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Optimization of the cultivation medium for natamycin production by *Streptomyces natalensis*

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The effects of certain nutrients on natamycin production by *Streptomyces natalensis* in submerged batch culture were studied. The production of this antibiotic required glucose in the cultivation medium with a concentration of 20 g/l. On the other hand, the highest antibiotic production was obtained in a cultivation medium containing 0.05 g/l of potassium dihydrogen phosphate. Further increase in phosphate concentration resulted in a significant increase in biomass concomitant with lower antibiotic production. Among different N-sources tested, only ammonium sulphate, sodium nitrate and beef extract were the suitable nitrogen sources in supporting the antibiotic production. Furthermore, a mixture of beef extract and yeast extract (8 g/l and 2 g/l, respectively) exhibited a synergistic effect in enhancing the natamycin production reaching about 1.5 g/l.

Natamycin (Pimaricin) is a commercially important polyene antifungal compound produced by microorganisms belonging to actinomycetes such as *Streptomyces natalensis* and *Str. chatanoogensis*. This antibiotic is predominantly a strong antifungal agent, inhibiting the growth of both yeasts and molds (PEDERSEN 1992) and prevent the formation of aflatoxin in filamentous fungi (RUSUL and MARTH 1988). Because of the low toxicity of natamycin to the mammalian cells compared to other antifungal compounds (LEVINSKAS *et al.* 1966), it found many applications in the treatment of many fungal diseases such as bronchopulmonary aspergillosis (CURRIE *et al.* 1990) and mycotic keratitis (GUPTA *et al.* 1999). Moreover, the low solubility of natamycin makes it suitable for use as surface treatment on foods and increasing the shelf time of many food products. Therefore, the use of natamycin as a natural product food preservative for certain cheese, sausage, fruits and beverages is permitted and used in many food industries (KIERMEIER 1973, DAVIDSON and DOAN 1993). Natamycin is one of the few antibiotics which are still recommended by FDA as food additive and classified as a GRAS (Generally regarded as a Safe) compound.

The biosynthesis of polyene antibiotics is regulated by the type and concentration of different medium components such as carbon, nitrogen, phosphate, metal ions and other medium ingredients (MARTIN and MCDANIEL 1977). Moreover, the ratio between the different medium constituents can shift the metabolic pathway of the producer strain to favor the production of secondary metabolites on the cost of primary metabolites and vice versa. Therefore, medium composition plays a critical role in both volumetric and specific antibiotic production which is reflected directly on the process economics.

With the exception of few patents (EISENSCHINK and OLSON 1993, OLSON 1993), little information is available in the literature on the effect of cultivation medium on natamycin production. This paper deals with the effect of medium composition on natamycin production by *Str. natalensis* NRRL 2651 in batch cultures. The aim of the present work is to increase volumetric production and yield of the antibiotic by selecting the suitable medium components.

Materials and methods

Microorganism and cultivation media: These studies were carried out with *Streptomyces natalensis* NRRL 2651 (USDA, ARS culture collection, Peoria, Illinois, USA). The strain was maintained on slants of medium ISP-2 composed of (g/l): Malt extract, 10.0; yeast extract, 4.0; glucose, 4.0 and agar, 20.0. The pH of the medium was adjusted to 7.2 before sterilization. The arisen spores after 7 days cultivation at 30 °C were collected in saline solution (NaCl 0.9% w/v) and diluted until it reached a countable level of 10^3-10^5 spore. Spore counts were performed by using a haemocytometer. Unless otherwise stated, the antibiotic production medium was composed of (g/l): glucose, 20; beef extract (DIFCO), 2.0; yeast extract (DIFCO), 2.0; asparagine, 0.5 and potassium dihydrogen phosphate, 0.05. The pH of the medium was adjusted to 7.0 before sterilization by autoclaving at 121 °C for 15 minutes. Glucose was autoclaved separately and added to the medium before inoculation.

Cultivation conditions: Unless otherwise mentioned, cultivation was carried out in 250 ml ERLENMEYER flasks containing 50 ml liquid medium. Inoculum was in the form of spores (2×10^8 spores/ml) obtained from a densely sporulating culture grown on ISP-2 medium for 7–9 days as described before. The flasks were incubated at 30 °C on a rotary shaker at 200 rpm for 96 h. Samples, in the form of three flasks each, were withdrawn intermittently for cell dry weight, antibiotic and glucose determination.

Analysis: Determination of cell dry weight: Cell dry weight was determined by filtering 50 ml of the culture fluid using a dry and perweighed filter paper (WHATMAN filter paper No. 1). The filtered sample was washed twice by distilled water and subsequently dried in an oven at 100 °C to constant weight.

Determination of glucose concentration: In the supernatant fluid, glucose concentration was determined enzymatically using a glucose enzyme determination kit (Glucose kit Cat. No. 4611, BIOCON Diagnostik GmbH, Burbach, Germany).

Determination of natamycin: The quantitative determination of natamycin was carried out using an Agar diffusion method (EGOROV 1985). A biological standard curve was done between the logarithm of different concentrations of standard natamycin (kindly donated from Gist-Brocades Dairy Ingredients Group, Delft, the Netherlands) and the inhibition zone diameter of the susceptible strain of *Saccharomyces cerevisiae.*

Results

Suitability of different carbon sources for natamycin production

Various mono-carbohydrates (including different C_3, C_5 and C_6 compounds), di-saccharides and polysaccharides (all used in equal concentrations of 20 g/l) were tested in growth experiments for their ability to support the production of natamycin by *Str. natalensis.* Results for growth after 96 h on these different substrates, in shake flask cultures are given in Fig. 1. In case of mono-carbohydrates, with the exception of glycerol and xylose, monosugars supported the growth of microorganism and increased the cell growth to about 4 folds or more compared to control (medium without carbohydrate). On the other hand, all dissachride and polysaccharide carbohydrates supported only cell growth.

In fact, the microorganism was able to grow on all carbon sources tested, but natamycin was only produced on using glucose, glycerol, ribose, galactose, xylose, starch, dextrin and malt extract. In case of disaccharides, the production of natamycin was very weak. For better understanding the relation between cell growth and antibiotic production, the antibiotic yield based on biomass [$Y_{P/X}$] was calculated. As shown in Fig. 1A, the maximal cell efficiency for antibiotic production was of the following manner glucose > glycerol > xylose > malt extract > ribose > dextrin > starch > galactose. On the other hand, neither fructose nor inulin (fructose polymer) supported antibiotic production. The cultivation medium supplemented with glucose was thus employed in all further experiments.

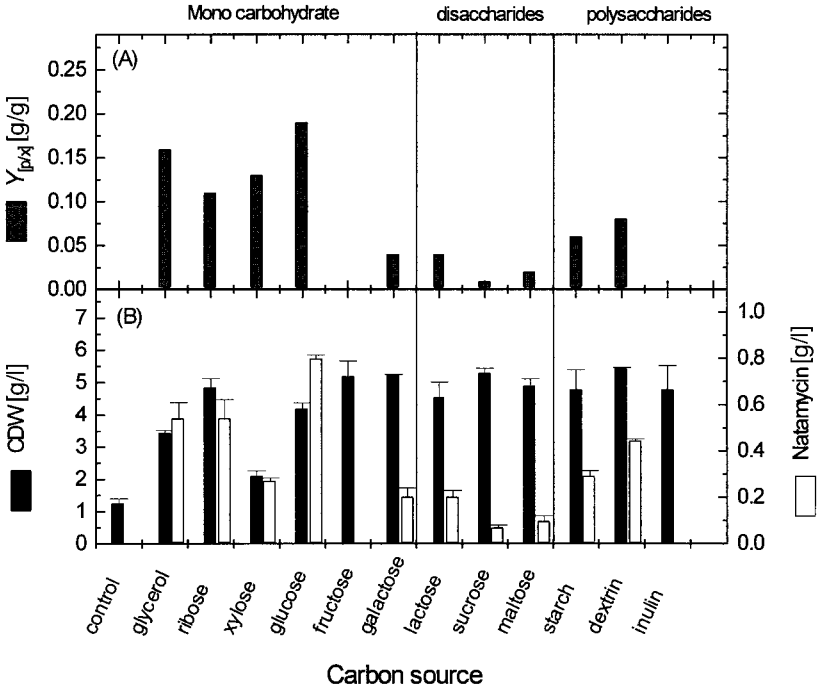

Fig. 1
Effect of different C-sources on the production of natamycin by *Streptomyces natalensis.* Data are the average of values taken from three shake-flask cultures. The standard error based on these three values was calculated and expressed as the error bar in the figures

Cell growth and natamycin production with different concentrations of glucose

Earlier experiment has shown that glucose is the best carbon source for natamycin production. It was thought, therefore, to test whether natamycin production could be improved by varying glucose concentration in the cultivation medium. For this purpose glucose was applied in different concentrations varied from 10 to 60 g/l. The results in Fig. 2 showed that both of volumetric and specific production of natamycin reached a maximal value at 20 g/l. Above this concentration, the volumetric and specific production decreased. As glucose concentration increased from 20 to 60 g/l the volumetric natamycin production decreased by approximately 55%. On the other hand, glucose was completely consumed when used at a concentration of 20 g/l or lower. The increase in glucose concentration above this level resulted in the accumulation of glucose in the cultivation medium and the remained amount depended on the initial concentration. Therefore, glucose in a concentration of 20 g/l was used in the subsequent experiments.

Effect of different phosphate concentrations on natamycin production

In general, phosphorus is essential for cell growth and regulation of several metabolic process. On the other hand, phosphate is one of the most important regulatory elements in the production of antibiotics. As shown in Fig. 3, phosphate addition to the cultivation medium in the concentration of 0.05 g/l increased the antibiotic production by about 8%. On further increasing phosphate concentration to 1.0 g/l, natamycin production decreased by about 64% even though cell dry weight significantly increased (Fig. 3). Glucose was completely consumed in all cultures. The value of the specific antibiotic production based on the glucose

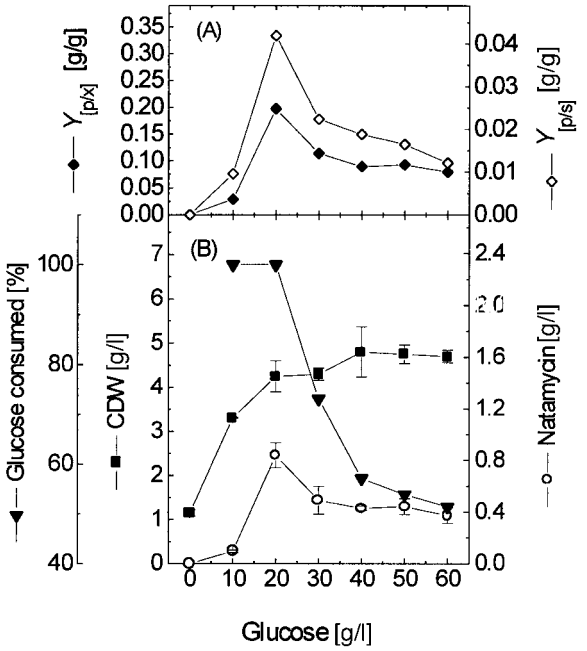

Fig. 2
Effect of different glucose concentrations on cell growth and natamycin production after 96 h cultivation

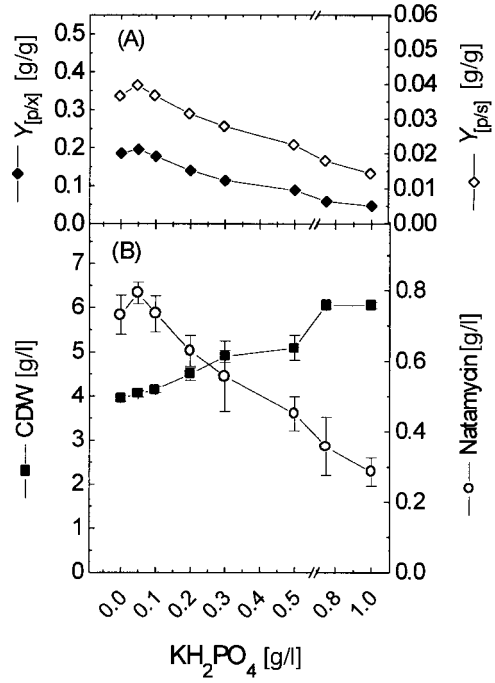

Fig. 3
Effect of different phosphate concentrations on cell growth and natamycin production after 96 h cultivation

consumption [$Y_{p/s}$] decreased with increasing the phosphate concentration. This also indicated that the presence of excess inorganic phosphate in the culture medium supported cell growth on the expense of antibiotic production.

Effect of nitrogen source on growth and natamycin production

Different nitrogen sources, inorganic ($NaNO_3$, NH_4Cl and $(NH_4)_2SO_4$) and organic (urea, yeast extract and beef extract) were tested. The results (Fig. 4) show that nitrogen source exhibited a significant effect on the natamycin production. The best nitrogen source for supporting antibiotic production was beef extract. Next, in order of suitability for natamycin production were sodium nitrate and ammonium sulphate. Ammonium chloride and urea did not support the antibiotic production. On the other hand, yeast extract was the best nitrogen source for supporting the cell growth. Therefore, an experiment have been done using a combined nitrogen source (yeast extract and beef extract). This mixed nitrogen source supported both cell growth and the antibiotic production and nearly doubled natamycin production compared to the other culture containing beef extract alone.

Effect of beef extract and yeast extract in combination on the production of natamycin

As shown in the previous experiment, the concomitant addition of beef extract and yeast extract to the cultivation medium significantly influences the antibiotic production. Therefore, an experiment have been designed to find out the optimal concentration of each component when applied in combination.

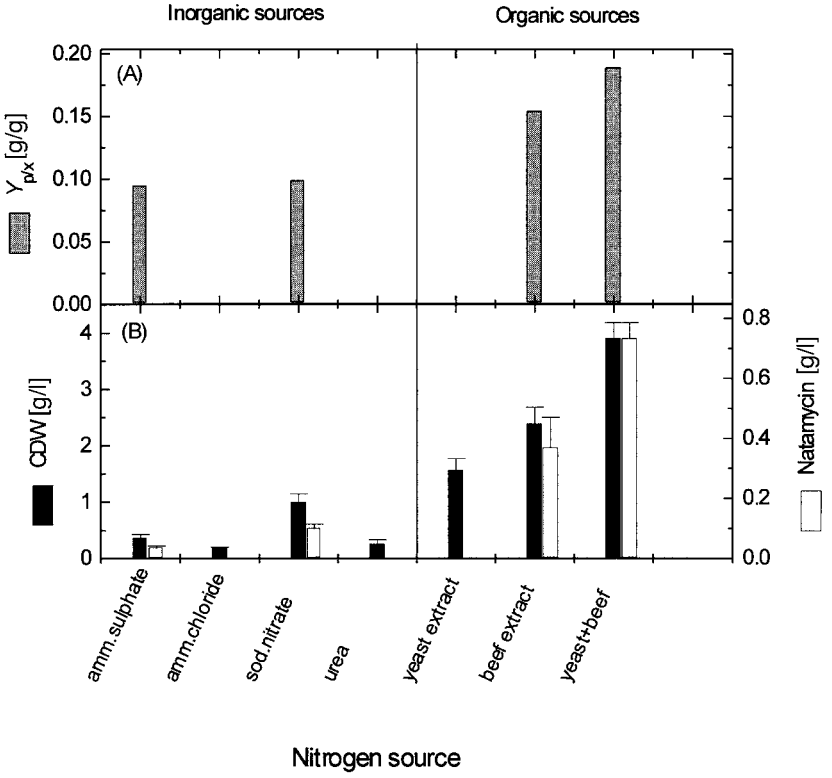

Fig. 4
Effect of different N-sources on cell growth and natamycin production after 96 h cultivation

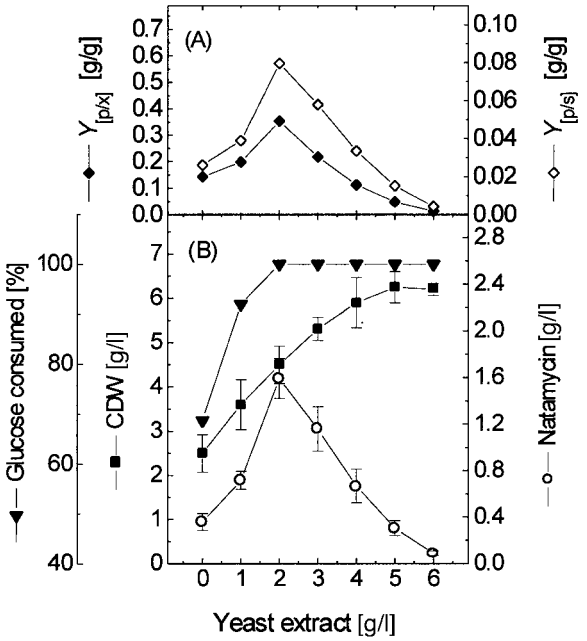

Fig. 5
Effect of different yeast extract concentrations on cell growth, glucose consumption and natamycin production after 96 h cultivation

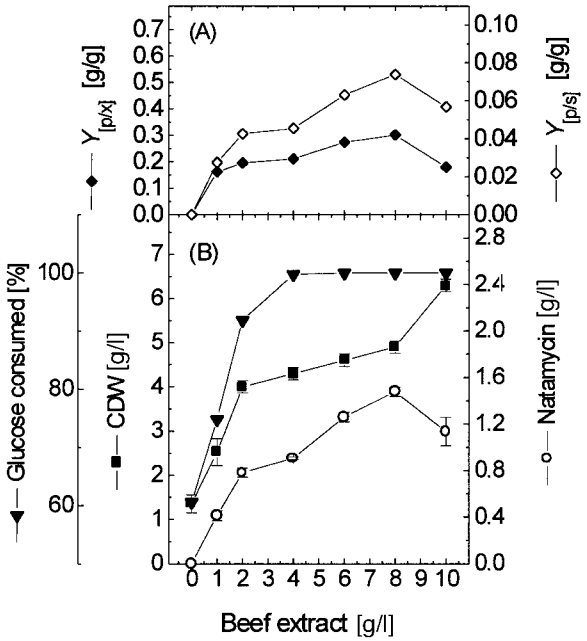

Fig. 6
Effect of different beef extract concentrations on cell growth, glucose consumption and production of natamycin after 96 h cultivation

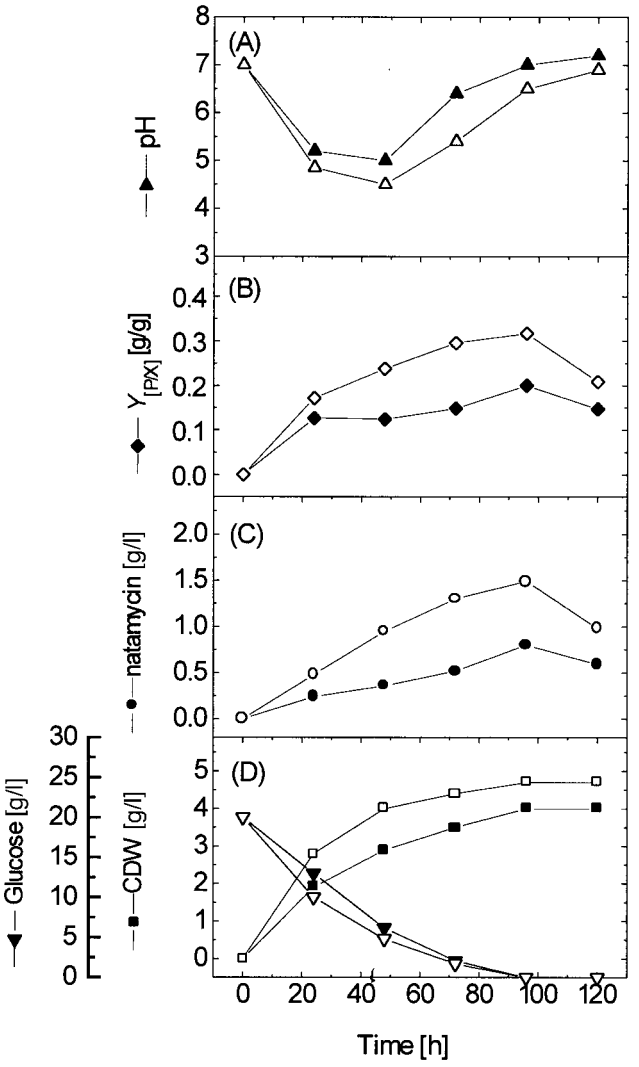

Fig. 7
Time course of cell dry weight, natamycin, glucose consumption, specific antibiotic production and pH in the culture broth of optimized and non-optimized medium. The open and closed symbols represent the optimized and non-optimized medium, respectively

Str. natalensis cultivation was carried out in a medium supplemented with 20 g/l glucose with varying concentrations of the complex nitrogen sources beef extract (BE) and/or yeast extract (YE). The results in Fig. 5 showed that, the presence of BE (2 g/l) with different concentrations of YE (0−2 g/l) resulted in more than 3 times increase in natamycin production. Further increase in YE concentration resulted in a gradual decrease in both volumetric and specific antibiotic production. On the other hand, the increase in YE concentration enhanced cell growth where it reached more than 6 g/l. In all cultures of 2 g/l YE or more, glucose was completely consumed after 96 h. Lower YE concentrations resulted in an incomplete consumption of glucose even after long cultivation time (data not shown).

On varying BE concentration in the presence of YE 2 g/l (Fig. 6), a significant relation between BE concentration and antibiotic production was observed. The production of natamycin increased gradually with the increase of BE concentration up to 8 g/l concomitant with an increase in cell growth. However, the increase in antibiotic titer was not only due to the increase in the microbial growth but also due to the increase of cell productivity while the value of specific production [$Y_{p/s}$] reached also a maximal at 8 g/l BE. Further increase in BE concentration enhanced cell growth and did not support the antibiotic production.

From the previous results, it is clear that both beef and yeast extract are indispensable not only for the cell growth but also to support good antibiotic production.

For better understanding how far the medium composition influenced the cell growth and antibiotic production, a comparative growth curve of *Str. natalensis* in both optimized and non-optimized medium was done. Fig. 7 shows that, in spite of the cell growth did not increase significantly in the optimized medium compared to the initial medium, the antibiotic production reached about 1.5 g/l (about two-folds higher than the initial medium) after 96 h cultivation. Further incubation decreased the titer of antibiotic. In both cultures, natamycin production was produced throughout the period of active growth and decomposed after C-limitation.

Discussion

The biosynthesis of secondary metabolites by *Streptomycetes* is controlled by several external (cultivation conditions) and internal factors (medium composition) involved a highly complex regulation. Carbon source studies on natamycin production by *Str. natalensis* revealed that, among different mono-carbohydrates tested, glucose was the best source to support the total volumetric and specific antibiotic production. This may be attributed to the fact that, glucose is incorporated in the natamycin molecule (GIL *et al.* 1984). These results are also in agreement with MARTIN and MCDANIEL (1977). They reported that glucose is the best carbon source for most polyene antibiotic production. The optimal concentration of glucose in the cultivation medium was 20 g/l, above which a significant decrease in natamycin was observed. MARTIN and MCDANIEL (1977) and GIL *et al.* (1984) reported that, in candidin fermentation higher initial glucose concentration retarded cell growth and resulted in a significant decrease in antibiotic production. The negative effect of high glucose concentrations may be explained on the basis of carbon catabolite regulation mechanism.

Upon studying the effect of medium supplementation with inorganic phosphate, results revealed that higher phosphate concentrations, above 0.05 g/l potassium dihydrogen phosphate, decreased the production of antibiotic which accompanied by a significant increase in cell growth. The biosynthesis of many antibiotics is controlled by the concentration of phosphate in the cultivation medium and most of them are produced only at inorganic phosphate concentrations that are suboptimal for growth and sometimes under phosphate limiting condition (WEINBERG 1974). Several authors reported also that polyene macrolid antibiotics are suppressed within a range of permissive phosphate concentration such as nystatin and amphotericin B (DONOVICK and BROWN 1965). Also, BELOUSOVA *et al.* (1970) found that inorganic phosphate decreased the biosynthesis of levorin produced by *Streptomyces levoris.* Also, our results are in agreement with LIU *et al.* (1975) who found that the addition of 10 mM inorganic phosphate to the candicidin production medium resulted in a 2-fold increase in mycelial growth concomitant with a significant decrease in antibiotic production.

Among different nitrogen sources investigated, results revealed that inorganic nitrogen sources did not support both cell growth and natamycin production. On the other hand, beef extract (BE) combined with yeast extract (YE) gave the highest amount of natamycin. In

such case, the natamycin volumetric production was about 2-fold higher than that obtained upon using BE alone. These results are in agreement with those observed by GIL *et al.* (1984) where they reported that the utilization of inorganic nitrogen source resulted in a poor production in case of polyene macrolid antibiotic. Complex nitrogen sources are generally recommended for large scale antibiotic production. Also, OLSON (1993) reported that natamycin cultivation medium should contain protein nitrogen source for better antibiotic production. EISENSCHINK and OLSON (1993) used a mixture containing a non-yeast protein nitrogen compound and a yeast protein to increase the production of natamycin.

In general, yeast extract is a complex nitrogen source which contains amino nitrogen (amino acids and peptides), water soluble vitamins, and carbohydrates. Moreover, the stimulatory effect of YE on natamycin production may be due to the presence of certain trace elements which exhibit a stimulatory effect on certain antibiotic production. Also, KAWAGUCHI *et al.* (1984, 1988) reported that the B-factor isolated from YE was found to act as inducer for rifamycin production in non-producing mutants. Similarly, BE provides the culture with adequate amounts of trace elements, vitamins, fatty acid precursors and their low phosphate content is necessary for the biosynthesis of antibiotic (GIL *et al.* 1984). Also, BE provides certain metal ions (Zn, Fe and Mg) that act either as activators for the enzymes which are necessary for the biosynthesis of secondary metabolites or as binding agent for the inhibitory effect of high phosphate concentration (MARTIN and MCDANIEL 1977).

Concluding, our results revealed that optimization of medium composition allowed a significant increase in natamycin production by *Str. natalensis*. The highest concentration of natamycin reaching about 1.5 g/l was produced under nutritional conditions when glucose was used as a C-source at 20.0 g/l and supplemented the medium with beef extract and yeast extract at 8 g/l and 2.0 g/l, respectively.

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