Semisolid state fermentation: effects of beet sugar root : peptone ratio on erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462

Mohamed A.M. Farid[a], Hoda M.A. Shata[b], Azza M. Noor El-Deen[a], Nayera A.M. Abdelwahed[a]

Departments of [a]Natural and Microbial Products [b]Microbial Chemistry, National Research Centre, Dokki, Egypt

Correspondence to Mohamed A.M. Farid, PhD, Department of Natural and Microbial Products, National Research Centre, Dokki, Giza, PO Box 12622, Egypt
Tel: +20 122 761 8859; fax: +20 233 370 931; e-mail: nrcfarid@yahoo.com

Received 19 February 2015
Accepted 02 March 2015

Egyptian Pharmaceutical Journal
2015, 14:94–102

Background and objectives
Erythromycin, a prominent member of the macrolide antibiotics, is commercially produced by submerged fermentation. However, this process requires high energy expenditures. The objective of the present work was to improve and optimize the cultural conditions of *Saccharopolyspora erythraea* NCIMB 12462 grown under semisolid state fermentation, with less energy requirements, less waste water generation, and easier product recovery, for the production of erythromycin using beet sugar root (BSR) and peptone.

Materials and methods
Chemical analysis of BSR was carried out according to the guidelines of AOAC and using high-performance liquid chromatographic. The concentration of erythromycin was measured using the agar diffusion bioassay method. Evaluation of different nitrogen sources for erythromycin production was carried out and the effect of different BSR : peptone ratio on erythromycin production was determined. The impact of initial moisture content (75–88%), size of inoculum, sodium chloride and calcium carbonate concentrations, and incubation period (1–12 days) on erythromycin production using solid state fermentation by *S. erythraea* NCIMB 12462 was evaluated.

Results and discussion
Optimization of environmental and culture parameters, concentration of nitrogen sources (ammonium sulfate, yeast extract, and peptone), BSR/peptone ratio, inoculum size, moisture content, and incubation time exhibited a significant increase in erythromycin production compared with the production before optimization. The concentration of erythromycin in optimized medium was 735.65 ± 8.58 μg/g dry BSR (1.36 times more than that of the control medium). The optimal conditions for erythromycin production using solid state fermentation for BSR were a initial moisture level of 77.78%, inoculum size of 2×10^6–2×10^7 spores/10 g dry BSR, incubation period of 10 days, and peptone at a concentration of 0.8 g/100 g BSR.

Keywords:
beet sugar root, erythromycin production, optimization, semisolid state fermentation

Egypt Pharm J 14:94–102

1687-4315

Introduction

Erythromycin is an important antibiotic, which belongs to the macrolide family, and is produced by fermentation of *Streptomyces erythreus* or *Saccharopolyspora erythraea*. It is widely applied in pharmaceutical preparations for local applications and in veterinary practice. Erythromycin is widely used clinically in treating respiratory infectious diseases [1] and in the treatment of many acute infections caused by bacteria belonging to *Staphylococci* spp. and *Neisseria* spp. [2]. It acts as an antimalarial drug in combination with other drugs in reducing pathogen resistance [3]. Besides all these medical applications, erythromycin is also used as a prophylactic and curative therapeutic agent for many diseases of poultry and farm animals. It also has many applications in animal feeding and cultivation of marine microalgae [4,5]. Erythromycin is commercially produced by submerged fermentation (SmF). However, this process requires high energy expenditures. In the search for more economical fermentation processes with high antibiotic activity, solid state fermentation (SSF) has gained interest in recent years due to the advantages that presents over SmF, such as higher product yields, less energy requirements, easier aeration, less waste water generation, reduced bacterial contamination, and easier product recovery [6]. With SSF, it is possible to utilize renewable and low-cost natural resources, such as agricultural and wood remains, energy crops, and byproducts of the food industry [6].

Hence, in the present paper the production of erythromycin by *S. erythraea* NCIMB 12462 grown under semisolid state fermentation conditions was investigated. This type of fermentation is a sort of SSF in which the free liquid content has been increased to facilitate nutrient availability and fermentation

control [7]. To the best of our knowledge, the production of erythromycin under semisolid state fermentation conditions has not been reported before this study.

Materials and methods

Microorganisms

S. erythraea NCIMB 12462 obtained from National Collection of Industrial and Marine Bacteria Limited (Aberdeen, Scotland, UK) was used in the present study. The spores of this strain had been preserved in a dormant state in starch–nitrate agar medium slants composed of 20 g/l of starch, 2 g/l of $NaNO_3$, 0.5 g/l of K_2HPO_4, 0.5 g/l of $MgSO_4{\cdot}7H_2O$, and 24 g/l of agar. The pH was adjusted to 7.2 before sterilization using 1 N NaOH and 1 N HCl. Subculturing was carried out once in 2 weeks, and the culture slants were stored at 4°C. The spores from a fully sporulated 10-day-old slant of *S. erythraea* NCIMB 12462 were dispersed in 10 ml of sterile distilled water by dislodging them with a sterile loop under aseptic conditions. The spore suspension was used as inoculum for each 250 ml Erlenmeyer flask containing the solid medium. Unless otherwise stated, for pure culture, each flask containing 10 g fresh beet sugar root (BSR) substrate was inoculated with 2 ml spore suspension (10^6–10^7 spores/ml). Spore count was measured using the dilution plate count method [7].

Bacillus subtilis NRRL B-543 was obtained from Northern Regional Research Laboratory (NRRL, Peoria, Illinois, USA). This bacterium was used as a test organism for the determination of the erythromycin produced by *S. erythraea* NCIMB 12462 using the agar diffusion method [9].

Inoculum preparation

The spores from a fully sporulated 10-day-old slant of *S. erythraea* NCIMB 12462 grown on ISP-2 agar slants at 28°C were dispersed in 10 ml of sterile distilled water by dislodging them with a sterile loop under aseptic conditions. The spore suspension was used as inoculum for each 250 ml Erlenmeyer flask containing the solid medium. Unless otherwise stated, for pure culture, each flask containing 10 g fresh BSR substrate was inoculated with 2 ml spore suspension (10^6–10^7 spores/ml). Spore count was measured using the dilution plate count method [8].

Substrate

BSR, from local market and a local sugar company in Kaffr El-Sheikh, Egypt, was pretreated by thoroughly washing with tap water and was homogenized in an electric mixer to small pieces and used as basic carbon source for semisolid fermentation media.

Analysis of beet sugar root components

Chemical analysis of BSR was performed. Moisture content, crude protein, crude fiber, fat content, and total ash were determined according to the method described by AOAC [10]. The amount of total carbohydrates was obtained by the difference between the weight of the sample taken and the sum of its moisture, ash, total lipid, protein, and fiber contents [11].

Determination of sugar contents

Extraction and determination of total water-soluble sugar, reducing sugar, and nonreducing were carried out according to the methods described by Miller [11]. The amount of nonreducing sugar was obtained by subtraction of the amount of reducing sugar from the total amount of water-soluble sugar.

High-performance liquid chromatographic determination of fructose, glucose, and sucrose in beet sugar root

The HP1100 system equipped with autosampler, quaternary pump, online degasser, and refractive index detector, controlled with ChemStation software (Hewlett Packard, Waldbronn, Germany), was used for chromatographic determination of fructose, glucose, and sucrose in BSR using ultrapure water as mobile phase (0.6 ml/min) and Shimadzu Shim-Pack SCR – 101N column (Tokyo, Japan).

Solid state fermentation

Ten grams of homogenized fresh BSR in a 250 ml Erlenmeyer flask was moistened with 10 ml mineral salt solution composed of 5 g/l of $CaCO_3$, 3 g/l $(NH_4)_2SO_4$, and 2.5 g/l NaCl, at pH 7, thoroughly mixed, and autoclaved at 121°C for 30 min. Each flask was inoculated with 2 ml of spore suspension (containing 1×10^6–1×10^7 spores/ml) and incubated at 28–30°C for 10 days. The moisture content of the medium after inoculation was 84.33%, including the moisture content of BSR. Unless otherwise specified, these fermentation conditions were maintained throughout the experiment. All experiments were performed in duplicate.

Optimization of the culture condition for erythromycin production

Factors affecting the production of erythromycin by *S. erythraea* NCIMB 12462 were optimized in 250-ml Erlenmeyer flasks containing 10 g of fresh BSR. The effect of incorporation of additional nitrogenous compounds (ammonium sulfate, ammonium phosphate

dibasic, sodium nitrate, potassium nitrate, meat extract, peptone, and yeast extract) to the production medium was studied. The nitrogen content of each added source was equivalent to the amount of nitrogen content in ammonium sulfate (3 g). Moreover, the different physicochemical parameters required to maximize the yield of erythromycin by *S. erythraea* NCIMB 12462 under SSF were investigated. The optimized parameter was incorporated at its optimized level in the subsequent optimization experiments. The impact of initial moisture content (75–88%), size of inoculum, sodium chloride and calcium carbonate concentrations, and incubation period (1–12 days) on erythromycin production using SSF of *S. erythraea* NCIMB 12462 was evaluated. All experiments were conducted in duplicate and the mean values were considered. After incubation of each fermentation sample, the crude extract was prepared.

Beet sugar root/peptone ratio

For optimization of the ratio of BSR and peptone as nitrogen source, *S. erythraea* NCIMB 12462 was grown in solid media prepared with different fresh BSR concentrations (10, 20, 30, and 40 g BSR/250 conical flask) and with different concentrations of peptone. The concentrations of peptone were proportional to the concentration of BSR. For 10 g BSR, peptone was added at a concentration of 0.2, 0.4, 0.6, 0.8, and 1.2 g/100 g BSR. For 20 g BSR, peptone at a concentration of 0.4, 0.8, 1.2, 1.6, and 2.4 g/100 g BSR was added. The other concentrations of BSR (30 and 40 g BSR) were also supplemented with the equivalent ratios of peptone. At the same time, different inoculum sizes of *S. erythraea* NCIMB 12462 were used to inoculate the sterilized medium based on BSR weight (2 ml/10 g BSR). In another set of experiments, each flask of the prepared media with different BSR and peptone concentrations (0.2, 0.4, 0.6, and 0.8 g/100 g BSR) was inoculated with 2 ml of spore inoculum of *S. erythraea* NCIMB 12462. The extraction volume of distilled water was also considered. All experiments were carried out at the optimized moisture content and with the equivalent amounts of other constituents optimized previously in the medium and extraction process. The flasks were incubated in static conditions at 28–30°C.

Erythromycin extraction

At the end of fermentation, the harvested biomass of each flask (10 g BSR/flask) was treated with 20 ml of water and shaken in an orbital shaker at 200 rpm at room temperature for 1 h. The whole content of each flask was centrifuged resulting in clear supernatant, and the final clear supernatant was used as the antibiotic source.

Erythromycin assay

The agar diffusion bioassay method [9] that utilizes the antibacterial property of erythromycin to produce a zone of inhibition against *B. subtilis* was used. A volume of 100 μl of filtrate was filled in the agar hole (0.9 mm diameter) punched in the nutrient agar plates [the antibiotic assay medium (Difco, Maryland, USA) comprised 10.0 g/l of glucose, 10.0 g/l of peptone, 2.5 g/l of meat extract, 5.0 g/l of yeast extract, 10.0 g/l of NaCl, and 20.0 g/l of agar) freshly seeded with 0.1 ml of *B. subtilis* NRRL B-543 strain as the test organism. The inhibition zone diameter was measured in mm after incubation of plates at 30°C for 24 h, and the concentration of erythromycin was calculated using standard erythromycin (Sigma Aldrich, St. Louis, Missouri, USA) calibration curve. All experiments were conducted in duplicate, and the mean of the two reading is presented as micrograms of erythromycin produced per gram dried BSR.

Results and discussion

Proximate composition of beet sugar root

The proximate composition of BSR is given in Table 1. The root of the beet contains about 65.5% water (g/100 g fresh weight) and 34.5% dry matter. The dry matter comprises about 1.5% fiber, 2% protein, 0.11% fats, 1.06% ash, and 29.83% total carbohydrates. The total sugars represent 81.16% of the root's dry matter. The sucrose content in sugar beet roots represents about 18.39%, whereas the content of glucose and fructose was 2.31 and 1.099%, respectively, as determined by high-performance liquid chromatographic (Fig. 1).

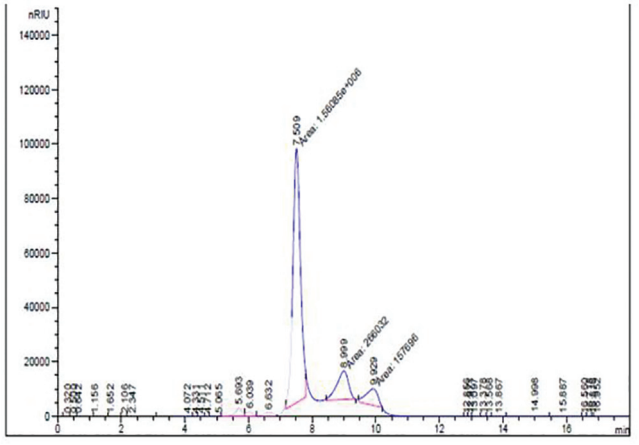
Effect of supplementary organic and inorganic nitrogen sources

The protein content in BSR is very low and thus the nitrogen levels as well as the commercial value decrease greatly when it is used as carbon source [12]. Hence, the exogenous addition of various nitrogen levels to the solid medium was studied. Supplementation of medium with organic, as well as inorganic nitrogen sources, resulted in the

Table 1 Chemical composition of beet sugar root

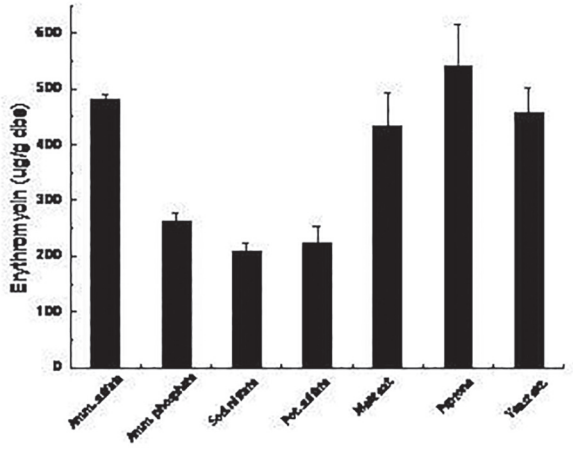
Components	%
Moisture	70
Protein	2
Fat	0.11
Ash	1.06
Total carbohydrates	25.33
Total sugars	23.8
Reducing sugars	3.8
Nonreducing sugars	20

Figure 1



High-performance liquid chromatographic separation chromatogram for sucrose, glucose, and fructose in BSR using ultrapure water as mobile phase (0.6 ml/min) and Shimadzu Shim-Pack SCR – 101N column.

Figure 2



Effect of various nitrogen sources in the presence of beet sugar root as carbon source on erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462 under solid state fermentation.

improvement in erythromycin production compared with control medium (1.1 μg/g dry BSR) without nitrogen source. In the present studies, nitrogen sources – namely, ammonium sulfate, ammonium phosphate dibasic, sodium nitrate, potassium nitrate, meat extract, peptone, and yeast extract – were used for growth and erythromycin production by *S. erythraea* NCIMB 12462. The results are shown in Fig. 2. All tested nitrogen sources supported erythromycin formation by the culture of *S. erythraea* NCIMB 12462, whereas ammonium sulfate, peptone, and yeast extract were proved to be superior to other nitrogen sources. A high titer of erythromycin (541.38 ± 74.35 μg/g dry BSR) was obtained in a medium containing peptone alone as organic nitrogen source, followed by yeast extract (457.27 ± 44.60 μg/g dry BSR). Among the different inorganic nitrogen sources tested, ammonium sulfate was the best source for erythromycin (482 μg/g dry BSR) production. The order of nitrogen source suitability was as follows: peptone>yeast extract>meat extract ≥ ammonium sulfate>ammonium phosphate dibasic > potassium nitrate>sodium nitrate. It was reported that ammonium salts did not favor the biosynthesis of novobiocin, actinomycin, neomycin, kanamycin, and others; however, for rapamycin, ammonium sulfate was the best nitrogen source [13,14]. Ammonium salts (nitrate, sulfate, chloride, acetate, and arginine) stimulate the formation of some components of IM-111-81 and azalomycin B, whereas ammonium succinate increased the productivity of AK-111-81 nonpolyenic macrolide antibiotic [15]. Hence, peptone, yeast extract, and ammonium sulfate were selected and used for subsequent studies.

Effect of different concentrations of the best nitrogen sources

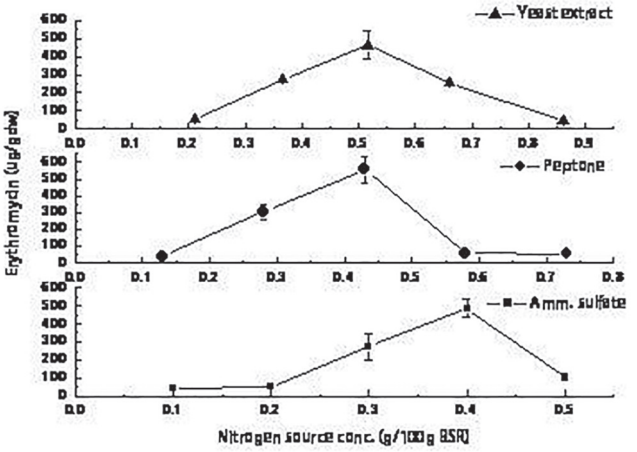
Experiments were performed with varying ammonium sulfate, peptone, and yeast extract concentrations (0.1–0.5, 0.13–0.73, and 0.21–0.86 g/100 g BSR, respectively) in basal medium with 10 g BSR/flask to study the effects of nitrogen sources on erythromycin production by *S. erythraea* NCIMB 12462 under SSF. Out of the concentrations of three nitrogen sources studied (ammonium sulfate, peptone, and yeast extract), the results in Fig. 3 show that there was a significant increase in the antibiotic production when the medium was supplemented with different ammonium sulfate concentrations. Peptone at 0.73% (w/w) and yeast extract at 0.52 (%w/w) recorded the maximum yield (557.54 = 8.03 and 463.25 = 7.74 μg/g dry BSR, respectively) for organic nitrogen sources. For ammonium sulfate, the maximum yield of erythromycin (492.25 = 4.88 μg/g dry BSR) was obtained at concentration 0.4% (w/w). Further concentration-dependent studies suggested that variation in ammonium sulfate, peptone, and yeast extract concentrations showed a negative impact on erythromycin production. Nitrogen sources have long been known to affect the antibiotic production, and suppress the biosynthesis of antibiotics and other secondary metabolites. The most common observation was a decrease in the levels of antibiotic produced in the presence of an excess of nitrogen source. Some researchers reported that antibiotic biosynthesis may be inhibited or repressed by ammonia and other rapidly utilized nitrogen sources [16,17]. The phenomenon of catabolic nitrogen repression of ammonium sulfate has been reported to affect many catabolic enzymes, which play a significant role in erythromycin biosynthesis [18]. High ammonium ion concentration had a negative

effect on macrolide production, and a strong correlation existed between macrolide production and the level of valine dehydrogenase [16,19–21]. The high activity of valine dehydrogenase enhanced the catabolism of branched chain amino acid and was in favor of producing important sources of the macrolide building blocks [17,19,20]. This regulation mechanism of ammonium ion was also proved in *Streptomyces viridochromogenes* AS4.126, the producer of avilamycin [22]. In contrast, some studies reported that ammonium impedes aminoglycoside synthesis, whereas others reveal stimulatory effects such as neomycin [23], gentamicin [24], and streptomycin production [14,25]. High nitrogen concentrations affect the synthesis of sensitive enzymes involved in primary and secondary metabolism and the utilization of different nitrogen sources from the fermentation medium as well. Therefore, several approaches have been reported to avoid the negative effect of the nitrogen on the fermentative production of metabolites [14].

Effect of different beet sugar root: peptone ratios on erythromycin production

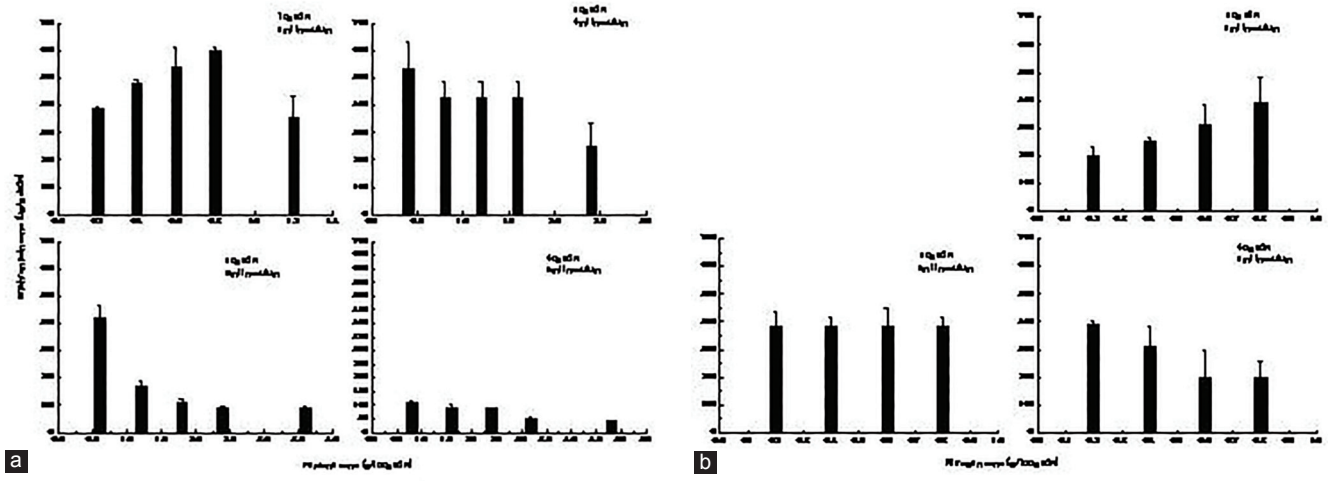
In general, the C : N ratio in the culture medium is a critical factor for cell growth and metabolite production. The effect of different ratios between BSR and peptone in culture medium on erythromycin production was studied with the controlling of BSR and peptone concentrations, inoculum size, and extraction process. As shown in Fig. 4a, the medium without peptone showed no erythromycin production. At low BSR concentration (10 g/flask), the increase in concentration of peptone resulted in a significant increase in erythromycin production up to 602.13 ± 11.56 μg/g dry BSR in culture containing 0.8 g peptone/l00 g BSR. In contrast, there was a reduction in erythromycin production as the additions of peptone were increased. In 20 g BSR/flask, the addition of peptone increases the erythromycin production up to 0.4 g peptone/l00 g BSR, and the maximal production was 536.61 ± 94.1 μg/g dry BSR. Moreover, the same behavior of reduction was observed with increasing peptone concentrations. Upon increasing the BSR concentration up to 30 g/flask, the maximal erythromycin production of about 418.58 ± 50 μg/g BSR was obtained in all values of peptone-supplemented culture. However, the repression effect was observed at a high BSR concentration of 40 g/flask with all concentrations of peptone. At higher BSR and peptone concentrations, erythromycin production was greatly influenced (108.48 ± 10–42.23 ± 3 μg/g dry BSR). In contrast, Fig. 4b shows the effect of different

Figure 3



Effect of different concentrations of $(NH_4)_2SO_4$, peptone, and yeast extract in the presence of beet sugar root on erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462 under solid state fermentation.

Figure 4



Effect of different beet sugar root : peptone ratios on erythromycin production in the presence of different inoculum sizes of *Saccharopolyspora erythraea* NCIMB 12462 under solid state fermentation. [Controlled inoculum size (4a) and fixed inoculum size (4b)]

BSR and peptone (0.2, 0.4, 0.6, and 0.8 g/100 g BSR) ratios using fixed inoculum size (2 ml/flask). In these series of experiments, the highest production of erythromycin was also achieved in the presence of 10 g BSR/flask and 0.8 peptone/100 g BSR. At higher BSR and peptone concentrations with 2 ml inoculum size, it was noticed that erythromycin production was slightly influenced (391.49 ± 13.07–201.13 ± 57.2 μg/g dry BSR). From the results of the two experiments, the decrease in erythromycin may be due to carbon source concentration, nitrogen source concentration, as mentioned before, and inoculum size as well.

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. More than 30 examples of secondary metabolites are reported to be suppressed by the presence of the carbon source. Glucose and other carbohydrates, such as glycerol, maltose, mannose, sucrose, and xylose, have been reported to interfere with the synthesis of secondary metabolites. For instance, glucose depresses the formation of aminoglycoside antibiotics (streptomycin, kanamycin, istamycin, neomycin, and gentamicin) through repression of biosynthetic enzymes [26–28]. However, production is frequently limited due to a negative effect exerted by the carbon source. This regulatory mechanism, termed carbon catabolite regulation (CCR), is widely distributed among microbial systems and functions primarily to assure an organized and sequential utilization of carbon sources, when more than one carbon source is present in the environment [14]. Actinomycetes (Gram-positive) bacteria are subjected to CCR. This group possessing a high guanine and cytosine (GC) content in DNA includes *Streptomyces*, a genus characterized by its ability to produce secondary metabolites. The synthesis of these compounds is usually sensitive to CCR. For example, glucose depresses the formation of many aminoglycoside antibiotics produced by actinomycetes (streptomycin, kanamycin, istamycin, neomycin) through repression of biosynthetic enzymes [14,26]. Polysaccharides (e.g. starch), oligosaccharides (e.g. lactose), and oils (e.g. soybean oil, methyloleate) are often preferable for fermentations yielding secondary metabolites [14]. Irrespective of the type of fermentation, whether it is a SSF or SmF, inoculum level also affects the yield of final product greatly [29,30].

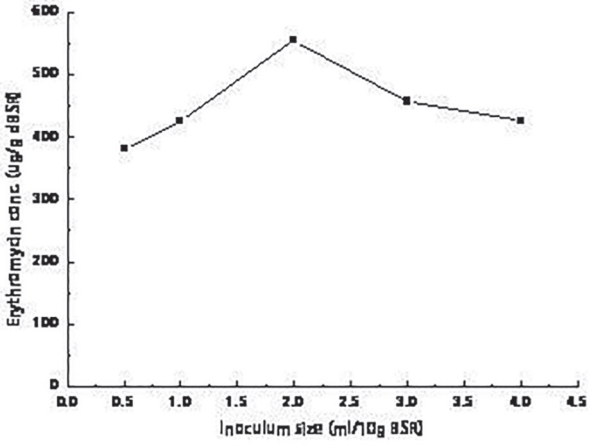
Effect of inoculum size

The effects of the inoculum on erythromycin production were studied by adding different concentrations (0.5 × 10^6–0.5 × 10^7, 1 × 10^6–1 × 10^7, 2 × 10^6–2 × 10^7, 3 × 10^6–3 × 10^7, and 4 × 10^6–4 × 10^7 spores/10 g BSR) of spores to the solid medium and fermentation was carried out for 10 days. The moisture content, pH of the substrate, and incubation temperature were kept at their optimum levels. The maximum production of erythromycin (616.50 ± 16.8 μg/g dry BSR) was obtained in the fermentation medium that was inoculated with 2 ml of spore suspension (2 × 10^6–2 × 10^7 spores/10 g BSR) of *S. erythraea* NCIMB 12462. Lower and higher inocula levels than the optimum level, resulted in decreased erythromycin activities (Fig. 5). In previous reports, authors have reported that adequate inoculum can initiate fast mycelium growth and product formation, thereby reducing the growth of contaminants. A decrease in antibiotic production was observed when the inoculum size was increased beyond the optimum level. Antibiotic production attains its peak when sufficient nutrients are available to the biomass. Conditions with a misbalance between nutrients and proliferating biomass result in decreased antibiotic synthesis [29,31]. A low inoculum density may give insufficient biomass causing reduced product formation, whereas an inoculum density higher than the optimum density may produce too much biomass and may deplete the nutrients necessary for secondary metabolite production [32].

Effect of different concentrations of sodium chloride and calcium carbonate

The effect of inorganic salt supplementation on erythromycin production is shown in Fig. 6. Addition of 0.5% (w/w) $CaCO_3$ resulted in maximal erythromycin secretion (616.15 μg/g dry BSR) with *Streptomyces* strains than with controls. $CaCO_3$ can significantly affect the pH of the medium during the course of fermentation, which in turn may influence

Figure 5



Effect of different inoculum sizes on erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462 under solid state fermentation. BSR, beet sugar root.

antibiotic production. Calcium carbonate has been used as a source of Ca^{+2} [33]. Moreover, it compensates lowering of the pH by consumption of carbon sources and maintains the pH of broth at optimum level for the production of erythromycin. A comparison of the concentration of the antibiotic in medium I and ISP-2 reveals that addition of this salt in the seeding media is not useful and that less pellet form of hyphae were observed in the media without calcium carbonate. The presence of 0.06% (w/w) NaCl also enhanced erythromycin production; thus, the salt requirements for the production of this antibiotic were apparently provided by the solid substrates.

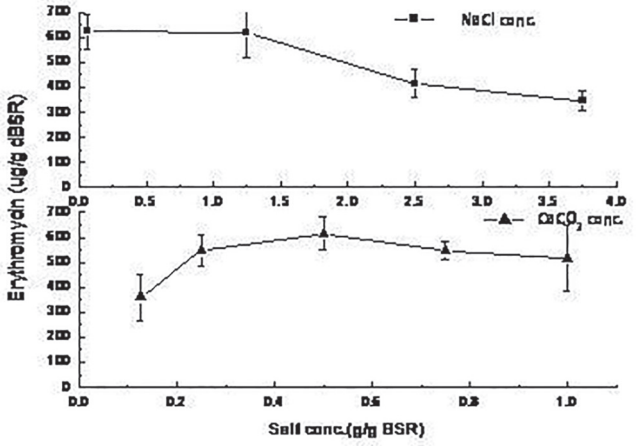
Effect of initial moisture content

The influence of initial moisture content of the solid substrate was investigated at various moisture levels (75, 77.77, 80, 82.85, 85, 86.36, and 88%) of BSR using distilled water before autoclaving, and the initial moisture percent was calculated. All experiments were carried out at initial pH 7, and excess distilled water was added to get the desired moisture percent. Figure 7 shows the yields of erythromycin under seven different moisture contents. The moisture content of 77.77% (w/w) provides the best environment for erythromycin production (715.8 μg/g dry BSR). A decrease in erythromycin yield was observed when the moisture contents were much higher or lower than the optimum level. However, any further increase in moisture level in SSF causes free water in the fermentation medium, which may lead to limit gas exchange and higher vulnerability to bacterial contamination, whereas low moisture leads to reduced solubility of nutrients and substrate swelling. For SSF, moisture is a key parameter to control the growth of microorganism and metabolite production [6]. The importance of substrate moisture level in SSF for the production and secretion of secondary metabolites has been well established [6]. In SSF, the intensity of microbial growth generally depends on the initial moisture level and it indirectly affects the production titer. This result was similar to the findings of Mahalaxmi *et al*. [29], who reported that an initial substrate moisture content less than 40% gave less rifamycin B production, but that of 50–56% could give the highest rifamycin B production. The highest tylosin production was obtained at 70% initial moisture contents [33]. Moreover, the maximum yield of neomycin production was obtained from 70% moisture at day 8. The results from the previous study stated that the ideal moisture content was 80% and the reduction in antibiotic yield could occur with low and with higher moisture levels [14]. Higher initial moisture in SSF leads to suboptimal product formation due to reduced mass transfer process, and decrease in initial moisture level results in reduced solubility, minimized heat exchange and oxygen transfer, and low availability of nutrients to the culture [34]. Low moisture levels decrease the solubility and availability of nutrients, minimize heat exchange and oxygen transfer rates, thus lowering the activity of microbial cultures and resulting in reduced productivity [34]. Higher substrate moisture in SSF resulted in less productivity because of reduced mass transfer process, such as diffusion of solutes and gases to the cells during fermentation process.

Effect of incubation period

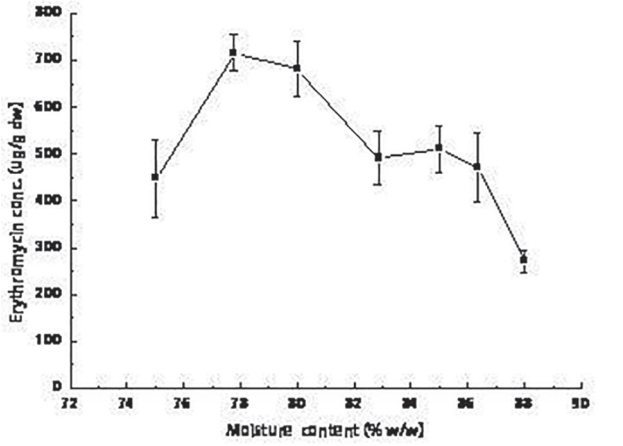
The culture of *S. erythraea* NCIMB 12462 was grown in time course studies to determine the optimum time for production of erythromycin under SSF using BSR as

Figure 6



Effect of different concentrations of sodium chloride and calcium carbonate in the presence of beet sugar root (BSR) on erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462 under solid state fermentation.

Figure 7

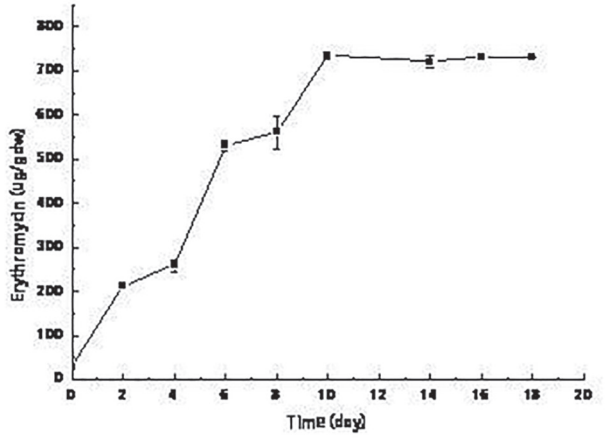


Effect of various initial moisture level (%) on erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462 under solid state fermentation. dw, dry weight.

the main carbon source, peptone as nitrogen source, and at optimized moisture level, inoculum size, and BSR/peptone ratio. Erythromycin production started earlier (i.e. during the lag phase) and antibiotic production was observed from second day of fermentation and reached maximum (735.65 ± 8.577 μg/g dry BSR) on the 10th day (Fig. 8). Further incubation after this time did not show any increment in the level of erythromycin production. The highest erythromycin produced after medium optimization represents 1.36-fold increase over the activities attained before optimization. It has been reported that antibiotic and secondary metabolite production are related to morphological characters of actinomycetes during the course of fermentation. It was reported that antibiotic production in liquid culture is correlated with mycelial fragment diameter in actinomycete cultures [35]. Smaller fragments appear to grow at the same rate as larger particles, but are incapable of significant antibiotic production. This phenomenon appears to account for loss of biosynthesis in liquid culture in species able to produce antibiotic on agar [36]. It was concluded also that, for *S. erythraea* cultivation in SmF, clump morphology is more suitable for erythromycin production compared with pellet morphology. Furthermore, a decrease in clump dimensions, together with lower non-Newtonian broth viscosities, probably as a result of decrease in mass transfer resistances, also enhances erythromycin productivity [37].

Erythromycin production by *S. erythraea* has been reported to take place in pellets 80–90 μm in diameter or larger, supporting the idea that the antibiotic is produced at a fixed distance from the hyphal end. Consequently, mycelia that are too small and have not developed to this length would be incapable of producing antibiotics [38].

Figure 8



Effect of incubation period on erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462 under solid state fermentation. dw, dry weight.

Conclusion

Overall, the present study revealed that *S. erythraea* NCIMB 12462 is effective in erythromycin production using BSR as carbon source under SSF.

It is interesting to notice that organic nitrogen (peptone and yeast extract) and the inorganic (ammonium sulfate) source had significant effect at individual level. The optimum production of erythromycin (735.65 ± 8.57694 μg/g dry BSR) was achieved by using BSR and peptone with optimized process parameters such as moisture level, inoculum level of 2 ml/10 g BSR v/w, incubation period of 10 days, 100 : 0.8 (w/w) ratio of BSR to peptone, and incubation temperature at 30°C. An overall 1.36-fold improvement in erythromycin production was achieved due to optimization.

Acknowledgements

Conflicts of interest

None declared.

References

1 Hoyt JC, Robbins RA. Macrolide antibiotics and pulmonary inflammation. FEMS Microbiol Lett 2001; 205:1–7.

2 Lesmana M, Lebron CI, Taslim D, Tjaniadi P, Subekti D, Wasfy MO, *et al*. In vitro antibiotic susceptibility of *Neisseria gonorrhoeae* in Jakarta, Indonesia. Antimicrob Agents Chemother 2001; 45:359–62.

3 Nakornchai S, Konthiang P. Activity of azithromycin or erythromycin in combination with antimalarial drugs against multidrug-resistant *Plasmodium falciparum* in vitro. Acta Trop 2006; 100:185–91.

4 Kim YK, Cerniglia CE. Influence of erythromycin A on the microbial population in aquaculture sediment microcosms. Aquatic Toxicol 2005; 73:230–241.

5 Campa-Córdova AI, Luna-González A, Ascenci F, Cortés-Jacinto E, Cáceres-Martínez CJ. Effects of chloramphenicol, erythromycin, and furazolidone on growth of *Isochrysis galbana* and *Chaetoceros gracilis*. Aquaculture 2006; 260:145–150.

6 Pandey A, Soccol CR, Mitchell DA. New developments in solid-state fermentation: I. Bioprocesses and products. Process Biochem 2000; 35:1153–1169.

7 Economou ChN, Makri A, Aggelis G, Pavlou S, Vayenas DV. Semi-solid state fermentation of sweet sorghum for the biotechnological production of single cell oil. Bioresour Technol 2010; 101:1385–8.

8 Parkinson D, Gray TRG, Williams ST. *Methods for studying the ecology of soil microorganisms. International Biological Programme no. 19*. Oxford: Blackwell; 1971. 116.

9 Perez C, Paul M, Bazerque P. An antibiotic assay by the agar well diffusion method. Acta Bio Med Exp 1990; 15:113–115.

10 Association of Official Analytical Chemists (AOAC). *Official methods of analysis of AOAC*. Virginia, USA: AOAC Inc.; 2003.

11 Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1972; 31:426–428.

12 Asadi M. *Beet-sugar handbook*. Published by John Wiley & Sons, Inc., Hoboken, New Jersey; 2007. 187.

13 Aharonowitz Y. Nitrogen metabolite regulation of antibiotic biosynthesis. Annu Rev Microbiol 1980; 34:209–33.

14 Sanchez S, Demain AL. Metabolic regulation of fermentation processes. Enzyme Microb Technol 2002; 31:895–906.

15 Gesheva V, Ivanova V, Gesheva R. Effects of nutrients on the production of AK-111-81 macrolide antibiotic by *Streptomyces hygroscopicus*. Microbiol Res 2005; 160:243–8.

16 Lebrihi A, Lamsaif D, Lefebvre G, Germain P. Effect of ammonium ions on spiramycin biosynthesis in *Streptomyces ambofaciens*. Appl Microbiol Biotechnol 1992; 37:382–7.

17 Wang P, Zhuang YP, Chu J, Zhang SL. Regulatory effects of ammonium ions on the biosynthesis of meilingmycin. Wei Sheng Wu Xue Bao 2005; 45:405–9.

18 Reeve LM, Baumberg S. Physiological controls of erythromycin production by *Saccharopolyspora erythraea* are exerted at least in part at the level of transcription. Biotechnol Lett 1998; 20:585–589.

19 Omura S, Tanaka Y, Mamada H, Masuma R. Effect of ammonium ion, inorganic phosphate and amino acids on the biosynthesis of protylonolide, a precursor of tylosin aglycone. J Antibiot (Tokyo) 1984; 37:494–502.

20 Omura S, Tanaka Y, Mamada R, Tanaka Y. Ammonium ion suppress the amino acid metabolism involved in the biosynthesis of protylonolide in a mutant of *Streptomyces fradiae*. J Antibiot 1984b; 37:1362–1369.

21 Beltrametti F, Jovetic S, Feroggio M, Gastaldo L, Selva E, Marinelli F. Valine influences production and complex composition of glycopeptide antibiotic A40926 in fermentations of *Nonomuraea* sp. ATCC 39727. J Antibiot (Tokyo) 2004; 57:37–44.

22 Zhu CH, Lu FP, He YN, Han ZL, Du LX. Regulation of avilamycin biosynthesis in *Streptomyces viridochromogenes*: effects of glucose, ammonium ion, and inorganic phosphate. Appl Microbiol Biotechnol 2007; 73:1031–8.

23 Okazaki H, Ono H, Yamada K, Beppu T, Arima K. Relationship among cellular fatty acid composition, amino acid uptake and neomycin formation in a mutant strain of *Streptomyces fradiae*. Agric Biol Chem 1973; 37:2319–23.

24 Gonzalez R, Islas L, Obregon AM, Escalante L, Sanchez S. Gentamicin formation in *Micromonospora purpurea*: stimulatory effect of ammonium. J Antibiot (Tokyo) 1995; 48:479–83.

25 Inoue S, Nishizawa Y, Nagai S. Stimulatory effect of ammonium on streptomycin formation by *Streptomyces griseus* growing on a glucose minimal medium. J Ferment Technol 1983; 61:7–12.

26 Demain AL. Carbon source regulation of idiolite biosynthesis. In: Shapiro S, editor *Regulation of secondary metabolism in Actinomycetes*. Boca Raton, FL: CRC Press; 1989. 127–134.

27 Ruiz B, Chávez A, Forero A, García-Huante Y, Romero A, Sánchez M, *et al*. Production of microbial secondary metabolites: regulation by the carbon source. Crit Rev Microbiol 2010; 36:146–67.

28 Sánchez S, Chávez A, Forero A, Garcıa-Huante Y, Romero A, Sánchez M, *et al*. Carbon source regulation of antibiotic production. J Antibiot (Tokyo) 2010; 63:442–59.

29 Mahalaxmi Y, Sathish T, Subba Rao CH, Prakasham RS. Corn husk as a novel substrate for the production of rifamycin B by isolated *Amycolatopsis* sp. RSP 3 under SSF. Process Biochem 2010; 45:47–53.

30 Venkateshwarlu G, Muralikrishna PS, Sharma G, Rao LV. Improvement of rifamycin-B production using mutant strains of *Amycolatopsis mediterranei*. Bioproc Eng 2000; 23:315–318.

31 Vastrad BM, Neelagund SE. Optimization and production of neomycin from different agroindustrial wastes in solid state fermentation. Int J Pharm Sci Drug Res 2011; 3:104–111.

32 Khaliq S, Rashid N, Akhtar K, Ghauri MA. Production of tylosin in solid-state fermentation by *Streptomyces fradiae* NRRL-2702 and its gamma-irradiated mutant (gamma-1). Lett Appl Microbiol 2009; 49:635–40.

33 Hamedi J, Malekzadeh F, Saghafi-nia AE. Enhancing of erythromycin production by *Saccharopolyspora erythraea* with common and uncommon oils. J Ind Microbiol Biotechnol 2004; 31:447–56.

34 Carrizales V, Rodrigues H, Sardina I. Determination of specific growth rate of molds as semi solid cultures. Biotechnol Bioeng 1981; 232:321–333.

35 Martin SM, Bushell ME. Effect of hyphal micromorphology on bioreactor performance of antibiotic-producing *Saccharopolyspora erythraea* cultures. Microbiology 1996; 142:1783–1788.

36 Pickup KM, Nolan RD, Bushell ME. A method for increasing the success rate of duplicating antibiotic activity in agar and liquid culture of *Streptomyces* isolates in new antibiotic screens. J Ferment Bioeng 1993; 76:89–93.

37 Ghojavand H, Bonakdarpour B, Heydarian SM, Hamedi J. The inter-relationship between inoculum concentration, morphology, rheology and erythromycin productivity in submerged cultivation of *Saccharopolyspora erythraea*. Brazil J Chem Eng 2011; 28:565–574.

38 Wardell JN, Stocks SM, Thomas CR, Bushell ME. Decreasing the hyphal branching rate of *Saccharopolyspora erythraea* NRRL 2338 leads to increased resistance to breakage and increased antibiotic production. Biotechnol Bioeng 2002; 78:141–6.