Enhancement in production of erythromycin by *Saccharopolyspora erythraea* by the use of suitable industrial seeding-media

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Received 15 July 2007; revised 17 Nov 2007; Accepted 1 Dec 2007

ABSTRACT

Background and purpose of the study: There is no report on the effect of seeding-medium ingredients on *Saccharopolyspora erythraea* growth and erythromycin production. In this study, the enhancing effects of seeding-media which have been used routinely for screening, isolation or identification of actinomycetes, on *Saccharopolyspora erythraea* growth and erythromycin production were investigated.

Methods: The control medium contained soybean meal, glucose, glycerol, $(NH_4)_2SO_4$ and $CaCO_3$ as the major constituents and several media (I, II, III, ISP2, ISP3 and ISP4) were used for production of the antibiotic. Concentrations of biomass and erythromycin were measured by spectrophotometry, HPLC and bioassay methods and the effects of the composition of seeding media on pH were also determined.

Results: The concentration of erythromycin in medium II was 2.71g/l (1.33 times more than that of the control medium) and trends in production were: medium II > ISP4 = ISP2 = Control > ISP2 = Control = ISP3 > ISP3 = medium III = medium I. In the media containing starch and casein, the hyphae of S. *erythraea* were star-shaped and much branched.

Major conclusion: By virtue of other technical and economical characteristics of the seeding media used for erythromycin production, it is concluded that medium II is the best seeding medium formulation for erythromycin in comparison to others.

Keywords: Casein, Erythromycin, *Saccharopolyspora erythraea*, Seeding-medium, Starch

INTRODUCTION

Actinomycetes are Gram-positive soil bacteria that produce a wide variety of antibiotics and other secondary metabolites (1). In general, the productivity of microbial metabolites is closely related to the method of fermentation. Physiological and genetic characteristics of the strain, the medium composition, changes in nutrients and their concentrations have various effects on the accumulation of different metabolites. The carbon and nitrogen sources may dramatically influence antibiotic formation (2,3).

While there are several reports on the effect of fermentation-media ingredients (4-7) and environmental conditions (8,9) on erythromycin production, there is no report on the effect of seeding-media ingredients on the strain growth and antibiotic production. In this investigation, impacts of various seeding media on the growth of *Saccahropolyspora erythraea* and erythromycin production were studied.

MATERIALS AND METHODS

Materials

The media ingredients were obtained from Merck Co. (Germany). $CaCO_3$, soybean meal and oatmeal were purchased from Poudrsazan, Behpak and Behnan Co. (Iran) respectively. Standard erythromycin was provided by Aldrich Co., USA.

Methods

Bacterial strains and media

Saccharopolyspora erythraea NUR001 from Russian National Researches Institute on Antibiotics was provided by the Shafa-e-Sari, Pharmaceutical Co., (Iran). *Micrococcus luteus* ATCC 9341 was employed for the microbiological assay of the produced antibiotic. Oatmeal agar (10) was used as the sporulation medium. Different seeding-media list in Table 1 were used (11). Seeding and medium for control contained soybean meal, glucose, glycerol, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $CaCO_3$ as the major

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constituents) and fermentation-medium contained soybean meal, dextrin, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, rapeseed oil and $CaCO_3$) (5).

Incubation condition
A lyophilized vial of *S. erythraea* NUR001 was suspended in sterile distilled water and inoculated on 250ml bottle containing 50 ml sporulation agar (10) and incubated at 30ºC for 10-14 days. Aqueous spore suspension was prepared and the numbers of spores was counted by spread plate method. The spore suspension could be stored at 4°C up to 14 days without any effect on the numbers or productivity of the strain. A volume of 2 ml of a spore suspension (ca. 10^7–10^8 spores/ml) was inoculated in a 1000 ml Erlenmeyer flask containing 100 ml of seeding medium and incubated at 30ºC for 48 h on a rotary shaker at 220 rpm. Then, the seed culture (5%, v/v) was inoculated into each 1000 ml Erlenmeyer flask containing 90 ml of fermentation medium and incubated at 30ºC for 11 days on a rotary shaker at 240 rpm. All experiments were performed in triplicate in four batches.

Assay
Samples of 5 ml, were removed at the end of fermentation and after measurement of the pH and biomass, they were kept at -70ºC for further analysis.

Biomass
The ratio of the packed-cell weight to the wet weight of the culture medium was measured after centrifuging broth samples of fermentation at 4,000 rpm for 20 min and results are reported as approximation of biomass (5, 12).

Determination of the erythromycin

Assay of total erythromycin
The concentration of total erythromycin, after removal of the biomass, residual oil and insoluble ingredients by centrifugation was measured by modified colorimeteric method (13). It is important to remove residual oil from fermentation broth, since it interferes with determination of erythromycin by colorimetric method. Aqueous fraction of the fermentation broth was diluted with 0.2 M carbonate/bicarbonate buffer, of pH 9.6, and extracted with chloroform. Extracted erythromycin was mixed with the bromophenol blue reagent (0.008% bromophenol blue in 0.2 M citrate–phosphate buffer, pH 4.2). The absorbance of the organic layer which was separated with great care was measured at 415 nm by a spectrophotometer.

Erythromycin potency
In order to confirm the production of biologically active erythromycin, fermentation broth samples were bioassayed against *M. luteus* ATCC 9341, using the cylinder plate assay method (14).
Assay of erythromycin A: The concentration of erythromycin A was determined by HPLC system (Adept 4900, Cecil, UK) equipped with a UV detector (CE4200, Cecil, UK) at 205 nm, C18 column (250×4.6 mm, Hichrom, UK). Acetonitrile:methanol:0.2 M ammonium acetate: water (45:10:10:35) was used as mobile phase at 1.0 ml/min, at column temperature of 40ºC (column oven, CE4601, Cecil, UK). Sample injection volume was 50 µl (15).

Morphology
In each slide, 30 fields of view were captured randomly by microscope (Olympus BX501, Japan, ×1000), and the most predominant morphology was reported.

Data analysis
SAS and Minitab soft wares were used for the analysis of data. A Duncan test was used for the comparison of the averages. All differences with more than 95% confidences of limits were considered as significant differences.

RESULTS

Effect of seeding-media ingredients on erythromycin production
The effect of various seeding-media on the production of erythromycin is presented in Fig. 1. Maximum concentration of erythromycin (2.71g/l) was obtained in the starch-casein medium (medium II) which was 1.33 times more than that of control (P<0.05). Minimum production of erythromycin was obtained in media I (1.37g/l) and III (1.55g/l).

Effect of seeding-media ingredients on growth and morphology of S. erythraea
As it is shown in Fig. 1, the highest concentration of biomass was in ISP-2 and control media and minimum concentration was in medium I. The morphology and life-span of *S. erythraea* NUR001 were affected by the seeding-media ingredients. As it is shown in Fig. 2, the hyphae of *S. erythraea* in the media containing starch and casein were more branched and star-shaped. In the I, III, ISP-2, ISP-4 media and control, they were longer and more branched than medium II. However in ISP-3, the hyphae were long and less branched.

Table 1. Composition, specification and some important technical and economical characteristics of the seeding media which were used for erythromycin production.

	Medium I	Medium II	Medium III	ISP-2	ISP-3	ISP-4	Control
Composition (g/l)	Yeast extract 4, Glucose 4, Malt extract 10, $CaCO_3$ 2	Starch 10, Casein hydrolysate 1, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 5	Starch 25, Yeast extract 2, Soybean meal 20, NaCl 5, $CaCO_3$ 3, $MgSO_4.7H_2O$ 1, $MnCl_2.8H_2O$ 0.008, $ZnSO_4.7H_2O$ 0.007, $CuSO_4.5H_2O$ 0.002, $FeSO_4.7H_2O$ 0.001	Yeast extract 4, Glucose 4, Malt extract 10	Oatmeal 20, $MnCl_2.4H_2O$ 0.001, $ZnSO_4.7H_2O$ 0.001, $FeSO_4.7H_2O$ 0.001	Starch 10, Glucose 20, $(NH_4)_2SO_4$ 2, NaCl 1, $MgSO_4.7H_2O$ 1, $CaCO_3$ 2, $MnCl_2.7H_2O$ 0.001, $ZnSO_4.7H_2O$ 0.001, $FeSO_4.7H_2O$ 0.001	Soybean meal 30, glucose 10, glycerol 10, $(NH_4)_2HPO_4$ 1, $(NH_4)_2SO_4$ 5, $CaCO_3$ 5
pH	7.2±0.1	7.3±0.1	7.2±0.1	7.3±0.1	7.2±0.2	7.2±0.2	7.0±0.1
Price ($ per kg)	0.025	0.012	0.044	0.024	0.013	0.056	0.055
Titer of erythromycin (g/l)	1.37	2.71	1.55	2.14	1.79	2.35	2.04
Biomass (wet cell weight -%w/w)	15.1	4.6	22.8	5.88	9.55	5.65	31.18
Yield $_{erythromycin/\ price}$	54.80	225.80	35.23	89.17	137.70	41.96	37.09
Yield $_{erythromycin/biomass}$	0.09	0.6	0.067	0.36	0.19	0.41	0.065

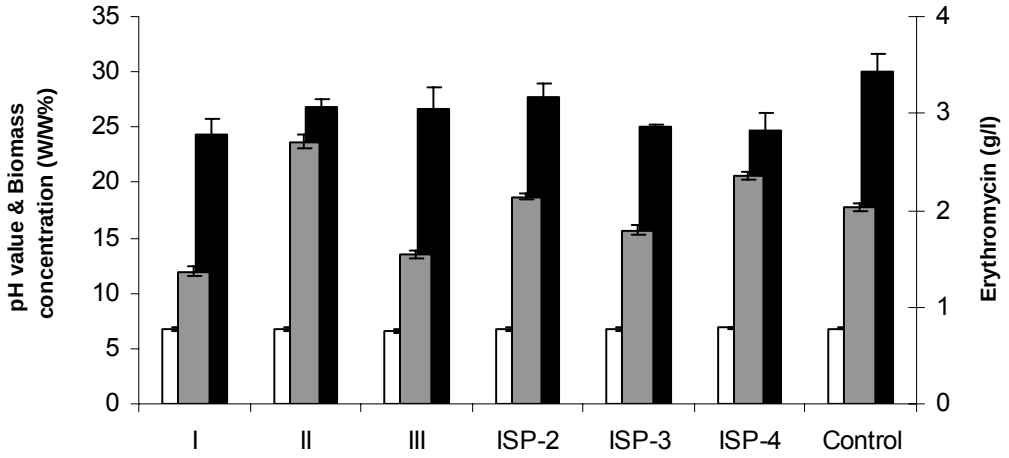

Figure 1. Effect of seeding-media ingredients on growth of *Saccharopolyspora erythraea* and erythromycin production. The fermentation media was inoculated with various seeding cultures. The value of pH (□), concentration of biomass (■) and erythromycin (■) are shown in the figure.

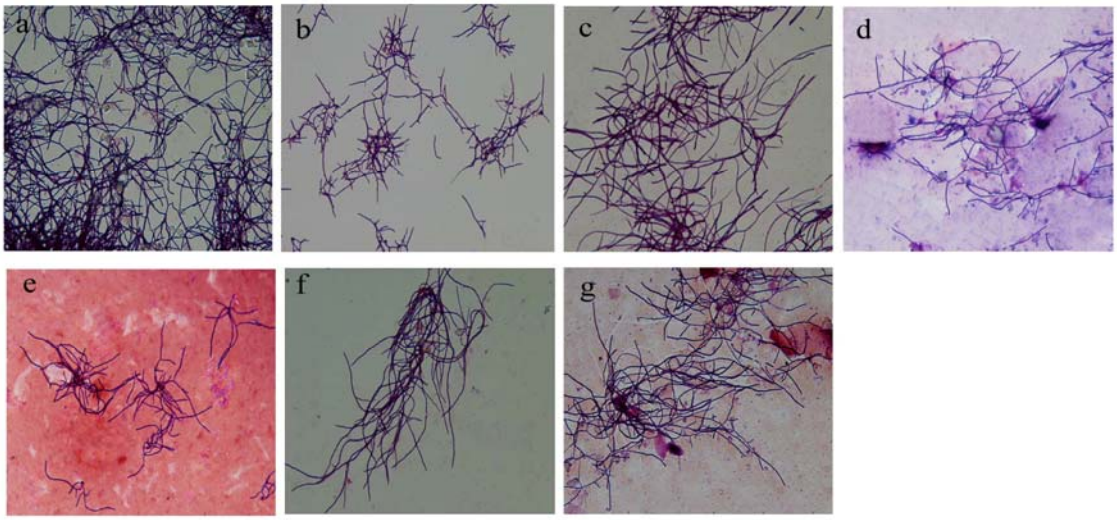

Figure 2. Effect of seeding-media ingredients on the morphology of *S. erythraea* NUR001. (a) seeding - medium I, (b) seeding medium II, (c) seeding-medium III, (d) ISP-2 medium, (e) ISP-3 medium, (f) ISP-4 medium, (g) control medium**.**

DISCUSSION

Optimization of fermentation conditions is necessary to improve secondary metabolites formation. In this research, effects of seeding-media ingredients on the growth of *Saccharopolyspora erythraea* and erythromycin production were evaluated. In medium II (containing starch and casein as the main substrates), production of erythromycin was maximum. Medium II has been used for screening and isolation of actinomyceytes (16). ISP media are the traditional media for cultural characterization of actinomycetes (11). However, there is no report on the use of these media for seeding in production of erythromycin. On the basis of technical and economical point of views of the seeding media which were used for production of erythromycin (Table 1), it may be concluded medium II was the best seeding-medium formulation for erythromycin in comparison to the other reported media.

Complex media are routinely used in fermentation industry. These media may contain inexpensive raw materials that are products of meat or grain processing. Starch has been used as seeding and fermentation media ingredient in production of clavulanic acid (17). Also, it has been used in the formulation of fermentation media of actinorhodin (18) and rapamycin (19). However, no report was found for using of casein as fermentation or seeding-medium ingredient. Traditionally, antibiotics are considered as secondary metabolites that produce at idiophase rather than trophophase. Several articles have shown that there is correlation between strain growth and production of erythromycin in certain situations such as in the absence of some oils (5) and limited amounts of oxygen (8), nitrate (6,7). It seems that correlation between growth of strain and erythromycin production not only was observed in the fermentation stage, but also it was observed in seeding process. Casein and starch are the complex medium ingredients which prolong the time of the down-time of the process, but enhance antibiotic production.

Calcium carbonate has been used as a source of Ca^{+2} (5). Also it compensates lowering of the pH by consumption of carbon sources and maintaine the pH of broth at optimum level for production of erythromycin. A comparison of the concentration of the antibiotic in the medium I and ISP-2 reveals that addition of this salt to in the seeding media is not useful and less pellet form of hyphae was observed in the media without calcium carbonate. The relationship between hyphal morphology, size and production of erythromycin has been revealed and it has been reported that long hyphae are the preferred morphology of *S. erythraea* in the fermentation media (20,21). While in the fermentation medium, at conditions which support rapid growth, a densely branched mycelium with a large hyphal diameter is observed, a less branched mycelium with small hyphal diameter is observed at poor growth conditions (22). Results of this investigation show that the preferred morphology of the strain for preparation of seeding material in medium II is more branched star-like hyphae. Further research is required to find out the relation between erythromycin production and the physiology of unusual star-like morphology of hyphae.

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