.8 and 6.7 fold higher, respectively, than SmF results.

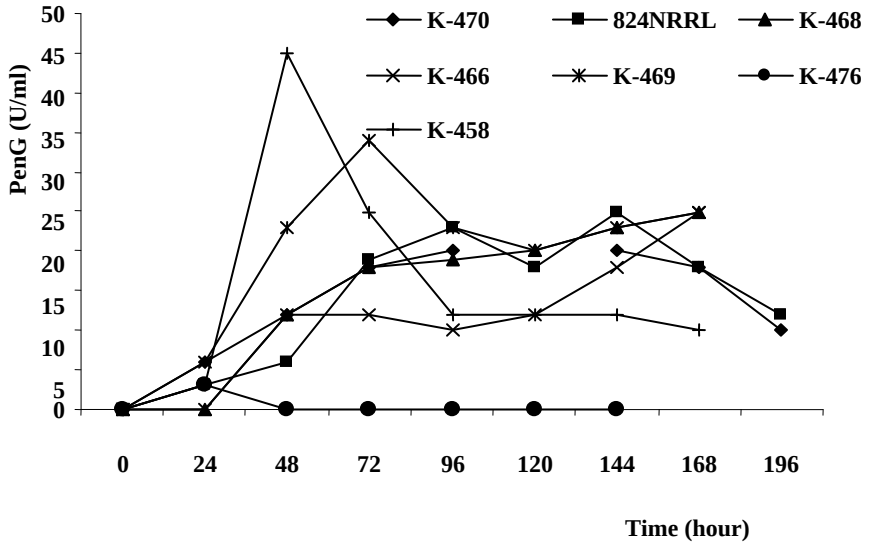

Fig. 1. PenG titers under submerged fermentation conditions with seven strains of *P. chrysogenum* for eight days

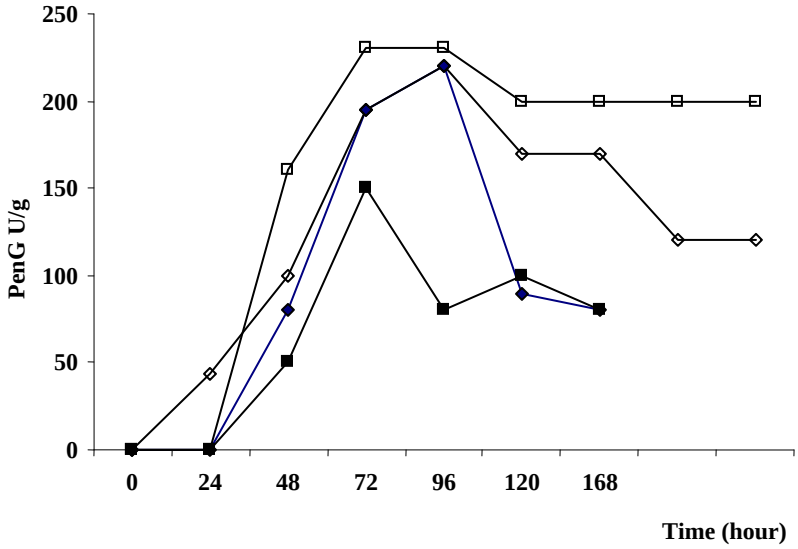

Fig. 2. Comparison of PenG productions of two strains under SSF conditions with two points of moisture level (60 % and 65 %) and two-fold (2X)concentration of CPM impregnated SBP media (-■- EGEK469, 60 %; -□- EGEK469 65 %;- ♦- EGEK458 60 %; - ◊- EGEK458 65 %)

Barrios-González et al. (1993) have compared the performances of SmF and SSF systems for the PenG production by the strain P. chrysogenum NRRL 1951, and 17 times

higher production was achieved in one third of the time under SSF conditions. They used sugarcane bagasse as an inert support for the production of PenG. A range of IMC (60 to 78%) was also investigated, and it was reported that IMC did not affect the time at which production started, but had an important influence on the maximum penicillin production reached and its stability. The present result was in consensus with their results. In their study, maximum production of 800 U/ml was obtained after 46 hrs of incubation with 70 and 73 % IMC. The IMC did not affect the onset of production in our study, as it started around 48 h for both levels of IMC.

Dominguez et al. (2001) have worked to develop an experimental design for optimization of PenG production under solid state fermentation conditions, using different ratios of bagasse, nutrients, and initial moisture by a mutant strain *P. chrysogenum* P2-4. They have reported that PenG production was affected by the proportions of these three components. However, water and nutrients concentration were not found as being responsible for the control of penicillin production in SSF, individually. They obtained two peaks for maximum production of PenG as 4 and 7 mg PenG /g from two media, which differed from each other with respect to the ratios of components. One of them had 'low moisture and high nutrients content (62 and 25.5 %, respectively) for the 4 mg PenG/g production, and the second had high moisture and a relatively low nutrients concentration (75.5 and 12.4 %, respectively) for the 7 mg PenG/g production.

By increasing the CPM concentration two- to three-fold for the fermentation with strain EGEK458, a three and a half-fold increase for the PenG production (286 U/g) was obtained near 50 hours (Fig. 3). PenG production reached a measurable level at 30 hours of fermentation. When comparing media with two-fold vs. four-fold CPM impregnation of CPM, the more highly concentrated condition was found to yield 6.3 times higher (as 507 U/g) PenG production. According to our results, the concentration of CPM greatly affected the time at which production started and the quantity of PenG produced for the strain EGEK458.

For the strain EGEK469, the effect of CPM concentration on PenG production did not correlate. As the concentration of CPM increased, the production of PenG decreased, and at four-fold concentration it was also delayed (Fig. 4). The best results were obtained with two-fold CPM impregnated medium for this strain. This result agreed with the previous results of Barrios-González et al. (1993). They have also compared the three concentration values of liquid media as two-, three-, and four-fold, which was used to impregnate the support. They obtained five times higher results (686U/mL), with the culture medium impregnated with two-fold high concentration of liquid medium, in comparison to the others. PenG production was a fair degree lower in two- and three-fold concentrated media impregnated cultures. We assumed that concentrated medium possibly affected this organism in another way than the EGEK458. Possible mechanisms to explain this result could involve decreased transportation and excretion of PenG to the fermentation medium due to reduced water activity (aw) of the medium, or the regulation of cellular permeability for PenG of this strain.

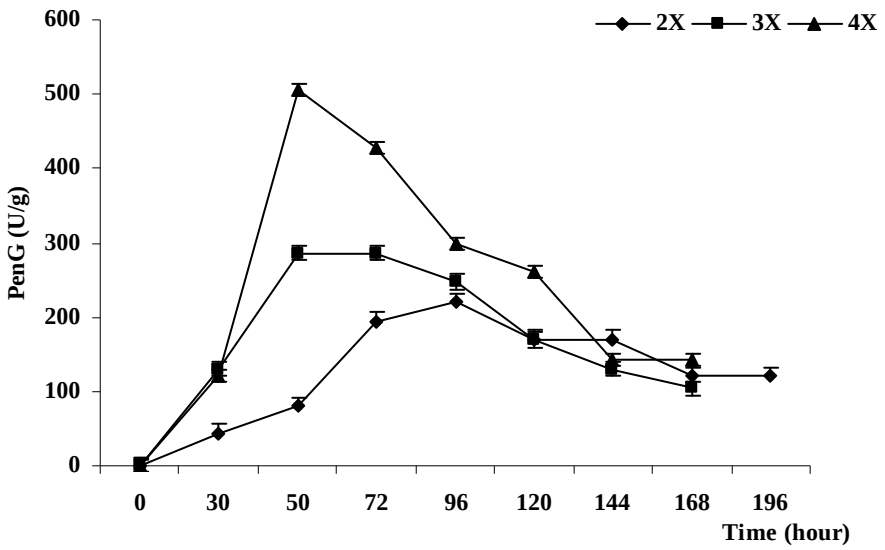

Fig. 3. PenG production yields by the strain EGEK458 under SSF conditions with constant moisture level (65 %) and three different concentrations of CPM (2X, 3X, 4X) impregnated to SBP

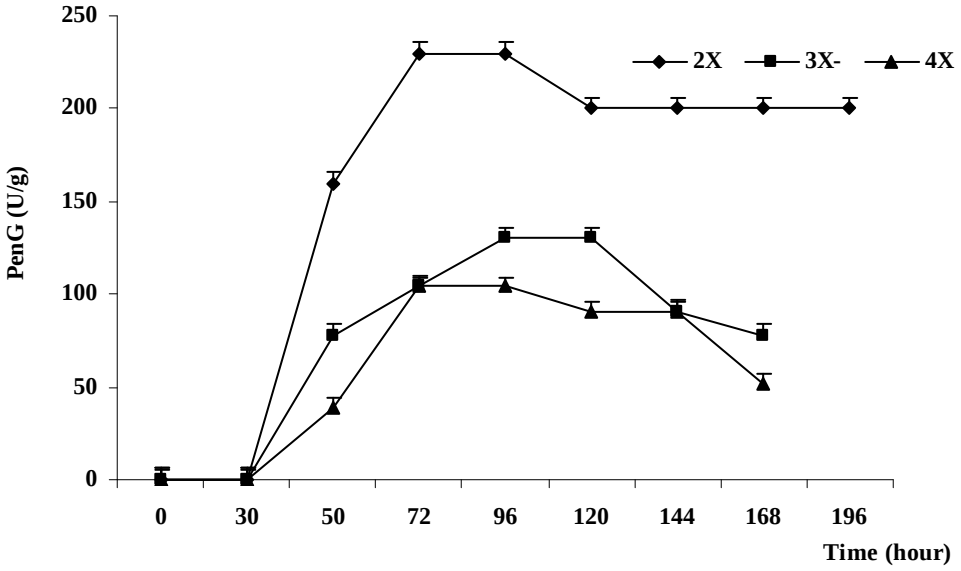

Fig. 4. PenG production yields by the strain EGEK469 under SSF conditions with constant moisture level (65 %) and three different concentrations of CPM (2X, 3X, 4X) impregnated to SBP

In another study by Barrios-González et al. (1988) the effect of support particle size on PenG production was investigated, and the use of large particle size support (14 mm) increased penicillin production by 37 %. In the present study, PenG production was investigated by different *P. chrysogenum* strains considering different aspects of SSF conditions on a different substrate. In our opinion, the variations from results obtained in previous studies were mainly related to the fungal strains which were used, the choice of

extraction procedure, and the concentration of liquid media used to impregnate the support. Under improved conditions of SSF, almost 15 times higher PenG production per g of substrate were obtained, compared to the production per ml of fermentation media by SmF conditions. Considering the yields for fermentation of PenG, the suitability of the sugar beet pulp as inert support for the production PenG was demonstrated.

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