THE GLUCOSE EFFECT ON LINCOMYCIN PRODUCTION BY STREPTOMYCES LINCOLNENSIS VAR. LINCOLNENSIS DSM 40 355 ON SYNTHETIC MEDIA

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ABSTRACT

The objective of this study was to obtain the information about the lincomycin production by type strain Streptomyces lincolnensis var. lincolnensis DSM 40 355 on the synthetic medium. In order to improve the productivity, the ways of inoculation, effects of the medium components and fermentation time were investigated. The production of lincomycin was carried out at 30 °C on rotary shakers at 180 rpm and was tracked on 3rd, 7th, 10th, 12th, 14th, and 16th day of cultivation by using HPLC analysis. The results show that the highest production was observed on 16th day of cultivation on production M2 medium at 30 ± 1 °C, after inoculation with 1 ml of preculture M1 medium, and after an additional supplementation of the media with 0.5 g of glucose per 50 ml medium on 7th day of cultivation. Preculture medium application and glucose addition had positive effect, resulting in a remarkable increase in the lincomycin production.

INTRODUCTION

Actinomycetes are important soil microorganisms. They can produce different kinds of secondary metabolites and are the main strains of producing biological active substances. Interestingly, the majority of the antibiotic-producing actinomycetes belong to the genus Streptomyces, which led to a growing economic importance for this group of organisms (Adegboye and Babalola, 2012). Secondary metabolites production in microbes is strongly influenced by nutritional factors and growth conditions. It is necessary to optimize each and every component of fermentation media by varying the concentration of media constituents in order to achieve the maximum antibiotic production. The manipulation of growth conditions of microorganisms is a common strategy used by pharmaceutical companies to improve the quantities and spectra of secondary metabolites with potential therapeutic interest (Tormo et al., 2003). Scientific and rational optimization of fermentation process can not only greatly improve the levels of eventual products but also reduce the fermentation costs. Actinomycetes fermentation is a complex of processes, it not only depends on the performance and fermentation medium, also requires the suitable environmental conditions (such as inoculation volume, medium capacity, fermentation time, temperature, agitation rate and initial pH). These factors may affect the antibiotics production (Song et al., 2012). The medium composition and ratio significantly affected fermentation process. Suitable nutrients can promote the synthesis of metabolites, cell growth, antibiotic fermentation unit, antibiotics extraction process, etc. (Kiers et al., 2000; Palmqvist and Hahn-Hagerdal, 2000).

Lincomycins form a small yet clinically important group of antibiotics. Lincomycin, natural lincosamide antibiotic, was firstly reported and isolated in 1962 from a soil actinomycete found near Lincoln, Nebraska, which gave origin to its name (Stratton, 1998; Špižek et al., 2004). This new streptomycte species was designated as Streptomyces lincolnensis var. lincolnensis DSM 40 355. As a lincosamide antimicrobial agent, lincomycin has gained clinical acceptance as a major antibiotics for the treatment of diseases caused by Gram-positive microbes, such as staphylococci and streptococci (Ye et al., 2009).

Various vegetable oils, natural nitrogen sources, and surfactants were investigated for efficient lincomycin production from Streptomyces lincolnensis (Choi and Cho, 2004). The aim of this study was to determine whether the glucose has effect on lincomycin production by type strain of Streptomyces lincolnensis var. lincolnensis DSM 40 355 on the synthetic media.

MATERIALS AND METHODS

Production strain

A type strain of Streptomyces lincolnensis var. lincolnensis DSM 40 355 (DSMZ, Germany) was used in this study.

Cultivation media and cultivation conditions

Spor suspensions of S. lincolnensis var. lincolnensis DSM 40 355, were inoculated into preculture M1 medium adopted from Nechaeva et al. (1974). This medium contained (per l): 1 g K2HPO4; 4 g NH4NO3; 8 g CaCO3; 0.05 g MnSO4; 0.02 g ZnSO4; 0.05 g FeSO4; 1 g MgSO4; 8 g glucose. The precultures were conducted in 150 ml Erlenmeyer flasks at 30 °C on rotary shakers at 180 rpm for 120 h and inoculated into the desired production medium. The production M2 medium (Wyss et al., 2001), synthetic minimal medium (carbon limited medium), used for lincomycin production contained (per l): 3.12 g KNO3; 750 mg KH2PO4; 750 mg KH2PO4; 500 mg MgSO4·7H2O; 100 mg NaCl; 100 mg CaCl2·2H2O; 0.396 g ZnSO4·7H2O; 0.079 mg CuSO4·5H2O; 0.0405 mg MnSO4·7H2O; 0.0175 mg MoO3; 0.0375 mg Na2B4O7·10H2O; 0.5355 mg FeSO4·7H2O; 5 g glucose. Production was carried out at 30 °C in 250 ml Erlenmeyer flasks on rotary shakers at 180 rpm on 3rd, 7th, 10th, 12th, 14th, and 16th day.

Inoculation experiments

In the first case, 1 ml of spore suspension of Streptomyces lincolnensis var. lincolnensis DSM 40 355 was used to inoculate 50 ml of preculture medium with 1 % of glucose. After 120 h of growth, 1 ml of preculture medium was used to inoculate the production medium (50 ml) with 2 % of glucose. In the second case, 1 ml of spore suspension of study strain S. lincolnensis var. lincolnensis DSM 40 355 was inoculated directly into the 50 ml of the production medium with 2 % of glucose.
Determination of pH and mycelia concentration (dry mycelia weight-DMW)
Values of pH were determined using a VARIO pH meter (WTW Wissenschaftlich-Technische Werkstätten GmbH, Germany). To measure the mycelia concentration in liquid medium after incubation, the cultures were autoclaved at 121 °C for 16 min to deactivate the actinomycete. DMW were obtained by harvesting the spores on pre-activated (at 80°C for 16 h), and pre-weighted filter paper (Qualitative filter papers, Grade 1288; Sartorius Stedim Biotech). The filtered biomass (mycelia mass) was dried at 80 °C for 16 h and weighed again. The weight of the dried biomass was then determined as a difference between initial and final weight, and determined as dry weight.

Quantitative determination of lincomycin production (HPLC conditions)
Samples were analysed according to a method adopted by Douša et al. (2006). HPLC analysis was performed on a Gemini NX C18 column, 100 mm x 4.6 mm, 3µ (Phenomenex), using an Ultimate 3000 HPLC-system (Dionex) with UV detection at a wavelength of 210 nm. The mobile phase was constituted by solvent A, 2 mM ammonium formate in water (pH was adjusted to 9 with ammonium hydroxide), and solvent B, acetonitrile. A linear gradient program was setup with 0-1.4 min 10 % B, 1.4-5.4 min 10-30 % B, then held at 30 % B for 4 min before coming back to 10 % B in 0.1 min (the HPLC column was reconditioned at 10 % B for an additional 3 min). The flow rate was 1.0 ml.min⁻¹, and 20 µl of the sample was injected onto the column. Data processing was carried out using Chromelieve software 6.8. Samples in a total amount of 1 ml were taken at a particular sampling period (3th, 7th, 10th, 16th and 16th) day from a cultivation medium and centrifuged for 5 minutes at 13 000 rpm (Biofuge pico, Heraeus Instruments) in order to get cell free supernatant. The supernatant was then filtered through a filter (13 mm Syringe Filter w/0.2 µm PTFE, VWR International, USA), and 500 µl of supernatant was directly used for HPLC analysis.

Lincomycin production on M2 medium
Based on the results obtained from HPLC analysis (a previous study and data not shown), the production of lincomycin on the M2 medium inoculated by 1 ml of M1 medium (seed medium) resulted in better yields in production of lincomycin. In the second trial a glucose quantity was changed and consequently compared with the other fermentations. The production of lincomycin was monitored after single adding 0.5 g of glucose on 7th day cultivation (feed batch). Cultures were cultivated under the fermentation conditions as stated above.

RESULTS AND DISCUSSION

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>M2 medium</th>
<th>M1/M2 medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH (g.l⁻¹)</td>
<td>HPLC (µg.l⁻¹)</td>
</tr>
<tr>
<td>0</td>
<td>6.46 ± 0.11</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>6.72 ± 0.30</td>
<td>1.26 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>6.88 ± 0.25</td>
<td>2.32 ± 0.20</td>
</tr>
<tr>
<td>10</td>
<td>7.09 ± 0.40</td>
<td>3.35 ± 0.38</td>
</tr>
<tr>
<td>12</td>
<td>7.24 ± 0.37</td>
<td>3.21 ± 0.32</td>
</tr>
<tr>
<td>14</td>
<td>7.78 ± 0.83</td>
<td>2.72 ± 0.45</td>
</tr>
<tr>
<td>16</td>
<td>7.90 ± 0.89</td>
<td>2.51 ± 0.44</td>
</tr>
</tbody>
</table>

Legend: M1 medium (Nechuena et al. 1974); M2 mineral medium with glucose and salt (Wysy et al. 2001); DMW - dry mycelia weight. HPLC - High-performance liquid chromatography. Values reported for lincomycin concentrations are the averages of duplicate flasks.

Spížek and Řezanka (2004) indicated that under optimum conditions, the wild-type strain S. lincolnensis strain produced 25 mg.l⁻¹ lincomycin A and negligible amounts of lincomycin B. In contrast, the strain S. lincolnensis 78-11 is a phage-resistant, lincomycin-producer-overproducing mutant of S. lincolnensis NRRL 2936, synthesising about 2.5 g.l⁻¹ lincomycin A and B. The ability of streptomycetes cultures to form antibiotics is not a fixed property but may be greatly increased or completely lost under different conditions of nutrition and cultivation (Waksman, 1961). Therefore, the medium constitution together with the metabolic capacity of the producing organism greatly affects antibiotic biosynthesis. Overproduction of secondary metabolites includes the physiological control, e.g. feed-back inhibition, carbon and energy source regulation, nitrogen source regulation, phosphatase regulation and the effect of autoregulatory compounds. Changes in the nature and type of carbon, nitrogen or phosphate sources and trace elements have been reported to affect antibiotic biosynthesis in streptomycetes (Barratt and Oliver, 1994; Spížek and Tichý, 1995; Lounès et al., 1996; Abbanat et al., 1999). According Spížek and Tichý (1995), Demain (1998) indicated that the regulatory mechanisms governing biosynthesis of secondary metabolites are extremely complicated.

Based on the results of HPLC analysis, the type strain Streptomyces lincolnensis var. lincolnensis DSM 40 355 is able to produce lincomycin on the synthetic medium, and thus consequently, the second study was also carried out with this strain and the effect of glucose addition on the production of antibiotic was studied. As Young et al. (1985) pointed out that once growth is halted by autoregulatory compounds, changes in the nature and type of carbon, nitrogen or phosphate sources and trace elements have been reported to affect antibiotic biosynthesis in streptomycetes.
depletion of one or more nutrients, the specific rate of lincomycin production falls rapidly. That was the reason for second fed-batch experiment, a total 0.5 g of glucose per 50 ml (total per l) was fed after 7 days of cultivation. An aim was to monitor the lincomycin production by using this way of fermentation (Figure 1, 2).

Carbon sources such as corn starch, glucose, sucrose and molasses are commonly used as growth substrates to produce enzymes, antibiotics and other secondary metabolites by fermentation. At the beginning of the fifties Epps and Gale (1942) showed that the synthesis of enzymes of primary metabolism, mainly of catabolic ones, and growth are negatively affected by glucose. Magasanik (1961) called it "catabolite repression" (glucose effect) and assumed that the enzyme inhibition is induced by intermediates of a rapid catabolism of glucose.

The examples of Gram-positive bacteria subject to carbon catabolite regulation (CCR) are the actinomycetes. This group possessing a high GC content in DNA are sensitive to aminoglycoside antibiotics, which act as catabolite repression repressors of biosynthetic enzymes (Sanchez and Demain, 2002).

The results given in Figure 1 and 2 showed that glucose could play an important role in lincomycin production. Glucose addition has positive effect, resulting in a remarkable increase in the antibiotic production. A total yield of lincomycin on 10th day was 1.7-fold higher, and 1.9-fold higher compared with that of the first study. Choi and Cho (2004) found out that when olive oil was used as a sole carbon source, the lincomycin yield was about 2.0-fold higher than that using starch medium. In fact, lincomycin accumulation was increased by about 95% on M1/M2 medium in the cultivation run with the glucose additions, which sheds new lights on the strategy for improving the production of antibiotic from Streptomyces lincolnensis var. lincolnensis.

On the 12th day, the lincomycin concentration was found to be higher than maximum amount obtained in the first study. The highest lincomycin titer (29.902 μg l−1) was observed on the synthetic M2 medium inoculated by precultured M1 medium on 16th day of cultivation.

These observations suggest that in all those cases where preculture medium was used and glucose was added, yields of lincomycin were obtained earlier and were higher than those from the first run and those that did not apply further glucose addition. This can be improved by further optimization of fermentation process by a feeding strategy as well as the composition of additional supplements.

The results obtained in our study are not at variance with the research results described by Young et al. (1985), who find that the glucose additions did not appear to inhibit lincomycin production of Streptomyces lincolnensis in chemically defined media, in fact lincomycin accumulation was increased by about 20% in the run receiving the additions. Semenova et al. (1994) have reported that the batch-type feeding of carbohydrates resulted in an increase of the lincomycin yield by 23-24% compared to the un-fed mode.

**Figure 1** Comparison of mycelia concentration (dry mycelia weight) and lincomycin production during glucose fed batch fermentation of Streptomyces lincolnensis var. lincolnensis DSM 40 355 on M2 medium (A) and M1/M2 medium (B). Starting medium composition was that of the standard fermentation. The fed batch received one glucose addition of 0.5 g on 7th day of cultivation. Production was carried out at 30 °C in 250 ml Erlenmeyer flasks on rotary shaker at 180 rpm on 3rd, 7th, 10th, 12th, 14th, and 16th day of cultivation.

**Legend:** dry mycelia weight (○); concentration of lincomycin (●). Reported data were the average of duplicate experiments.

The purpose of media optimization is to support the efficient growth of microorganisms. Different combinations of medium constituents and sequences of optimized fermentation conditions need to be investigated to determine the growth conditions, which produce the biomass with best suited physiological state constituted for antibiotic production (Nanjwade et al., 2010). A comparison of DMW results obtained in fed batch operation modes is depicted in Figure 1 and 2. The specific rate of lincomycin production is plotted as a function of biomass concentration also in Table 1. At the beginning the concentration of mycelia (dry weight) increased, that reached a maximum of 3.35 ± 0.38 g l−1, but did not increase further and continued to decline due to the decrease of growing lincomycin concentration. These results indicate that DMW decreased with increasing antibiotic concentration similarly as in Young et al. (1985).

**CONCLUSION**

The highest production of lincomycin was detected on the M2 medium inoculated with a preculture M1 medium (seed medium), and after a single additional supplementation of the fermentation broth with 0.5 g of glucose per 50 ml (10 g l−1) on the 7th day of cultivation. The pH during cultivation of Streptomyces lincolnensis var. lincolnensis DSM 40355 altered by the addition of glucose. Compared to the production of biomass, we observed the opposite development and raising the pH with increasing concentration of lincomycin in the medium. A widespread usage of the antibiotics, including lincomycin, may result in occurrence of pharmacologically active compounds in environment, and their traces can be consequently found in foods and/or feeds. Proper and accurate analytical method and mainly analytical calibrants (standards) are necessary for their reliable detection. Knowledge gained from this study will be used to produce fully 13C isotope labeled lincomycin as internal standards (IS) for the accurate determination of this antibiotic in food and/or feeds by HPLC electrospray (ESI) MS/MS.

**Acknowledgments:** The research leading to these results has received funding from the European Community under project no 26220220180: Building Research Centre „AgroBioTech“ and by the Ministry of Education by the Slovak Research Grant Agency from pro project No. VEGA 1/0476/13.

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