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Semi-solid-state fermentation: A promising alternative for neomycin production by the actinomycete *Streptomyces fradiae*

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ABSTRACT

The production of neomycin by the actinomycete *Streptomyces fradiae*, under semi-solid-state fermentation conditions was the main subject of this study. Two supports (nylon sponge and orange peelings) were tested in order to determine the most suitable one for the production of neomycin by the above-mentioned microorganism. Nylon sponge led to the highest neomycin production, reaching a maximum value of 13,903 μg/mL on the 10th day of cultivation. As a control, the same experiment was performed under submerged fermentation (SmF) conditions, without solid support. Here the production of neomycin by *S. fradiae* was about 55-fold lower (i.e. 250 μg/mL) than that obtained for SSF.

1. Introduction

Neomycin (Fig. 1) is an important antibiotic, which belongs to the aminoglycoside family. It is widely applied in pharmaceutical preparations for local applications and in the veterinary practice. It was discovered in 1949 by the microbiologist Selman Waksman and his student Hubert Lechevalier, who isolated the neomycin-producing bacterium *Streptomyces fradiae* (Waksman and Lechevalier, 1949). Much later, a new neomycin-producer identified as *Streptomycens marinensis* was found (Sambamurthy and Ellaiah, 1974). This antibiotic is effective against Gram-negative, Gram-positive and acid fast bacteria.

Neomycin has a spectrum of antibiotic activity higher than that of penicillin, streptomycin and bacitracin and, although not being active against fungi is, beyond bacteriostatic, bactericidal, killing the cells against which it acts (Waksman and Lechevalier, 1949). In its sulphate form, as mainly used, neomycin comprises a mixture of three structurally-related compounds: neomycin A (usually referred to as neamine), neomycin B (also known as framycetin) and neomycin C (Fig. 1). Neomycin B is the main component of the mixture and has the highest antibiotic activity. Neomycin C is the less active minor component. Neomycin A is the hydrolytic degradation product of neomycin B and C and has only 10% of the antibiotic activity of the major components (Yuan et al., 2006).

Neomycin is commercially produced by submerged fermentation (SmF). However, this process requires high energetic expenditures. In the search for more economical fermentation processes with high antibiotic activity, solid-state fermentation (SSF) appears as an attractive alternative. Thus, Ellaiah et al. (2004) showed that SSF led to higher neomycin production than SmF by a mutant strain of *Streptomyces marinensis*. In addition, SSF is claimed to be more cost-effective than SmF. Thus, Castilho et al. (2000) found that the production of lipase by *Penicillium restrictum* resulted economically more advantageous in SSF than in SmF. More recently, Osma et al. (2011) have shown that the cost of producing the enzyme laccase by the white-rot fungus *Trametes pubescens* was 50-fold lower in semi-solid-sate fermentation conditions than in SmF.

SSF is defined as any fermentation process in which microorganisms grow on solid support materials in the absence of free-flowing water (Pandey, 1992). Two types of solid supports can be used in SSF: natural supports (e.g. lignocellulosic wastes) and inert supports (e.g. plastic foams). SSF has gained much interest in recent years due to the advantages that presents over SmF such as higher product yields, less energy requirements,

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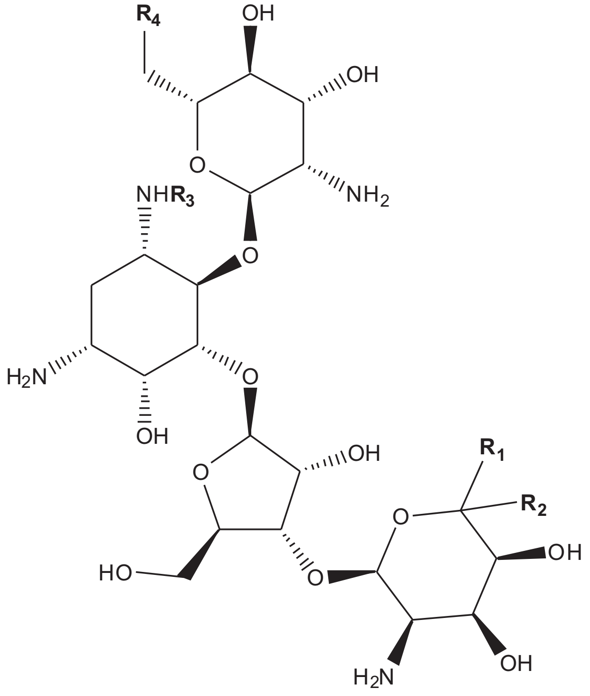

Fig. 1. Molecular structure of the main components of neomycin. Neomycin B when $R_1 = H$, $R_2 = CH_2NH_2$, $R_3 = H$, $R_4 = NH_2$; neomycin C when $R_1 = CH_2NH$, $R_2 = H$, $R_3 = H$, $R_4 = NH_2$; and neomycin A (or neamine) when the structure is only constituted by rings of R3 and R4, $R_3 = H$, $R_4 = NH_2$.

easier aeration, less wastewater generation, reduced bacterial contamination and easier product recovery (Pandey et al., 2000). Hence, in the present paper the production of neomycin by *S. fradiae* grown under semi-solid-state fermentation conditions was investigated. This type of fermentation is a sort of SSF in which the free liquid content has been increased in order to facilitate nutrient availability and fermentation control (Rodriguez-Couto et al., 2002; Economou et al., 2010). To the best of our knowledge the production of neomycin by *S. fradiae* under semi-solid-state fermentation conditions has not been reported before this study.

2. Materials and methods

2.1. Microorganisms

Streptomyces fradiae DSMZ 40063 was obtained from the German Collection of Microorganism and Cell Cultures (Germany). The bacterium was grown on Petri plates containing 4 g/L glucose, 4 g/L yeast extract, 10 g/L malt extract, 2 g/L $CaCO_3$ and 12 g/L agar agar at pH 7.2–7.4 for 5 days at 28–30 °C. Then, the plates were maintained at 4 °C and sub-cultured every 3 weeks.

Staphyloccocus epidermidis DSMZ 1798 was obtained from the German Collection of Microorganism and Cell Cultures (Germany). This bacterium was used as a test organism for the determination of the neomycin produced by *S. fradiae* applying the Kirby–Bauer test. *S. epidermidis* was grown on Petri plates containing 10 g/L peptone from caseine, 5 g/L yeast extract, 5 g/L glucose, 5 g/L NaCl and 5 g/L agar agar at pH 7.2–7.4 for 5 days at 28–30 °C. Then, the plates were maintained at 4 °C and sub-cultured every 1–2 weeks.

2.2. Supports

2.2.1. Inert support

Cubes (edge size 1.0 cm) of nylon sponge (Scotch Brite, 3 M Spain, S.A.) were used as inert supports. Prior to use, the cubes of nylon sponge were pre-treated by boiling for 10 min and washing thoroughly three times with distilled water (Linko, 1991). Thereafter, the cubes were dried in an oven at 30 °C.

2.2.2. Natural support

Orange fruits (*Citrus sinensis*) were obtained from a local market and the peelings were collected after the normal human consumption of the fruit flesh. The orange peelings are mainly composed of both soluble and insoluble carbohydrates. The soluble sugars in orange peelings are glucose, fructose and sucrose. The insoluble polysaccharides in cell walls of orange peelings are composed of pectin, cellulose and hemicellulose. They also contain minor amounts of organic acids, mainly citric, malic, malonic and oxalic, proteins, mineral ions, phenolic compounds and polyols (Grohmann et al., 1995). Prior to use, the orange peelings (size 1.5 cm × 1.5 cm) were pre-treated as follows: they were first soaked for 1 h in 30 mL of KOH 83.17 mM (10 g of fresh peelings) to neutralise organic acids (Stredansky and Conti, 1999). Then, they were thoroughly washed with distilled water and dried in an oven at 30 °C.

Prior to use, all the supports were autoclaved at 121 °C for 20 min.

2.3. Inoculum preparation

At the 5th day of grown of *S. fradiae*, 5 mL of KCl solution (20 g/L), previously sterilised, were added to the Petri plates. The spores were scraped and transferred into 100-mL cotton-plugged Erlenmeyer flasks containing 30 mL of inoculum medium, which was composed of 10 g/L glucose, 20 g/L soya peptone, 5 g/L meet extract, 5 g/L NaCl and 10 mg/L $ZnSO_4\ 7H_2O$ in tap water (initial pH 7.4). The flasks were incubated on an orbital shaker at 200 rpm and 30 °C for 48 h. After centrifugation (5000 rpm, 20 min), the resulting pellets were washed with a sterile solution of KCl (20 g/L) several times and re-suspended in 30 mL of the same saline solution. This cell suspension was used as inoculum in the experiments.

2.4. Submerged fermentation

The experiments were performed in 500-mL cotton-plugged Erlenmeyer flasks containing 90 mL of culture medium and 10 mL of inoculum. The composition of the culture medium was the same as that used for the inoculum. The flasks were incubated on an orbital shaker at 200 rpm and 28–30 °C for 10 days. Samples were only taken on days 4, 6, 8 and 10 of incubation. At the end of fermentation, the entire content of each flask was centrifuged at 5000 rpm for 20 min. The pellet was collected for biomass determination by means of its dry weight and the supernatant for the determination of neomycin by applying the Kirby–Bauer test.

2.5. Semi-solid-state fermentation

Experiments were conducted in 250-mL cotton-plugged Erlenmeyer flasks containing 2 g of nylon sponge cubes or 15 g of orange peelings, according to the experiment, 30 mL of culture medium and 3 mL of inoculum. The amount of support used was selected according to previous experiments. The composition of the culture medium was the same as that used for the inoculum. The flasks were incubated in static conditions at 28–30 °C and in complete darkness for 12 days. Samples were taken on days 4, 6, 8, 10 and 12.

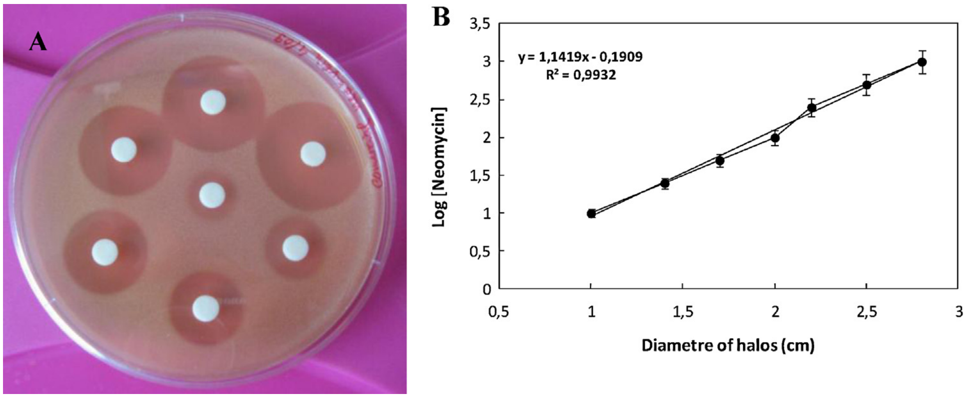

Fig. 2. (A) Kirby–Bauer test for the determination of the calibration curve from the inhibition zones of growth of *Staphylococcus epidemidis* with increasing concentrations of sulphate neomycin from 10 to 1000 μg/mL. (B) Determined calibration curve.

Neomycin was extracted by adding 5 mL of phosphate buffer (pH 8.0) in each Erlenmeyer flask (Adinarayana et al., 2003) in order to get the liquid retained by the supports. Then, the flasks were kept on an orbital shaker at 200 rpm for 1 h and afterwards the content of each flask was centrifuged (5000 rpm, 20 min) and filtered (0.22 μm). This filtrate was used to determine the content of neomycin by the agar diffusion method, also known as Kirby–Bauer test. In this case the biomass could not be determined since it was tightly bound to the support.

All the experiments were done in triplicate and mean values were reported.

2.6. Analytical determinations

Reducing sugars were measured by the dinitrosalicylic (DNS) acid method, using D-glucose as a standard, according to Miller (1959).

Neomycin concentration was determined by the standard agar diffusion method using *S. epidermidis* as a test organism (Grove and Randall, 1955). Antibiotic-assay discs (Aldrich Whatman® Schleicher & Schuell®) were impregnated with 50–90 μL of sample, placed on agar plates and the inhibition zones measured (in cm) after 24 h of incubation at 30 °C. Standard neomycin sulphate (Vetranal, Sigma–Aldrich) was used to construct the calibration curve (Fig. 2).

3. Results and discussion

3.1. Neomycin production by SmF cultures of S. fradiae

In Fig. 3A–C the halos corresponding to neomycin inhibition for the samples collected on days 4 to 10, can be observed.

Fig. 3D shows the evolution of the reducing sugars and the neomycin production by *S. fradiae* grown under SmF conditions. It can be seen that the neomycin production started after glucose, measured as reducing sugars, began to decrease. This indicates that neomycin production by *S. fradiae* is triggered by carbon depletion.

The maximal concentration of neomycin was detected on the 10th cultivation day with a value of about 250 μg/mL (Fig. 3D). However, the concentration determined on the 6th cultivation day was higher than that determined for day 8. This is due to bacterial growth that was lower on the 8th cultivation day than on the 6th one as showed the biomass values in Fig. 3E. Consequently concentrations of neomycin and reducing sugars were also lower on the 8th cultivation day.

Fig. 3C shows the halos corresponding to neomycin inhibition for the samples collected on the days 8 and 10. It can be seen that the bacterium grows forming independent colonies, unlike the other Petri plates where the growth is more homogeneous. This was likely due to the heterogeneous diffusion of the antibiotic on the Petri plates due to its high concentration.

3.2. Neomycin production by semi-solid-state fermentation cultures of S. fradiae

In the nylon sponge cultures, the first sample was collected on the 2nd day and the last one on the 12th day of incubation. The neomycin production started very early (2nd day) and glucose, measured as reducing sugars, was not totally depleted. Then, the neomycin production increased peaking on the 10th day with a value of 13,903 μg/mL (Fig. 4A). This value is 55-fold higher than that obtained in SmF. In addition, it is about 4-fold higher than that reported by Ohta et al. (1995) by *S. fradiae* grown in a stirred-tank reactor and by Vastrad and Neelagund (2011) by *S. fradiae* grown on apple pomace under optimised SSF conditions.

In the orange peelings cultures, neomycin production did not start until the 10th cultivation day (Fig. 4B). The increase in reducing sugars on the 2nd day was likely due to the hydrolysis of the sugars contained in the orange peelings during autoclaving. The consumption of glucose, measured as reducing sugars, was much slower than in the nylon sponge cultures. Glucose concentration, measured as reducing sugars, from day 4 onwards was higher in orange peeling cultures than in nylon sponge ones. This might be due to the reduction of some of the carbohydrates contained in the peelings by the bacterium.

The amount of neomycin produced (281.4 μg/mL on days 10 and 12), although slightly higher than that obtained in SmF, was much lower than the one achieved operating with nylon sponge as a support. In addition, the lag phase lasted 9 days. Therefore, orange peelings are not a suitable support for the production of neomycin by *S. fradiae* under semi-solid-state fermentation conditions. This indicates the importance of the support selection in semi-solid-state cultivation. Fig. 4C and D shows the halos corresponding to neomycin inhibition for the samples collected on days 2, 4 and 6 from nylon sponge (EN) and orange peeling (CL) cultures. The uneven growth of *S. epidermidis* on the agar plates was likely due to the heterogeneous diffusion of the antibiotic on the agar plates caused by its high concentration.

The higher neomycin production obtained using cubes of nylon sponge as supports was likely due to the high porosity of the nylon sponge which allowed a better diffusion of oxygen and nutrients into the cultures, thus favouring the production of the antibiotic.

The higher cost of using an inert support can be overcome by the advantages it presents over a natural one such as decreasing the cost of the downstream processing, improving process

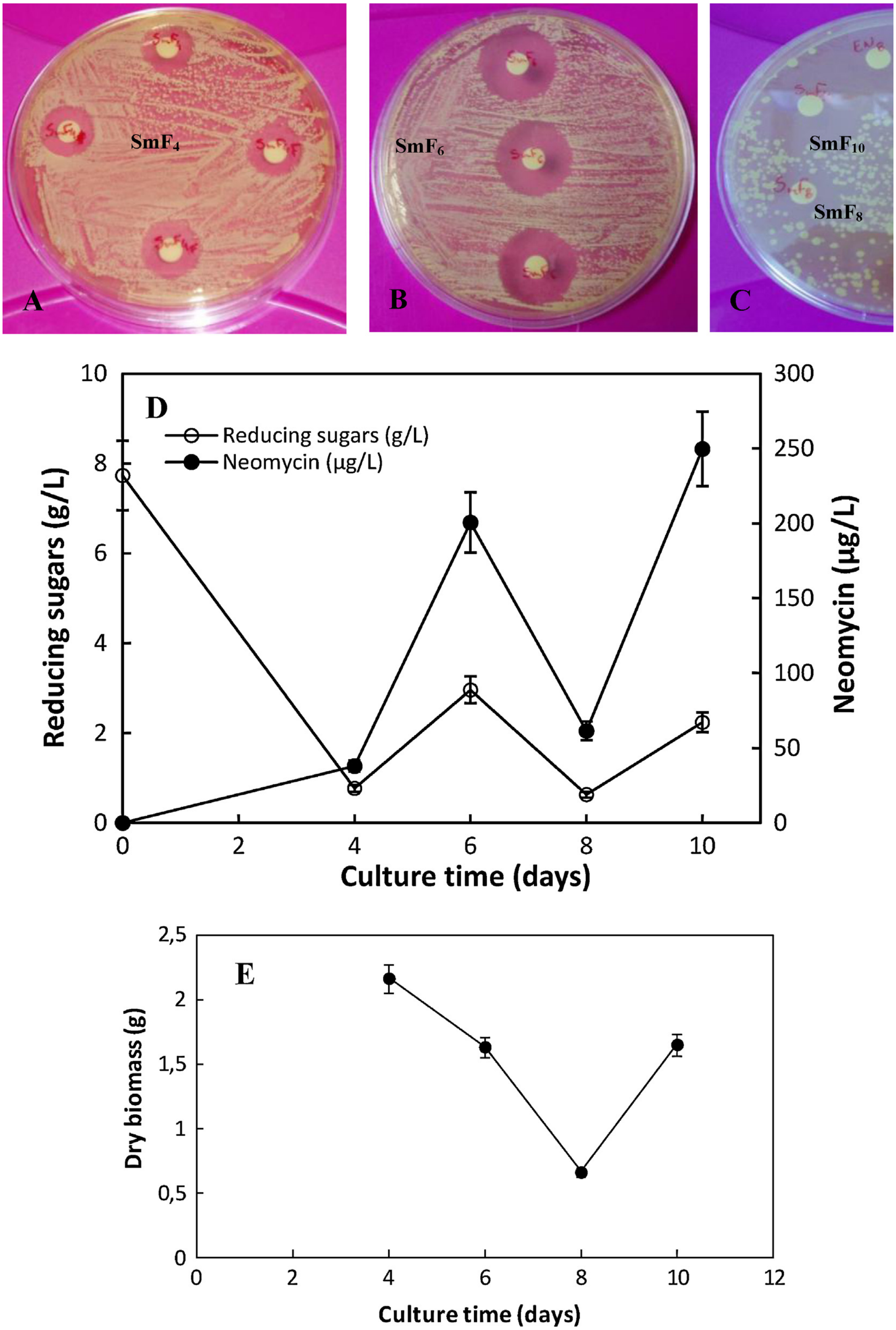

Fig. 3. Kirby–Bauer test for samples collected on days (A) 4; (B) 6; (C) 8 and 10 of incubation, from *Streptomyces fradiae* grown under submerged fermentation (SmF) conditions with *Staphyloccocus epidermidis* as a sensitive bacterium. (D) Glucose consumption, measured as reducing sugars, and neomycin production by *Streptomyces fradiae* grown under submerged fermentation conditions. (E) Biomass determined as dry weight.

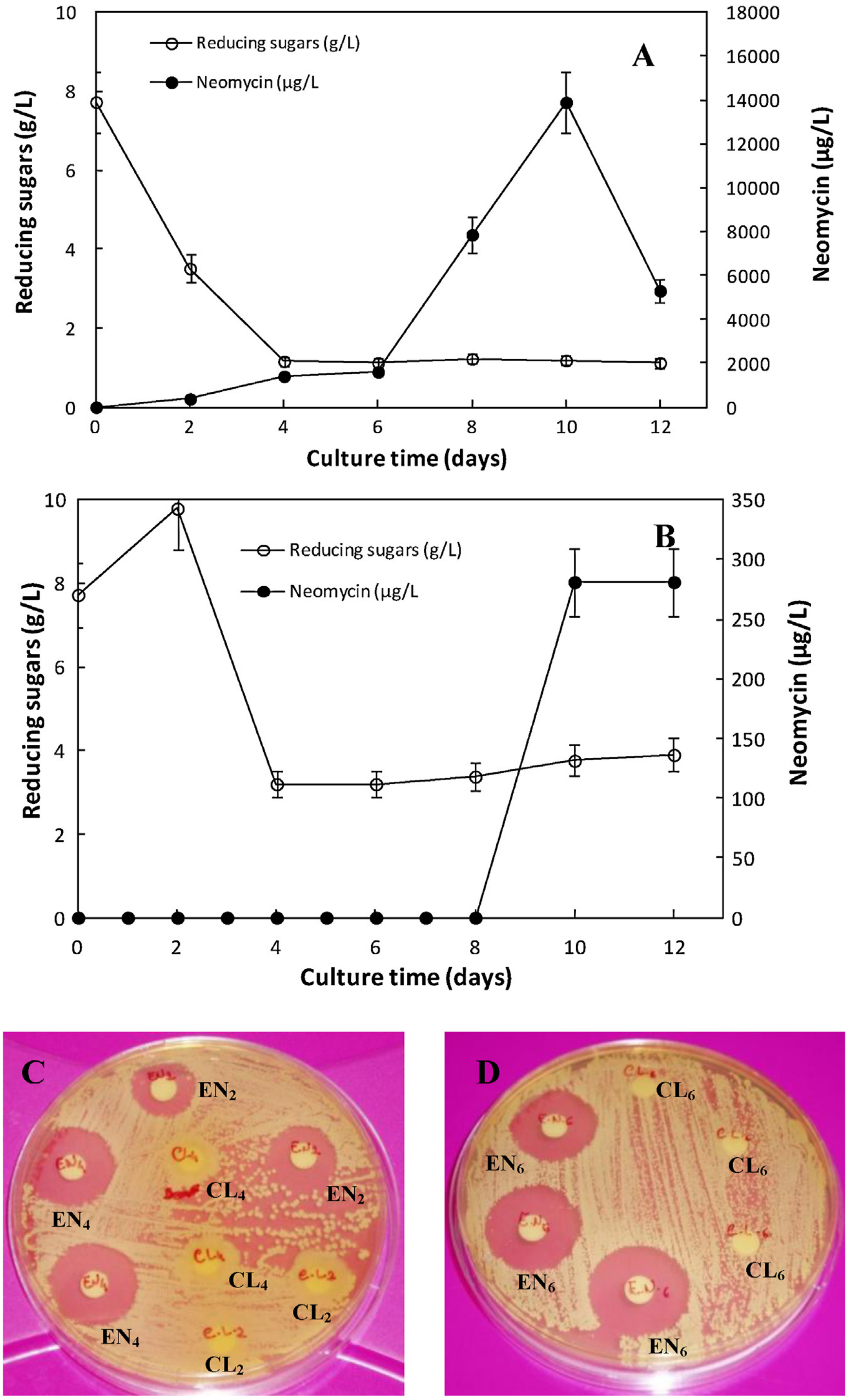

Fig. 4. Glucose consumption, measured as reducing sugars, and neomycin production by *Streptomyces fradiae* grown on: (A) cubes of nylon sponge and (B) orange peelings, under semi-solid-state fermentation conditions. Kirby–Bauer test for samples from *Streptomyces fradiae* grown on nylon sponge (EN) and orange peelings (CL) under semi-solid-state fermentation conditions for (C) the 2nd, 4th days and (D) 6th day.

control and monitoring and enhancing process consistency (Ooijkaas et al., 2000). In addition, it allows the design of suitable production media. Moreover, as the physical structure of the inert support is maintained throughout the process it can be reutilised (Rodríguez-Couto, 2012).

4. Conclusions

SmF is usually used for the commercial production of neomycin. The results of this paper show that semi-solid-state fermentation operating with an inert support, such as nylon sponge, increased considerably the production of neomycin by *S. fradiae* with a maximum antibiotic production of 13,903 μg/mL. This value is 55-fold higher than that obtained in SmF conditions. Therefore, it can be concluded that semi-solid-state fermentation holds great promise for neomycin production.

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