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Improvement of Biological Penicillin Production from *Penicillium Chrysogenum* Using Molasses as Carbone Source

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Penicillin as a secondary metabolite is produced by various fungus especially *Penicillium chrysogenum*. In order to study the affecting parameters on the growth and penicillin biosynthesis, a strain of *P. chrysogenum*; PTCC-5031 was used in this work. Since *P. chrysogenum* can consume different sources of carbon, in order to decrease the production expenses, molasses; as a viscous by-product of the processing of sugar cane or sugar beets was used with several ratios with synthetic carbon sources such as lactose and glucose in preparation of pre-culture and main cultivation media. The effect of molasses concentration in fermentation broth on the cell growth and penicillin production yield was studied. Obtained results showed that using 1:1 (w/w) ratio of molasses and lactose can incredibly decrease the fermentation time, increase the cell growth as well as penicillin production yield.

1. Introduction

Penicillin production by the filamentous fungus *P. chrysogenum* is one of the most extensively studied antibiotic processes. However, research for the improvement of existing penicillin fermentation processes continues to expand with improvement processes mainly focusing on providing better cultures, and using less expensive raw materials for production (Ariyo et al. 1996). Since the amounts and rates of penicillin formation during biosynthesis process are influenced by the substrate carbon and precursor and by their types and amounts added (Matelova et al. 1972) for instance, relatively few papers have been published on the carbon nutrition of *P. chrysogenum* and its effect on penicillin yield.

Molasses as a viscous by-product of the processing of sugar cane or sugar beets is very cheap in comparison with other synthetic carbon sources such as glucose and lactose. Addition of molasses to the cultivation medium would decrease the total price of product. There are a few reports in which molasses has been used as a carbon source mainly in the growth phase medium but no reports has mentioned about molasses utilization in the production phase medium (Rani et al. 2004). In the present work, we studied the effects of addition of molasses as carbon source on the biosynthesis of penicillin in shaken flask cultures of *P. chrysogenum*. The fermentation profiles of *P.*

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chrysogenum batch cultivation showed that using of molasses and lactose as readily and slowly utilizable carbohydrates, respectively in the fermentation medium have a more pronounced effect on penicillin yield.

2. Materials and methods

2.1 Strain and preparation of spores

Strain of filamus fungus *Penicillium chrysogenum* (PTCC 5031) was purchased from Persian Type Culture Collection (PTCC) of Iran. The spore suspension was prepared using solid state cultivation in Malt Extract Agar medium. Spores in each slant were suspended in 5ml sterile distilled water using. In order to prepare a uniform suspension of spores, they were sonicated for 3-4 min in an ultrasonic water bath at room temperature.

2.2 Preparation of seed

The components in seed medium containing Lactose monohydrate 40 g, corn steep liquor (CSL) 20 g, $NaNo_3$ 3 g, KH_2PO_4 0.5 g, $MgSO_4$. H_2O 0.25 g dissolved in distilled water and the volume was increased to 1 liter and pH was fixed at 7.0 by using 1mol l^{-1} NaOH. A spore suspension (usually containing 5 ml spores) was inoculated into the seed medium. In pre-cultivation step, the seed flasks were incubated on a rotary shaker (200 rpm with describing a circle of 2-in diameter) for 72 h. In order to compare the growth rate and penicillin induction between the above mentioned medium and our proposed media containing various amounts of molasses, cultivation was performed at 25 ° C for about 40-48 h. Five millilitres of the vegetative growth were used to inoculate into 50 ml of batch fermentation medium.

2.3 Fermentation conditions

All fermentations were carried out in triplicate on the rotary shaker at 25 ° C in 250 ml Erlenmeyer flasks containing 50 ml of medium. The reported data in all curves for cell growth and penicillin production were as average values obtained for three flasks. The composition of the fermentation medium was same as that described in seed medium. For penicillin G production phenyl acetic acid (PAA) as precursor was added. Our preliminary obtained results showed us that addition of PAA is more effective after 48 hr of starting time. The final concentration of PAA in culture was about 0.4 g l^{-1}.

3. Analytical methods

Samples of the broth were taken from the fermentations at desired intervals and after filtration; certain other analyses were made on the broth.

3.1 Dry biomass

Dry biomass was measured as the dry cell weight (DCW) per ml of culture. Biomass was estimated gravimetrically after filtration through Whatman No. 1 washed thoroughly with water. The cells were dried at 80 ° C for 24 h, and dry cell weight was determined.

3.2 pH

The pH of each culture sample was measured with a glass electrode before any analysis was performed. This pH was reported as an initial pH of the solution.

3.3 Antibiotic assay

The concentration of penicillin in culture broth was determined by Cylinder-Plate assay as described in USP method (Parkway and Rockvilla 2007). *Bacillus subtilis* ATCC 6633, was used as the test organism for a biological assay and thus penicillin production reported in U ml^{-1}, 1 mg of penicillin is corresponded to approx. 1670 units.

3.4 Total carbohydrate assay

The method of Lane-Eynon was used to measure the total sugars (Lane and Eynon 1968).

3.5 Total nitrogen assay

Nitrate nitrogen as total nitrogen present in fermented broth was measured by persulphate method (Clesceri et al. 1999).

4. Results and discussion

4.1 Effect of molasses substitution with lactose in fermentation medium on the cell growth and penicillin yield

In this study, cultures prepared with molasses as sole carbon source were used to cultivate the *P. chrysogenum* and two main parameters; dry cell weight and penicillin yield were measured and the obtained results were compared with control culture contained lactose as the sole source of carbon. As shown in Fig. 1, the penicillin production showed a trend of steady increase during the fermentation process in control culture. However, with molasses as sole carbon source, although a steady increase in penicillin production is observed during the initial step of fermentation however, after 72 hr of incubation, penicillin production is levelled off and started to decrease gradually until the end of process. The results indicated that substitution of lactose with molasses would result in 70% lower penicillin yield than control culture. It is also obvious in Fig. 1 that mycelium concentrations (DCW) in fermentation in which molasses was utilized as sole carbon source, was increased. It is apparent that addition of molasses helped the mycelium growth. Molasses is about 50% sugar by dry weight, predominantly sucrose, but also contains significant amounts of glucose and fructose. These sugars are readily utilizable carbohydrate, which support more rapid mycelium growth than does lactose (Davey and Johnson, 1953; Hosler and Johnson, 1953; Matelova et al., 1972). Sucrose can be easily consumed by *P. chrysogenum*, therefore the most of the sugar content in fermentation medium is used for cell growth (Jarvis and Johnson, 1947). However, in control culture containing lactose, which is known as slowly utilized carbohydrate (Davey and Johnson, 1953; Soltero and Johnson, 1953), the consumption rate was lower, which would result in lower dry cell weight and lower cell growth. Since the cell growth rate and the amount of carbon source are the most important affecting parameters on the penicillin yield in *P. chrysogenum* fermentation, increasing of cellular biomass would incredibly increase the apparent viscosity of the cultivation medium as well as the mass transfer resistance in aeration process, which would result in lower penicillin yield. The drop in mycelia weight occurs near the time of carbohydrate exhaustion.

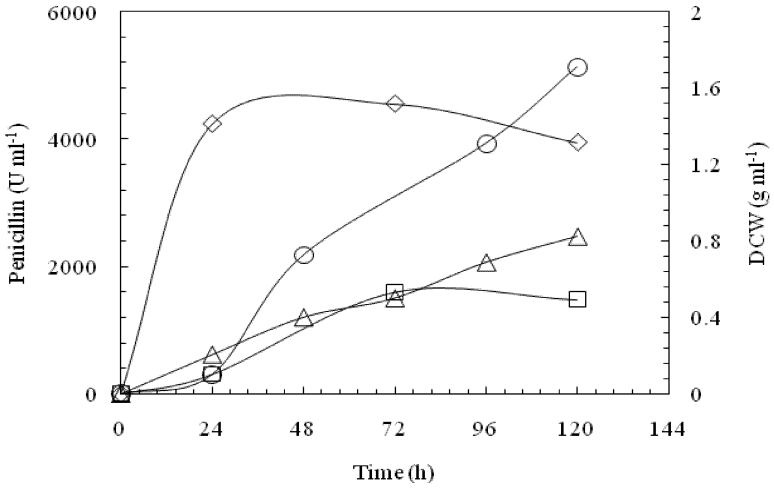

Figure 1. The effect of molasses substitution as carbon source on penicillin yield. With molasses addition, the mycelium concentration was enhanced, but amount of penicillin was decreased. Data were the average of 3 batches. Penicillin production (○, control; □, molasses), DCW (△, control; ◇, molasses).

4.2 Effects of molasses composition in medium on the penicillin production

It has been realized for some time that a maximum rate of penicillin production is obtained only under fermentation conditions which support a very slow rate of growth (Davey and Johnson, 1953). Although conditions which support a very low mycelia growth rate are desirable for penicillin formation, the growth phase of the fermentation should be characterized by the rapid development of a high concentration of mycelium. Thus, an ideal medium should support two distinct growth rates: a rapid rate throughout the growth phase and a much slower rate during the remainder of the fermentation. We postulated that the combined use of readily available carbohydrate, such as glucose which easily supplied by molasses, with slowly fermented carbohydrate, such as lactose, would be probably more beneficial to penicillin production. To examine this hypothesis, experiments with various ratios of lactose and molasses were carried out. Total sugar in medium was kept constant in all experiments. The obtained results in these series of fermentations are summarized in Table 1. A number of conclusions may be drawn from the data. As shown in Table 1, the maximum penicillin production is achieved in lower fermentation time, when the medium contains 50% molasses. According to the Table 1, in comparison with control culture, when the culture containing 50% molasses is used for cultivation, the yield of process increased about 60% and the required time to achieve this amount of produced penicillin was decreased about 20%. These results may attract a good attention from those industries which currently deal with penicillin production in large and industrial scale especially for those factories which molasses is easily available. In previous researches, it has been reported that enzymes responsible

for the synthesis of many secondary metabolites such as antibiotics, are often depressed in the presence of excess glucose (Chang et al., 1990). Although glucose is one of the easily utilized carbon sources for growth of most antibiotic-producing microorganisms, rapid utilization of glucose often leads to reduced rates of antibiotic production. This expression is agreed with our obtained results showed in Fig. 1. Revilla et al. (1984) reported that high glucose levels repress the synthesis of several penicillin synthesizing enzymes, including ACV synthetase and isopenicillin-N-synthetase (cyclase). However, optimised ration of glucose to lactose would result in remarkable increase in penicillin yield as shown in Table 1.

Table 1. Summary of results obtained from the fermentations

Medium component	Max. penicillin production (U ml^{-1})	Time of max. yield (h)	pH at time of max. yield	DCW at time of max. yield (g ml^{-1})	Penicillin production rate* (U ml^{-1} h^{-1})	Total sugar at time of max. yield (%)	Total nitrogen at time of max. yield (mg l^{-1})
Lactose (Control)	5140	120	7.85	0.82	43	3.156	90
25% Molasses	2928	72	7.95	1.81	41	2.97	69
50% Molasses	8221	96	8.15	1.41	86	2.41	45
75% Molasses	2045	72	8.55	1.9	28	2.34	39
100% Molasses	1951	72	8.58	1.52	22	1.16	38

* Penicillin production rate = (maximum penicillin production, U ml-1)/(time to reach maximum yield, h)

5. Conclusion

As the rate of mycelium production varies directly with the rate of sugar utilization during the growth phase, the use of a mixture of glucose and lactose in the medium will produce suitable rate conditions for the fermentation. However, Lactose was known to be the best carbohydrate for penicillin production. This superiority was attributed to its slow rate of fermentation. In order to produce the optimal amount of penicillin, we chose various compositions of lactose and molasses to be used in the fermentation process. The obtained results in the present work showed that the use of 50% molasses concentration (1:1 (w/w) ratio of molasses and lactose) in preparation of medium could be considered as potential concentration for higher production of penicillin in lower fermentation time.

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Total sugars in molasses as invert sugar: Lane-Eynon constant volume volumetric method AOAC official method 968.28, 1968.