J. Sci. I. R. Iran

Vol.10 No.3
Summer 1999

STREPTOMYCES FRADIAE, A POTENT PRODUCER OF CHOLESTEROL OXIDASE

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Abstract

Thirteen species of *Streptomycetes*, *Arthrobacters* and *Mycobacterium* were studied for the determination of a potential cholesterol oxidase producer. Only *Streptomyces fradiae* and *Streptomyces rishiriences* were found to be capable of producing this enzyme. *Streptomyces fradiae* was selected for the production of cholesterol oxidase and cultured in different media to find optimum media and conditions for enzyme production. The optimum media was found (g/lit): glucose, 12; starch, 9; yeast extract, 6; peptone, 4; $(NH_4)_2SO_4$, 7.5; Tween 80, 0.5 with cholesterol, 2 as an enzyme inducer. 34°C was the optimum temperature for the production of cholesterol oxidase.

Introduction

Cholesterol oxidase [EC: 1.1.3.6] is an oxidoreductase enzyme that catalyses cholesterol into 4-cholesten-3-one in two stages. The main application of this enzyme is cholesterol measurement in a coupled reaction with cholestrol esterase and peroxidase[1,6,14,15,16,25,31] which is very important in clinical diagnosis. It has also been known in recent years as a potent insecticidal protein [17]. Due to its commercial importance, many studies for the production and purification of cholesterol oxidase have been carried out. Production of the enzyme was first reported in 1973 from *Brevibacterium* by Uwajima [26] and from *Nocardia* by Richmond [19]. The purification of the enzyme from *Streptomyces violascens* [4, 7,18, 22, 24] and *Brevibacterium sterolicum* [8,26,27,28] has also been studied. The production of this enzyme from *Streptomyces* sp. [10] and *Rhodococcus* (sp.) [9, 11, 23, 29, 30] are among the latest reports.

Since *Streptomyces spp.* have been known as the best producers of cholesterol oxidase[4,10,14], the ability of cholesterol oxidase production by some species of *Streptomyces*, *Arthrobacters* and *Mycobacteria* was reevaluated and optimization of media for enzyme production was carried out.

Keywords: Streptomycetes; Optimization; Cholesterol oxidase

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Materials and Methods

All chemicals were purchased from Sigma (USA) and the following microorganisms were received from Persian Type Culture Collection (PTCC): *Streptomyces murinus* (1136), *S. aerofaciens* (1118), S. *hygroscopicus* (1132), *S. fradiae* (1121), S. noursei (1140), S. *nitrosporous* (1138), *S. griseus* (1126). They were routinely maintained in solid media containing (g/lit): Yeast extract, 2: Peptone,

2; Glucose, 10; $FeSO_4$, 4; Agar, 15. pH was adjusted to 7.2 [13].

After 48 hours, the microorganisms were transferred into a production liquid medium which has reported as a suitable medium for the production of cholesterol oxidase by *Streptomyces sp.* [10]. Production medium consisted of (g/lit): starch, 10; glucose, 10; peptone, 6; yeast extract, 5; Cholesterol, 2; k_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 1; $MnSO_4.H_2O$, 0.008; $CuSO_4.5H_2O$, 0.002; $ZnSO_4.5H_2O$, 0.02 and $CaCl_2$, 0.0002. Cholesterol was dispersed by sonication of the medium for 10 minutes and pH was adjusted to 7.2 before sterilization. Samples were taken every 24 hours from each media for 120 hours.

Cholesterol oxidase activity was measured by a modified method of Sasaki et al. [20] as follows: 0.5 ml of enzyme solution was added to a mixture of 0.4 ml phosphate buffer (0.5M, pH 7.0), 0.3 ml peroxidase 20 u/ml in phosphate buffer (0.1M, pH 7.0), Triton-100 4% in phosphate buffer, (0.1 M, pH 7.0), 0.1 ml of o-dianizidine 0.5% in water and 0.05 ml cholesterol 1% in ethanol. The mixture was incubated at 37°C for 10 min. The mixture was boiled 5 minutes to deactivate the enzyme and the absorption of the sample was measured against blank at wavelength 460 nm using LKB ultrospec II. Growth of the microorganisms was determined as packed cell volume (PCV) by centrifugation of the fermented broth at 3000 RPM for 5 minutes as described by Hara et. al. [5].

Results

To find the best cholesterol oxidase producer, 100 ml of production medium was inoculated with the above microorganisms. It was found that five of the microorganisms: *S. fradiae*, *S. rishiriences*, *S. hygroscopicus*, *S. nitrosporous*, and *S. griseus* were capable of producing extracellular cholesterol oxidase (Fig.1). Among them, *S. fradiae* and *S. rishiriences* showed a relatively higher potential for enzyme production (0.03 and 0.025 U/ml respectively). Enzyme production by *S. rishiriences* was approximately as high as *S. fradiae*, but due to secretion of black pigments in the medium

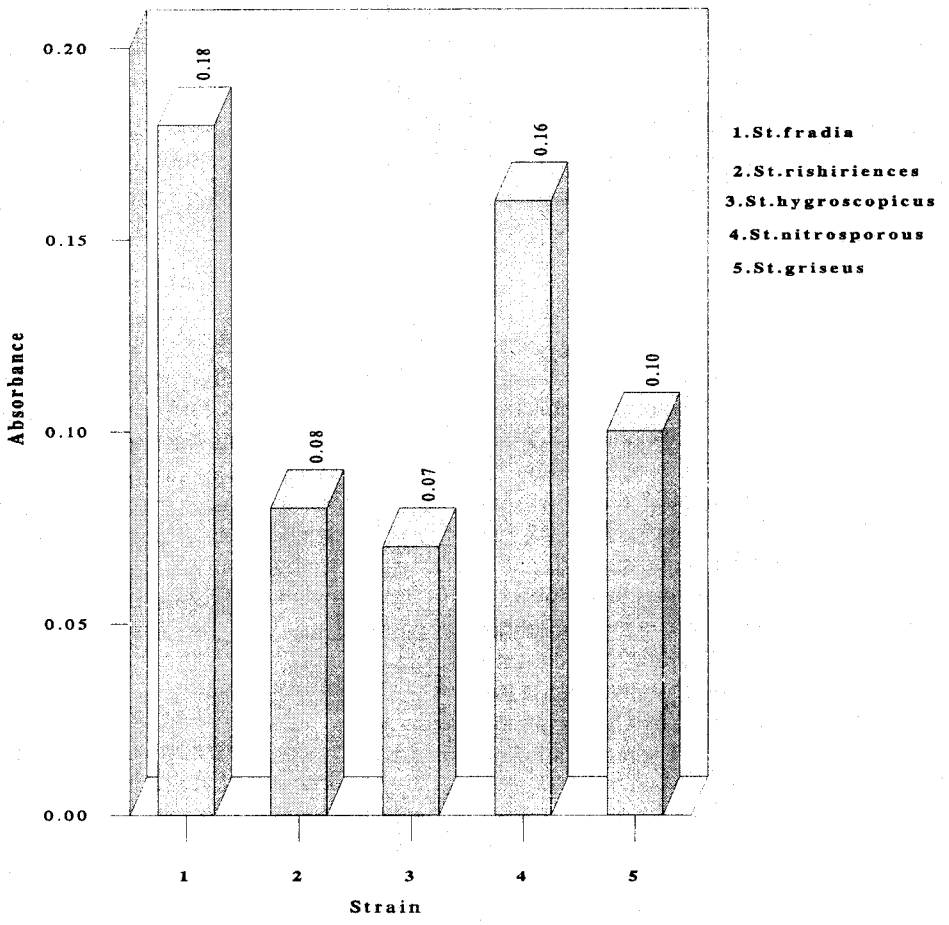

Figure 1. The ability of cholesterol oxidase production by different *streptomyces*

which interfered with the assay procedures, it was not selected for more studies. Due to the relatively high production level of extracellular cholesterol oxidase by *S. fradiae*, it was chosen as a superior source for further experiments.

The effects of various carbon sources on enzyme production by *S. fradiae* were investigated in the production media with replacement starch and glucose with 2% of the following carbon sources: glucose, maltose, sucrose, lactose, starch and starch plus glucose (1% of each). The results are shown in Figure 2. Cells grown on fructose showed a slightly higher level of enzyme production, but starch plus glucose were selected due to their availability. The effects of glucose concentrations in media containing 1% starch and also the effect of various starch concentrations in media with 1% glucose on enzyme production were also studied. It was found that the optimum concentration for highest enzyme production at conditions stated above was 1.2% glucose and 0.9% starch (Figs. 3,4).

The effects of different nitrogen sources on the enzyme production was investigated by adding some inorganic or complex nitrogen sources (1%) in basic medium such as: $(NH_4)_2SO_4$, NH_4NO_3, yeast extract, peptone and 0.5% yeast extract plus 0.5% peptone. The results showed that peptone, $(NH_4)_2SO_4$ and yeast extract pluspeptone caused the highest level of growth and production (Fig. 5). Optimum concentration of the nitrogen sources was also investigated, showing that the optimum concentrations for $(NH_4)_2SO_4$, yeast extract and peptone were 0.75%, 0.6% and 0.4% respectively (Figs. 6,7).

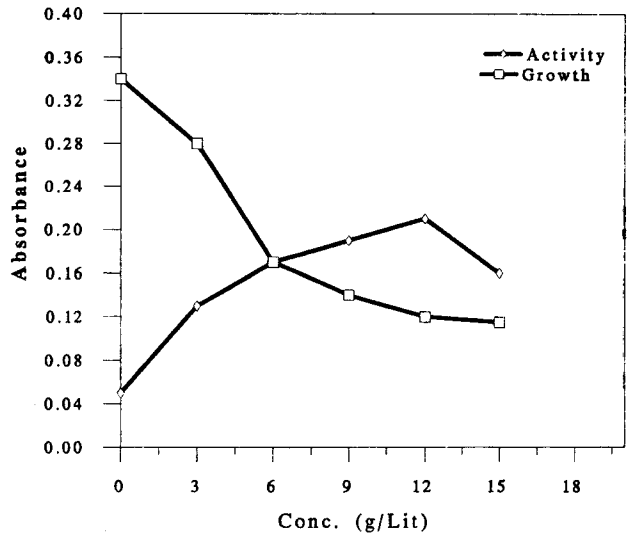

Figure 3. Effect of glucose concentrartion on cholesterol oxidase production and growth of *S. fradiae*

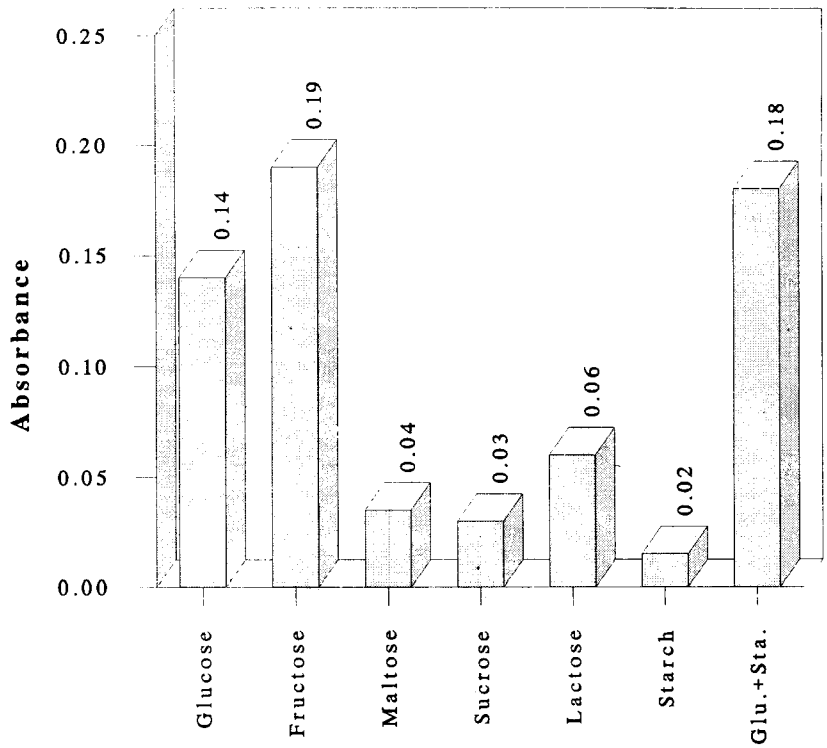

Figure 2. Cholesterol oxidase production by *St. fradiae* on different carbon sources.

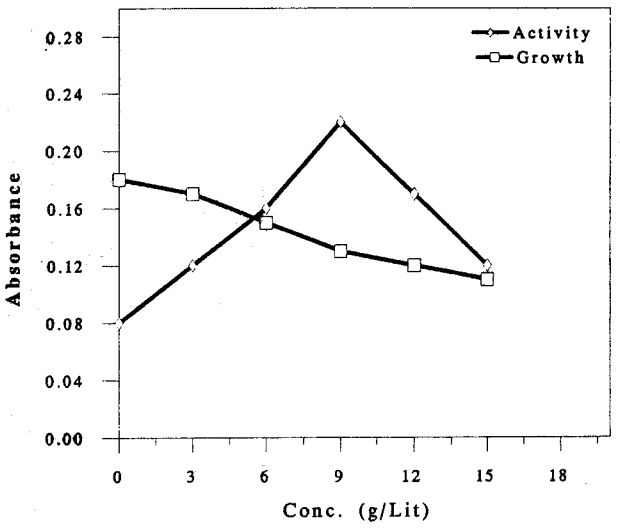

Figure 4. Effect of starch concentration on cholesterol oxidase production and growth of *St. fradiae*

The optimum concentration of cholesterol performed showed that 0.2% was optimum for the enzyme production (Fig. 8). While there was scarcely any production in the absence of cholesterol, at concentrations higher than 0.2% enzyme, production decreased significantly. This could be due to cholesterol coagulation, which makes it unavailable for microorganism.

The effect of Tween 80 and Triton X-100 (0.05%) on the enzyme production in optimized medium was investigated. The results showed that Tween 80 gives the highest yield of enzyme production. In the presence of Triton X-100, a reduction in enzyme production and growth was observed, which could possibly be due to changes in cell membrane (Fig. 9).

The effect of temperature on enzyme production and growth was also studied. There was no significant differences in growth at temperatures between 26° to 37°C. However, the optimum temperature for enzyme production was 34°C. (Fig. 10).

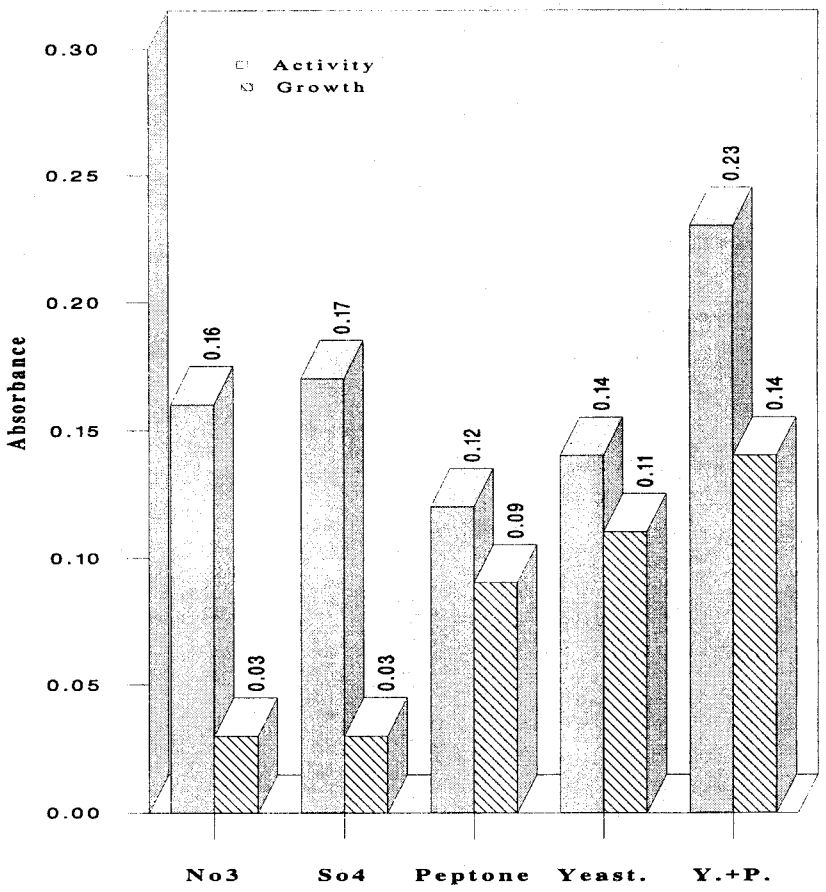

Figure 5. Effect of different nitrogen sources on cholesterol oxidase production and growth of *St. fradia*

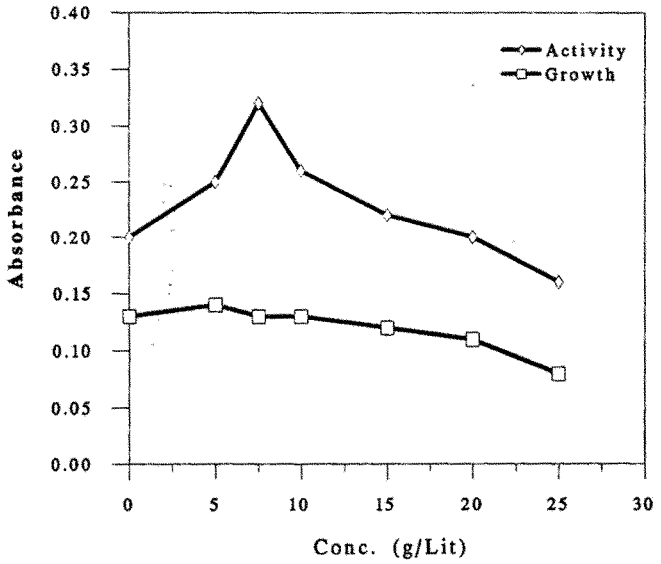

Figure 6. Effect of Ammonium sulfate concentration on cholesterol oxidase production and growth of *St. fradiae*

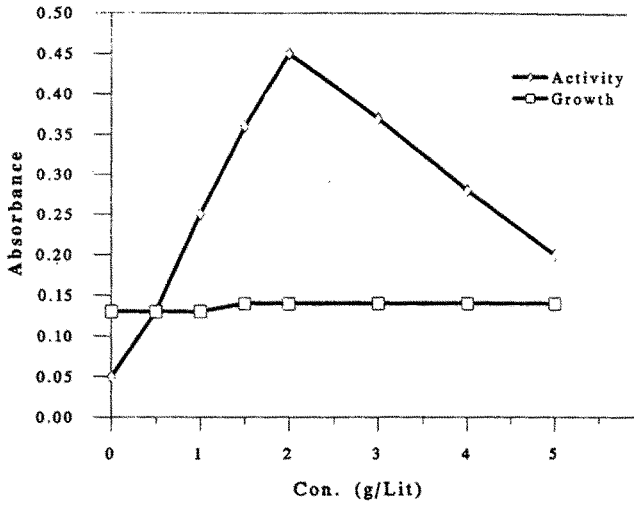

Figure 8. Effect of cholesterol concentration on cholesterol oxidase production and growth of *St. fradiae*

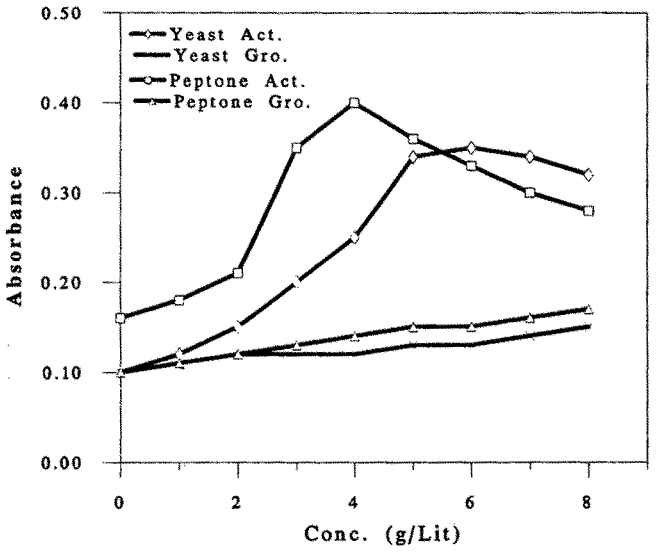

Figure 7. Effect of yeast extract and peptone concentration on cholesterol oxidase production and growth of *St. fradiae*

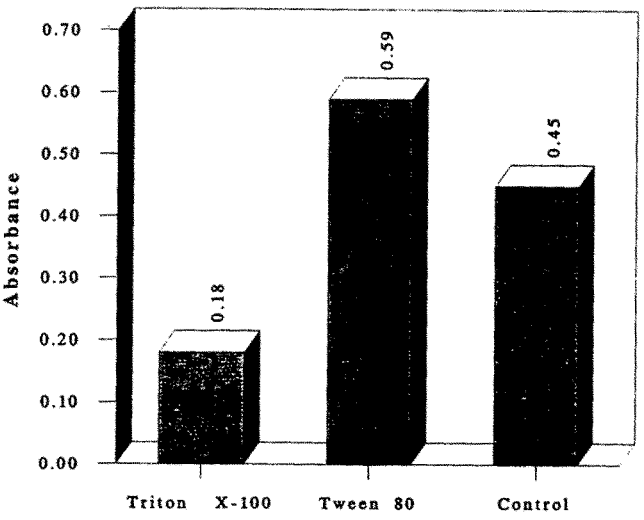

Figure 9. Effect of different detergents in cholesterol oxidas production by *St. fradiae*

Discussion

There are many reports on cholesterol oxidase production by *Streptomyces* spp. [3, 4, 10, 23] and other microorganisms such as *Brevibacterium* sp. [25, 26] and *Rhodococcus* sp. [9, 11, 23, 28, 29], but, there is no report on cholesterol oxidase production by *S. fradiae*. In this study, *S. fradiae* was found to be a good source for extracellular producton of cholesterol oxidase, which in this regard, is similar to *S. violascens* [7, 24] *Brevibacterium sterolicum* [8, 26] and *Arthrobacte simplex* [12, 18]. Cholesterol oxidase may be existed i both intra and extra-cellular locations, as reported i *Corynebacterium cholesterolicum* [20], but due to th economical importance of extracellular production c cholesterol oxidase, intracellular enzymes were n considered. Optimization of media for production of th

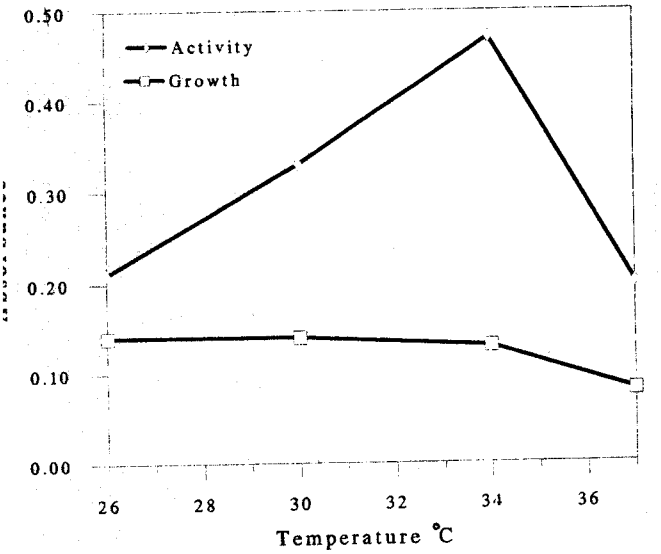

Figure 10. Effect of temperature on cholesterol oxidase production and growth of *St. fradiae*

highest level of cholesterol oxidase in *Stereptomyces* sp. has been the subject of many studies [10]. The basic medium in this study was the same medium which was reported by Lartillot as the optimized media for *Streptomyces* sp. [10]. Our results showed that *S. fradiae* requires nearly the same chemicals as other *Sterptomyces* sp. for maximum enzyme production. The only major additional requirement in the optimized medium in comparison with basic media was 0.5% $SO_4(NH_4)_2$ which increased enzyme production up to 50% without any change in growth. Higher concentration of $SO_4(NH_4)_2$ up to 2% did not affect on growth, but decreased enzyme production. It has been shown that some of non-ionic detergents increase cholesterol oxidase production in *Nocardia* sp. [2, 21], *Streptomyces* sp. [10] and *Rhodococcus* sp. [11, 23, 30]. This could possibly be due to the increasing solubility of cholesterol in the presence of detergent, which makes it more accessible for microorganisms. Our results showed that Tween 80 (0.05%) increased the enzyme production up to 30%, while Triton X-100 decreased enzyme production significantly.

According to these results, *S. fradiae* can be considered a potent source for cholesterol oxidase production and requires a further comprehensive investigation on the purificantion and properties of enzymes.

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