IMPROVEMENT IN AUREOFUSCIN PRODUCTION BY *Streptomyces aureofuscus* WITH THE ADDITION OF ACETATE AND PROPIONATE SODIUM

MELHORIA NA PRODUÇÃO AUREOFUSCIN POR Streptomyces aureofaciens *COM A ADIÇÃO DE ACETATO E PROPIONATO DE SÓDIO*

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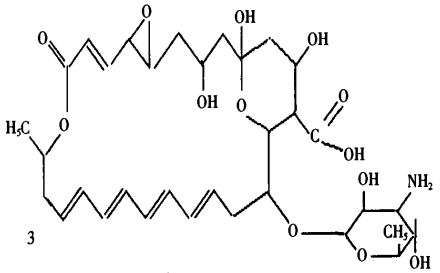
ABSTRACT: Aureofuscin is an important tetraene macrolides antibiotic produced in submerged culture by *Streptomyces aureofuscus* isolated from the soil in China. In the present work, the effects of the addition of precursor on cell growth and the kinetics of aureofuscin production were investigated during submerged cultivation of *Str. aureofuscus*. The sodium acetate and sodium propionate are more suitable precursor than alcohol, sodium butyrate, n-propanol, n-butanol. The addition of acetate and propionic sodium at a ratio of 5:1 at a total concentration of 1.5mg mL^{-1} after 24 h showed stimulatory effects on aureofuscin production, reaching 3.708 mg mL^{-1} (approximately 267% increases in aureofuscin production, compared with the control culture). Moreover , A further enhancement in aureofuscin production was achieved by cultivation in a 5-L stirred-tank bioreactor under controlled pH conditions under the above optimum condition. The optimal fermentation conditions were that 4L/min ventilatory capacity, 220r/min rotational speed, dissolves oxygen not be lower than 20%, fermentation period 84h and pH should control nearby 5.5, and the maximum yield of aureofuscin of 3.99 mg mL^{-1} was achieved after 84 h When the sodium acetate and sodium propionate were added as a mixture of acetate and propionate at a ratio of 5:1 and a final concentration of 1.5 mg mL^{-1} after 24 h cultivation.

KEYWORDS: Aureofuscin. Precursor. *Streptomyces aureofuscus.* Antibiotic production.

INTRODUCTION

Aureofuscin is an important antifungal antibiotic produced by *Streptomyces aureofuscus* isolated from the soil in China. Its structure is similar to natamycin (natamycin = pimaricin). Both are tetraene macrolides antibiotic. Figure1 shows the Chemic structure of Aureofuscin and Natamycin. However, aureofuscin has more antibacterial activity than natamycin against *Saccharomyces cerevisiae*, *Aspergillus niger, Penicillium chrysogenum*, and *Fusarium.* Aureofuscin has the chemical formula $C_{25}H_{37}NO_{10}$, a molecular mass of 511(IPRS 1975).



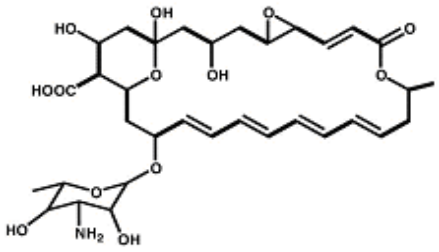


Figure 1-1. Chemic structure of Aureofuscin Figure 1-2: Chemic structure of Natamycin

Aureofuscin belongs to the polyene antibiotic group and the biosynthesis of the aureofuscin basic chain follows the biosynthesis of fatty acids, which is composed of the decarboxylative condensation of simple carboxylic acids (APARICIO et al 2004). Therefore, the addition of low-molecular weight precursors such as carboxylic acids and amino acids was considered to be an inexpensive, scalable and easy approach to increase the production of different antibiotics, such as in the case of rapamycin(CHENG YR et al 1995), tylosin(NGUYEN et al 1995), pneumocandin (Petersen et al 2001), rampolanin (BRUNATI et al 2005), glycopeptide antibioticA40926 (JOVETIC et al 2008), bitespiramycin (LI ZL et al 2009), and natamycin (ELSAYED et al *2013*). To our knowledge, natamycin has been extensively used as a food preservative. However, aureofuscin has not been well researched or industrially developed because of its low yields from fermentation since 1975. Therefore, if the yield of aureofuscin could be enhanced, it could be a promising antifungal agent to be used industrially as a food preservative.

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In this study, we attempt to increase the production of aureofuscin by adding precursors. We investigated the effect of concentration and adding time of sodium acetate, sodium propionate, sodium butyrate, alcohol, n-propanol on aureofuscin production. After full optimization of the precursor addition strategy, the optimal fermentation condition (stir speed, the kinetics of cell growth and aureofuscin production) was investigated to increase the production of aureofuscin.

MATERIALS AND METHODS

Microorganisms and Cultivation conditions

Str.aureofuscus was obtained from the strain preservation center in Shenyang Agricultural University (strain numbers SYAU0709).

Str.aureofuscus was incubated at 29◦C.

2×YT medium, yeast extract/malt extract liquid medium (YEME), and agar minimal medium (MM) were prepared as described. Yeast extract/amidulin (YSA) medium was used for aureofuscin fermentation (J. Wei et al 2011).

Sample preparation and dry cell weight determination

Sample was 10 mL fermentation broth in the case of bioreactor cultures, it was in the form of flask. All samples were taken at 72h during cultivation and were centrifuged at 4000 rpm for 20 min. The supernatant was frozen at −20◦C for antibiotic determination, and the remaining centrifuged cells were washed twice using distilled water, followed by centrifugation. The centrifuge tubes were then dried to a constant weight at 80◦C to determine the dry cell weight.

Aureofuscin bioassay and HPLC analysis (JIE WEI et al 2008)

For the detection of aureofuscin, the maximum absorption wavelength was measured from 200nm to 400 nm with an ultraviolet spectrophotometer. To analyze aureofuscin, a 1 ml portion of the culture broth was extracted by adding 9 ml of methanol, shaking vigorously, centrifuging at high speed (9200g,10000 r/min, 10 min), and saving the supernatant.

High Performance Liquid Chromatography (HPLC) analysis was performed using a Waters C18 column as the stationary phase and methanol-water-glacial acetic acid (48:32:1, v/v) as the mobile phase. The flow rate was 1.0 ml/min, UV wavelengh at 302.5 nm. A known, pure sample of aureofuscin was used as the internal standard.

RESULTS AND DISCUSSION

Effect of the different addition on aureofuscin production

In this experiment, six parallel cultivations were conducted to investigate the effect of the different addition on aureofuscin production. The sodium acetate, sodium propionate, sodium butyrate, alcohol, n-propanol, n-butanol were added separately at the beginning of the cultivation time at concentrations 0.5mg mL^{-1}. Figure2 indicates that aureofuscin production increased upon supplementation of the fermentation medium with sodium acetate or sodium propionate.

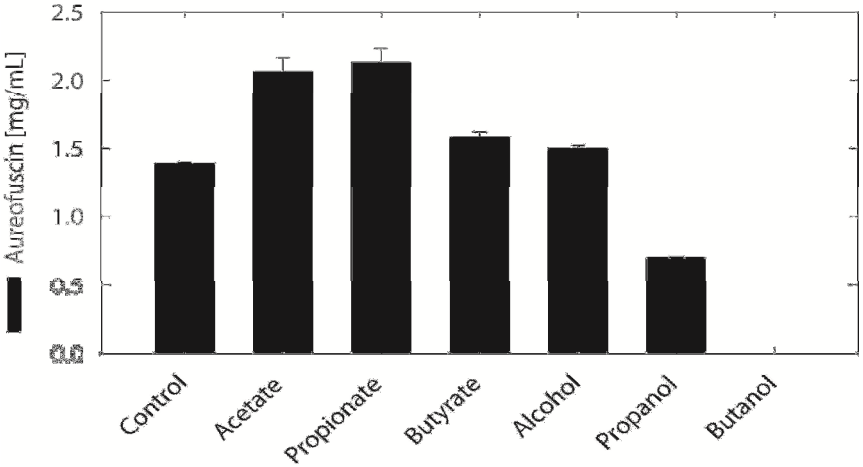

Figure 2. Effect of different addition on aureofuscin production

The maximal aureofuscin production of 2.133 mg mL^{-1} was achieved in sodium propionate supplemented culture. The addition of acetate to the fermentation medium also showed a stimulatory effect on aureofuscin production. The maximal aureofuscin production of 2.065 mg $mL^{-1}$was obtained. On the other hand, the stimulatory effect of butyrate and alcohol on volumetric aureofuscin

production was much lower compared with acetate and propionate. The specific aureofuscin production was lower for n-propanol and n-butanol than the control. Based on these results, the addition of sodium acetate or sodium propionate to the fermentation medium at a low concentration of 0.5mg mL^{-1} was chosen as the best precursors for aureofuscin production in the subsequent experiments.

Effect of the addition concentrations on aureofuscin production

To better understand the stimulatory effect of different carboxylic acids on aureofuscin production, the antibiotic-specific production yields are reported in Figure 3. As shown, the yield of aureofuscin production increased gradually by increasing either sodium acetate or sodium propionate concentrations in the culture from 0.5 to 3 mg mL^{-1} and decreased thereafter. The increase in aureofuscin production in acetate- and propionate-supplemented cultures was not related to the change in the biomass but to the increased cell productivity. The maximal values of aureofuscin prouction of 3.125 mg mL^{-1} and 3.247 mg mL^{-1} in cultures supplemented with 1.5 mg mL^{-1} sodium acetate and sodium propionate, respectively, were higher than those reported in previously published work using the same strain in batch culture under optimal cultivation conditions in medium without carboxylic acid addition (2.94 mg mL^{-1} and 2.897 mg mL^{-1}) (WANG HUA et al 2007; JIE WEI et al 2009).

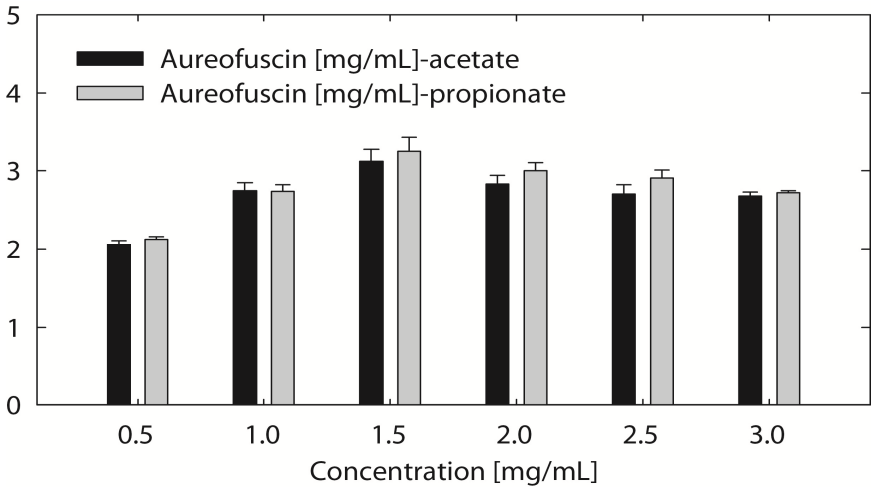

Figure 3. Effect of the addition concentrations of sodium acetate and sodium propionate on aureofuscin production

Addition at different sodium acetate: sodium propionate ratios

Experiments were conducted to investigate the effect of concomitant addition of acetate and propionate at different ratios (sodium acetate: sodium propionate ratios ranging from 1:9 to 9:1). The concentration of total acetate and propionate supplemented for all ratios used was 1.5 mg mL^{-1}. As shown in Figure4, cell growth decreased gradually when the acetate ratio in the mixture was increased from 1:9 to 9:1. On the other hand, the effectiveness of acetate and propionate supplementation on aureofuscin production was more pronounced at all applied ratios when a mixture was used compared with single acetate and propionate experiments. The maximal aureo fuscin production of 3.575 mg $mL^{-1}$was achieved in culture supplemented with sodium acetate: sodium propionate at a ratio of 5:1. This value was higher than the control culture by approximately 157% and was also higher than in cultures individually supplemented with acetate or propionate by approximately 14% and 9.9%, respectively.

The observed stimulatory effects of the addition of a carboxylic acid mixture of acetic and propionic acids at a ratio of 7:1 were also reported in the previous study of Elsayed Ahmed et al.,in which acetate-propionate increased natamycin production in a submerged culture of *Str. natalensis* by approximately 150% (3.98 g L^{-1})when added to the production medium at a concentration of 2 g L^{-1}.

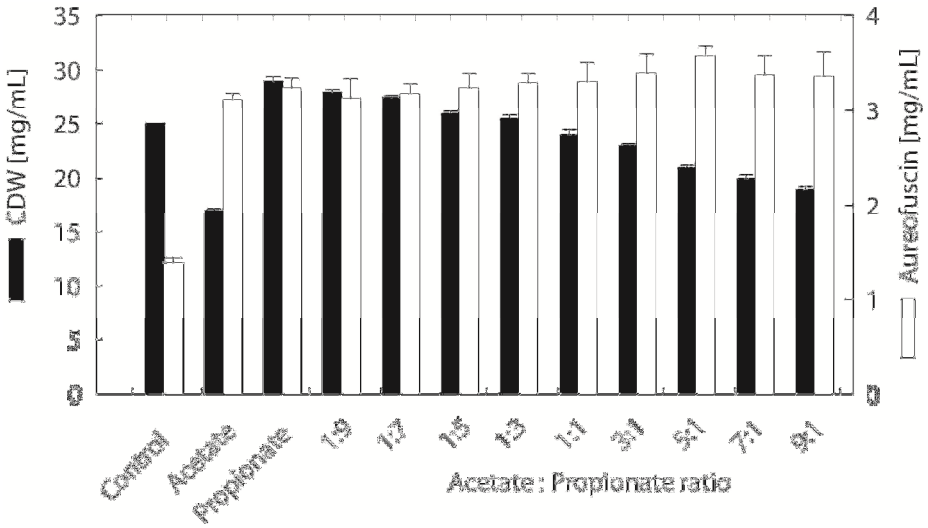

Figure 4. Effect of different sodium acetate : sodium propionate ratios on cell growth and aureofuscin production by *Str. aureofuscus* in shake-flask cultivation.
(Both acetate-propionate addition were added simultaneously as a mixture to a final concentration of 1.5 mg mL^{-1}at the beginning of the cultivation time before inoculation.)

Effect of the addition time of acetate and propionate on aureofuscin production

In addition to the effect of the ratio of acetate: propionate ratios on aureofuscin production, the time of addition also played a significant role in this enhancement effect. The maximal yield of aureofuscin production was achieved when sodium acetate and sodium propionate (ratio 5:1) were added at the beginning or during the first 24 h of cultivation. If sodium acetate and sodium propionate were added after 72 h (during the late exponential growth phase-early stationary phase), the positive effect of the addition of acetate and propionate on aureofuscin biosynthesis was almost negligible (Figure 5). The maximal aureofuscin production of 3.708 mg mL^{-1} was achieved when the acetate-propionate mixture was added after 12h. However, cell growth was slightly influenced by the addition of sodium acetate-propionate. It was clearly observed that the earlier the addition time, the higher the negative effect on biomass production. Once cells entered the stationary phase (after 72 h), no effect of acetate-propionate mixture on biomass was observed.

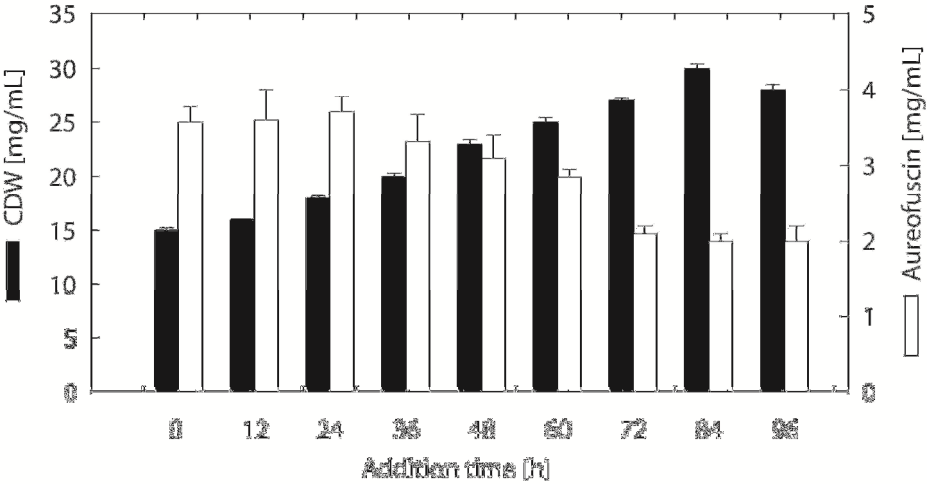

Figure 5. Effect of addition time on cell growth and aureofuscin production by *Str. aureofuscus* in shake-flask cultivation. (The sodium acetate and sodium propionate were added as a mixture of acetate and propionate at a ratio of 5:1 and a final concentration of 1.5 mg mL^{-1}.)

The results also showed that to achieve the stimulatory effect of acetate-propionate mixture, they should be added to the cultivation medium during the first 24h of cultivation. This maybe because the aureofuscin biosynthesis in this strain started during the exponential growth phase parallel

to cell growth. This was attributed to the fact that propionic acid needs to be converted into propionyl-CoA to act as a precursor for polyene ring synthesis. The enzyme system controlling this step is more active during the exponential growth phase (JING K et al 2011; BIBB MJ 2005; GIL; CAMPELO DIEZ 2003; MARTIN JF; MCDANIEL LE 1976).

Aureofuscin production during batch cultivation in a 5-Lstirred-tank bioreactor with and without sodium acetate-propionate addition

Based on the above studies, cultivations were transferred to bioreactor level to investigate the possibility of industrialization of this process. Medium composition and cultivation conditions were the same as in shake flasks. The pH of the culture was controlled at 7.2 at the beginning by the addition of NaOH to the culture medium. Figure 6 shows cell growth, aureofuscin production, pH value, dissolved oxygen (DO) and reducing sugar (%) in culture medium in a 5-L bioreactor. During cultivation without sodium acetate-propionate addition, the maximal cell dry weight (CDW) was 32 mg mL^{-1}. This value was higher than the biomass obtained in sodium acetate-propionate supplemented culture by approximately 45%.

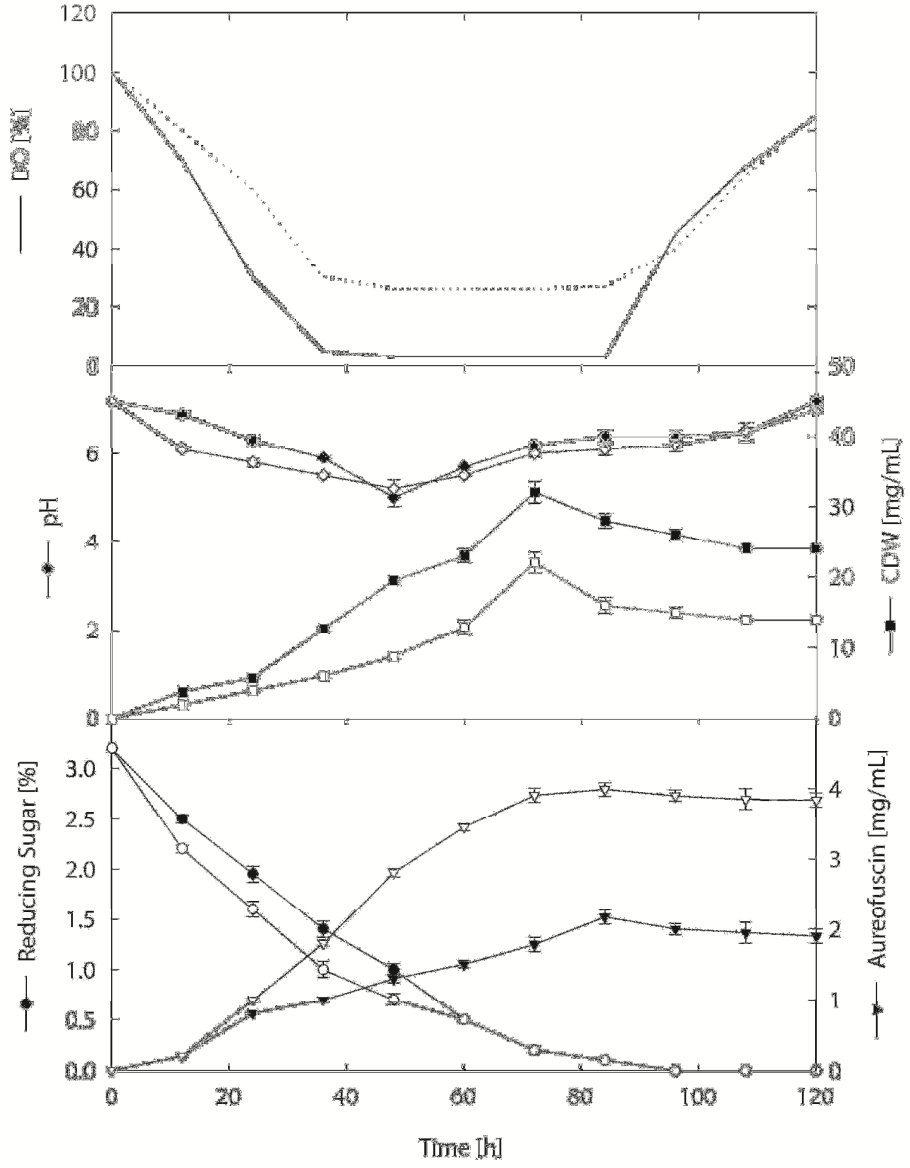

Figure 6. Kinetics of cell growth and aureofuscin production by *Str. aureofuscus* in a 5-L bioreactor (working volume 3 L, agitation 220 rpm, aeration 1 v $v^{-1}min^{-1}$, temp.29◦C) with and without sodium acetate-propionate. (Closed and open symbols correspond to results obtained in cultivations without and with sodium acetate-propionate addition, respectively. The sodium acetate and sodium propionate were added as a mixture of acetate and propionate at a ratio of 5:1 and a final concentration of 1.5 mg mL^{-1} after 24 h cultivation.).

The maximal value of aureofuscin in acetate-propionate supplemented culture was 3.99 mg mL^{-1} after 84 h (approximately 183% higher than the control culture). The increase in aureofuscin production was directly related to higher cell productivity in the sodium acetate-propionate supplemented culture. However, the changes in the pH in both cultures were similar. The pH value decreased gradually from 7.2 to approximately 5.2 during the first 48h of cultivation time and increased again gradually to neutral pH after 120 h. During the growth phase, the pH drop as a result of excretion of TCA organic acids such as pyruvic ,α- ketoglutaric and lactic acids in low concentration. The excess carbon in medium could be used to produce organic acids if not oxidized aerobically to CO_2 for energy production (El-Enshasy et al 2003,2008; Ayar-Kayali H and Tarhuan L 2006). In the subsequent incubation period, the pH increased gradually as a result of the decrease of cell growth and the increase in aureofuscin production.

In both cultures, the reducing sugar concentration in the bioreactor decreased at a faster rate and the reducing sugar was completely consumed after 84 h. This was because bioreactor cultivation provides better oxygen availability in the cultivation vessel due to the continuous aeration and better agitation; this increased oxygen availability is positively reflected on the physiological activity of aerobic microorganisms. The higher cell metabolic activities led to higher cell growth and antibiotic production rates. In general, the changes in dissolved oxygen DO in both cultures were similar, decreasing successively during the exponential growth phase (the first 72h of cultivation), increasing gradually thereafter and reaching approximately 85% saturation by the end of the cultivation.

From the obtained results, we infer that the addition of a mixture of acetate and propionate sodium at a ratio of 5:1 in a total concentration of 1.5 mg mL^{-1} at the beginning 24h of the cultivation is an easily applicable and inexpensive strategy to increase aureofuscin production in submerged culture by *Str.aureofuscus*. This approach could also be scalable for large-scale production of aureofuscin as it also showed higher aureofuscin yields in small-scale bioreactor culture. Production of other polyene antibiotics, which share the same pathway of polyene ring synthesis via the condensation of short-chain carboxylic acids, could also be improved through the addition of a short-chain carboxylic acid mixture as shown in this research. To the best of our knowledge, this is the first report using an acetate-propionate mixture as a precursor to enhance aureofuscin production in *Str.aureofuscus* at both the shake-flask and bioreactor levels.

Significance and Impact

The production of aureofuscin is very low in the wild-type strain. We attempt to increase the production of aureofuscin with adding precursors. To our knowledge, this approach has not been attempted in *S. aureofuscus* before.

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Conflict of Interest

There is no potential conflict of interest to declare.

RESUMO: Aureofuscin is an important tetraene macrolides antibiotic produced in submerged culture by *Streptomyces aureofuscus* isolated from the soil in China. In the present work, the effects of the addition of precursor on cell growth and the kinetics of aureofuscin production were investigated during submerged cultivation of *Str. aureofuscus*. The sodium acetate and sodium propionate are more suitable precursor than alcohol, sodium butyrate, n-propanol, n-butanol. The addition of acetate and propionic sodium at a ratio of 5:1 at a total concentration of 1.5mg mL^{-1} after 24 h showed stimulatory effects on aureofuscin production, reaching 3.708 mg mL^{-1} (approximately 267% increases in aureofuscin production, compared with the control culture). Moreover , A further enhancement in aureofuscin production was achieved by cultivation in a 5-L stirred-tank bioreactor under controlled pH conditions under the above optimum condition. The optimal fermentation conditions were that 4L/min ventilatory capacity, 220r/min rotational speed, dissolves oxygen not be lower than 20%, fermentation period 84h and pH should control nearby 5.5, and the maximum yield of aureofuscin of 3.99 mg mL^{-1} was achieved after 84 h When the sodium acetate and sodium propionate were added as a mixture of acetate and propionate at a ratio of 5:1 and a final concentration of 1.5 mg mL^{-1} after 24 h cultivation.

PALAVRAS-CHAVE: Aureofuscin. Precursor. *Streptomyces aureofuscus.* Antibiotic production.

REFERENCES

APARICIO, J. F.; MENDES, M.V.; ANTÓN, N.; RECIO, E.; MARTIN, J.F. Polyene macrolide antibiotic biosynthesis. **Current Medicinal Chemistry**, v. 11, p. 1645-1656, 2004. http://dx.doi.org/10.2174/0929867043365044

Ayar-Kayali, H.; Tarhuan, L. Vancomycin antibiotic production and TCA-glyoxalate pathway depending on the glucose concentration in Amycolatopsisorientalis. **Enzyme and Microbial Technology**, v. 38, p. 727-734, 2006. http://dx.doi.org/10.1016/j.enzmictec.2005.07.023

BIBB, M. J. Regulation of secondary metabolism in Streptomycetes. **Current Opinion Microbiology**, v. 8, p. 208-215, 2005. http://dx.doi.org/10.1016/j.mib.2005.02.016

Brunati, M; Vava, A; Marinelli, F; Lancini, G. Influence of leucine and valineon ramoplanin production by Actinoplanes sp. ATCC 33076. **Journal of Antibiotics,** v. 58, p. 473-478, 2005. http://dx.doi.org/10.1038/ja.2005.63

Cheng, Y. R; Fang, R; Demain, A. L. Effect of amino acids on rapaymcin biosynthesisby Streptomyces hygroscopicus. **Applied Microbiology Biotechnology**, v. 43, p. 1096-8, 1995. http://dx.doi.org/10.1007/BF00166931

El-Enshasy, H. A; Abdel Fattah, Y; Atta, A; Anwar M; Omar H; Abou El Magd S, et al. Kinetics of cell growth and cyclosporine A production by Tolypocladiuminflatum during process scaling up from shake flask to bioreactor. **Journal of Microbiology Biotechnology**, v. 18, p. 128-134, 2008.

El-Enshasy, H. A; Beshay, U. I; El-Diwany, A. I; Omar, H. M; El-Kholy, A. E; El-Najar, R. Improvement of rifamycins production by Amycolatopsis mediter-ranei in batch and Fed-batch cultures. **Acta Microbiologica Polonica**, v. 51, p. 301–313, 2003.

HA, EL-E.; NA, M.; MA, F.; AI,EL-D. Improvement of erythromycin production by Saccharopolyspora erythraea in molassesbased medium through cultivation medium optimization. **Bioresource Technology**, v. 99, p. 4263-4268, 2008. http://dx.doi.org/10.1016/j.biortech.2007.08.050

Elsayed Ahmed Elsayed; Mohamed Abdel Fattah Farid; Hesham Ali El Enshasy. Improvement in natamycin production by Streptomyces natalensiswith the addition of short-chain carboxylic acids. **Process Biochemistry**, v. 48, p. 1831-1838, 2013. http://dx.doi.org/10.1016/j.procbio.2013.09.009

GIL, J.; CAMPELO-DIEZ, A. Candicidin biosynthesis in Streptomyces griseus. **Applied Microbiology Biotechnology**, v. 60, p. 633-642, 2003. http://dx.doi.org/10.1007/s00253-002-1163-9

Institute of Pharmaceutical Research in Shanghai (IPRS) in China. Aoriginal antimycotic antibiotic-Aureofuscin. **China Journal Microbiology**, v. 15, p. 180-187, 1975.

WEI, J. , MENG, X-j. , LI, H. Extraction and Identification of Aureofuscin from Fermentation Broth. **China Food Science**, v. 29, n. 10, 254-256, 2008.

WEI, J.; MENG, X-j , JIANG, Q.; LIU, H. Study on the Breeding of High Aureofuscin Producing Strains and Their Fermentation Conditions. **Journal of Food Science and Biotechnology (China)**, v. 28, n. 2, 240- 242, 2009.

Jing, K.; Hao, X.; Lu, Y. Effect of propionate on the production of natamycin with Streptomyces gilvosporeus XM-172. **African Journal of Biotechnology**, v. 10, p. 10030-10033, 2011.

JOVETIC, S.; FEROGGIO, M.; MARINELLI, F.; LANCINI, G. Factors influencing cell fatty acidcomposition and A40926 antibiotic complex production in Nonomuraea sp.ATCC 39727. **Journal of Industrial Microbiology Biotechnology**, v. 35, p. 1131-1138, 2008. http://dx.doi.org/10.1007/s10295-008-0392-z

WEI, J.; MENG, X.; WANG, Q. Enhanced Production of Aureofuscin by Over-expression of AURJ3M,Positive Regulator of Aureofuscin Biosynthesis in Streptomyces aureofuscus. **Letters in Applied Microbiology**, v. 52, n. 4, p. 322-329,2011. http://dx.doi.org/10.1111/j.1472-765X.2011.03003.x

Li, Z. L.; Wang, Y. H.; Chu, J.; Zhuang, Y. P.; Zhang, S. L. Effect of branched chain aminoacids, valine, isoleucine and leucine on the biosynthesis of bitespiramycin 4-O-acylspiromycins. **Brazil Journal Microbiology**, v. 40, p. 734-746, 2009. http://dx.doi.org/10.1590/S1517-83822009000400003

Martin, J. F.; McDaniel, L. E. Biosynthesis of candicidin by phosphate limited restingcells of Streptomyces griseus. **Europe Journal of Applied Microbiology**, v. 3, p. 135-144, 1976. http://dx.doi.org/10.1007/BF00928432

Nguyen, L. T.; Nguyen, K. T.; Spizek, J.; Behal, V. The tylosin producer, Streptomyces fradiae, contains a second valine dehydrogenase. **Microbiology**, v. 141, p. 1139-1145, 1995. http://dx.doi.org/10.1099/13500872-141-5-1139

PETERSEN, L. A.; Hughes, D. L.; Hughes R.; DiMichele, L.; Salmon, P. Connors N. Effects of amino acid and trace element supplementation on pneumocandinproduction by Glarea lozoyensis: impact on titer, analogue levels, and the identification of new analogues of pneumocandin B0. **Journal of Industrial Microbiology Biotechnology**, v. 26, p. 216-221, 2001. http://dx.doi.org/10.1038/sj.jim.7000115

HUA, W.; YONGSHENG, G. Optimization of Fermentation Condition for Aureofuscin. **Acta Agriculturae Universitatis Jiangxiensis (China)**, v. 29, n. 2, 292-296, 2007.