Enhanced production of streptomycin and hydrolytic enzymes by *Streptomyces griseus* strains using different types of organic solvents and detergent compounds

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Different organic solvents and detergents showed profound effects on the production of streptomycin (SM), amylases and proteases by *Streptomyces griseus* strains. The maximum SM production (1128 and 780 mg/L) was obtained using *Streptomyces* NRRL-strain when ethanol (1.0% v/v) and Tween-80 (0.1%v/v) were added to the medium. Production of amylases was also enhanced using the strain with the addition of benzene at decline phase, n-butanol at the trophophase and isopropanol at the idiophase with values, 10.66, 8.28 and 8.05 U/mL for $\alpha$-amylase (EC-3.2.1.1) and 6.28, 4.68 and 4.55 for $\beta$-amylase (EC-3.2.1.2), respectively. While the use of other solvents and detergents reduced the production of amylases specially activities by *S*. *griseus* DSM-40759, characterized by higher productions of $\alpha$- and $\beta$-amylases in different growth phases especially at the death phase. Worthily, proteases secretion was only induced to 1.02 U/mL at the idiophase by culturing DSM-strain with the addition of Triton X-100 and SDS to the fermentation medium. Moreover, the solvents shifted the pH values in the acidic range by the two strains at different phases compared to the control.

**Keywords:** *Streptomyces griseus*, streptomycin, amylases, proteases, organic solvents, detergent compounds

**Introduction**

The changes affecting permeability of the cytoplasmic membrane acts on the transport of nutrients on the production of natural products\(^1\). The streptomycin (SM) biosynthesis was stimulated by barbital\(^2\), which did not act as a precursor or a coenzyme in the process of biosynthesis\(^3\). The surfactants were initially used for the production of antibiotics in order to activate *Streptomyces viridochromogenes* spores by changing the physiology of sporulation and germination\(^4\). So, the role of the cytoplasmic membrane was studied in the transport of nutrients of *Streptomyces* and the efflux of antibiotics into the medium\(^5\). In addition, the leakage of alkaloids from the cells of *Claviceps* sp-58 occurred when the medium was supplied with Tween-80; this leakage was due to the permeability of the cytoplasmic membrane\(^6\). The effect of tunicamycin as cell wall inhibitor utilized D-glucose-amine for the production of SM\(^7\). Therefore, organic solvents such as ethaol, $n$-propanol, acetone and ethyl acetate were used to inactivate the production of antibiotics, mitoimycin\(^8\). At the same time, methanol was used as an inducer of nucleoside antibiotics in *Streptomyces* species\(^9\). In addition, antibiotic, pyoluteorin, was produced by *Pseudomonas fluorescens* S-272 when grown on ethanol as a single carbon source in shake flasks\(^10\) and it increased the antibiotic production when a high ethanol, NaCl concentration was used after a high temperature shock\(^11\). Similarly, mutants of *S*. *streptomyces noursei* resistant to benzyl alcohol selected for the formation of antibiotic without the yield of spores\(^12\). At the same time, *S. griseus* increased the $\beta$-glucosidase level when Tween-80 and Triton X-100 were added to the growth media\(^13\) as well as ten-fold increase in extracellular proteolytic activity was obtained when Tween-80 was added\(^14\). At the same time, nonionic surfactants used to enhance rumen microbial proteases and cellulases activity\(^15\).

Several studies emphasized that glycosyltransferase gene plays an important role towards the alcohol substrate for high yields of oligosaccharide antibiotics by actinomycetes\(^16\). The organic solvents actually used to produce new secondary metabolites or for the enhancement of active metabolites. In the present study, the use of different kinds of organic solvents and detergent compounds were used by different strains of *S. griseus* for the enhancement of SM.

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streptomycin production and related hydrolytic enzymes at various production phases.

Materials and Methods

Chemicals

The chemicals used were CPC and Tween-80 (BDH), sodium taurocholate (bile salts) (Riddle de Han) and Triton X-100 (Rasayan) and several organic solvents (ADWIC Company). Strains of the following microorganisms, Streptomyces griseus-DSM-40759, S. griseus-NRRL-3754 and Bacillus subtilis NRRL-B-543 were used.

Media and Cultural Conditions

S. griseus strains (DSM and NRRL) were cultivated at 28°C on slants containing soybean meal, 20; mannitol, 20; agar, 15 and distilled water 1.0 g/L. Vegetative cells (preculture) were obtained by using 1/3 of weight/L of previous medium without agar, prepared in a 250 mL Erlenmeyer flask containing 50 mL medium and incubated for 48 h.

Vegetative cells (preculture) were obtained by using 1/3 of weight/L of previous medium without agar, prepared in a 250 mL Erlenmeyer flask containing 50 mL medium and incubated for 48 h. The SM produced in submerged culture (basal medium) contained: soybean meal, 20; glucose, 20; NaCl, 2.5; CaCO3, 3; (NH4)2SO4, 3; K2HPO4, 0.5 g/L; and pH 6.8-7.0. The culture (25 mL) prepared in a 250 mL Erlenmeyer flask was sterilized at 121°C for 20 min. Each flask was inoculated with 2 mL of preculture cells having OD, 0.945 for DSM-strain or OD, 1.5 for NRRL-strain and used as the production medium. The inoculated flasks incubated for different incubation periods at 28°C on a rotary shaker at 200 rpm. The experiments were performed in triplicate.

Minimum Inhibitory Concentration (MIC) of Detergent Compounds

Detergent compounds grouped into anionic detergents [sodium dodecyl sulfate (SDS) and cetylpyridinium chloride (CPC)] and nonionic detergents (Triton X-100 and Tween-80) as well as sodium taurocholate (bile salts). In the experiments, the MIC of detergent compounds was carried out against B subtilis NRRL-B543 with the same bioassay for SM potency (Table 1).

The detergent compounds at the optimum concentrations were added individually into the selected fermentation medium at zero time before sterilization. After cooling, each flask was inoculated with 2 mL of vegetative cells (48 h) of NRRL-3754 or DSM-40759 strains and incubated at 28-30°C and rolled on rotary shaker at 200 rpm for the proper periods. After each growth phase (48 h), 3 flasks were taken to determine production of streptomycin SM (mg/L), α- and β-amylases and proteases (U/mL) as well as final pH values.

| Table 1—Effect of different concentrations of detergents against B. subtilis NRRL-B543 strain |
|----------------|----------------|----------------|
| Detergents     | Concentrations % |
| SDS (w/v)      | 5.00            | 0.50           | 0.050          |
| CPC (w/v)      | 0.05            | 0.005          | 0.0005         |
| Tween-80 (v/v)| 0.50            | 0.100          | 0.010          |
| Triton-X100 (v/v) | 0.50          | 0.100          | 0.010          |
| Sod. taurocholate (bile salt) (w/v) | 1.00 | 0.500 | 0.050 |

Organic Solvents

The organic solvents methanol, ethanol, isopropanol, n-butanol, n-hexane, cyclohexanone, amylacetate and benzene (2%)% after sterilizing with bacterial hydrophobic filter Millipore (Filter size 0.22 μ) were added individually into the fermentation medium at zero time. The control expressed as a medium without organic solvents.

Bioassay of Streptomycin

At neutralization pH of medium, the SM yield was assayed by the agar-diffusion method in the filtrated broth using B. subtilis NRRL-B-543 strain as the test organism and expressed as mg/L.

Enzymatic Activity

α-amylase

The activity of α-amylase EC (3.2.1.1) in the cultivation medium measured using the potato starch as a substrate and the D-glucose liberated was determined enzymatically by glucose kit (Biocon, Cat. No. BD-461100-1, Germany). The enzymatic activity was expressed as U/mL and defined as μmole D-glucose/mL of the culture filtrate/min under the optimum reaction mixture condition.

β-amylase

The β-amylase EC (3.2.1.2) activity in the cultivation was measured using the potato starch as a substrate and the reducing sugar was determined according to the Miller’s method with some modifications. The enzymatic activity was expressed as U/mL and defined as μmole maltose/mL of the culture filtrate/min.

Proteases

The extracellular proteases of the filtrated broth were measured using soluble casein as a substrate. The enzymatic activity was expressed as U/mL and defined as μmole tyrosine/mL of the culture filtrate/min.

Results and Discussion

A fermentation period for SM production could be classified into three phases each one extending 48 h
by using the microbial strains under study. The SM production began at the trophophase. While, most of the SM production occurred in idiophase, which extended another 48 h, and after that the decline phase took place.

**MIC of Detergent Compounds**

The detergent compounds tested for the MIC using *B. subtilis* NRRL-B-543 strain as test organism of SM potency has been presented in Table 2. The concentration sub-MIC added individually to the fermentation medium for SM and hydrolytic enzymes production before sterilization.

**Role of Organic Solvents and Detergent Compounds on SM Production and Final p**H **by *S. griseus* Strains at Different Growth Phases**

No variation could be observed at the trophophase by *S. griseus* strains (Fig. 1) where the maximum yield of SM (753 mg/L), was obtained using Tween-80 compared to the control. The NRRL-strain yielded 616, 503 and 411 mg/L SM using CPC, (Triton- X-100=ethanol=methanol) and (n-hexane=isopropanol), respectively; it was reduced by DSM-strain using some solvents whereas amylacetate, n-butanol and cyclohexanone completely inhibited the production. The pH values were maintained at neutral range by the used strains using most of the solvents while ethanol for NRRL-strain and benzene for DSM-strain the pH were changed towards acidic range (Fig. 2). In addition, for all detergents, the pH values were changed towards acidic range by the culturing of used strains.

At the idiophase, most detergents and solvents enhanced the SM production compared to the trophophase and control and peaked at 780 mg/L by the culturing of *S. griseus* NRRL-strain on medium containing Tween-80, ethanol and methanol with values 753 mg/L which kept the pH of culture at neutral range, the important biochemical parameter for SM production in this phase (Fig. 2). While the final pH shifted towards alkaline range (8.18) when methanol was used. Many

<table>
<thead>
<tr>
<th>Detergents</th>
<th>MIC%</th>
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<tr>
<td>CPC</td>
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<tr>
<td>SDS</td>
<td>0.50</td>
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<td>Triton-X100</td>
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<td>Tween-80</td>
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<tr>
<td>Sodium taurchulate (Bile salts)</td>
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![Fig. 1](image1.png)  
Fig. 1—Effect of organic solvents and detergent compounds on SM by *S. griseus* strains at different SM production phases.

![Fig. 2](image2.png)  
Fig. 2—Effect of different organic solvents and detergent compounds of final pH values by *S. griseus* strains at different SM streptomycin production phases.
Role of Organic Solvents and Detergent Compounds on the Production of Amylases and Proteases by *S. griseus* Strains at Different Growth Phases

Organic solvents and detergent compounds had varied effects on the production of amylases and proteases by *S. griseus* strains through different growth phases where most microbial extracellular enzymes produced simultaneous growth and these enzymes were necessary for the true secondary metabolism. At the trophophase (Figs 3 & 4) DSM-strain characterized with the high production of α- and β-amylases and proteases in control medium as compared to NRRL-strain. However, production of amylases was enhanced only by the culturing of NRRL-strain compared to the control in the submerged culture containing: *n*-butanol, cyclohexanone, ethanol, *n*-hexane and isopropanol, yielded 8.28, 7.78, 6.78, 6.52 and 5.41 U/mL for α-amylases while, 4.68, 3.87, 3.84, 3.68, 3.06 and 2.77 U/mL for β-amylases, respectively. The methanol did not affect amylases production. On the other hand, all solvents reduced production of amylases by culturing DSM-strain besides all the detergent compounds reduced the amylases production by the culturing of *S. griseus* strains compared to the control cultures and have the same yield of amylases except SDS differed. However,
addition of methanol enhanced production of proteases with a yield of 0.87 U/mL using DSM-strain. While, methanol, ethanol and cyclohexanone enhanced the production of proteases partially with values of 0.56, 0.50 and 0.40 U/mL, respectively. On the contrary, all the used detergent compounds reduced the production of proteases by culturing of DSM-strain compared to the control. Where, interaction of solvents with lipid membranes such as n-butanol and other used alcohols disrupt certain lipoprotein membranes with the release of water soluble proteins at these concentrations used and the concentrations of each one of the alcohols and detergent compounds appear to be of critical quantity and lyses may occur at the concentration which produces a surface pressure.

Generally, during the idiophase, the production of hydrolytic enzymes was enhanced by culturing of NRRL-3754 strain using all the solvents compared to the control and trophophase where n-butanol and isopropanol yielded 8.17 and 8.05 U/mL α-amyrase and 4.62 and 4.55 U/mL β-amyrase, respectively. While, benzene, n-hexane, methanol, ethanol, cyclohexanone and amylactate yielded 5.25, 5.10, 4.95, 4.75, 4.65 and 4.14 U/mL α-amyrase and 2.97, 2.88, 2.80, 2.69, 2.58 and 2.34 U/mL β-amyrase, respectively. However, Tween-80 slightly enhanced production of amylases by culturing of NRRL-3754 strain as compared to its control and yielded 5.63 and 3.11 U/mL α- and β-amylases, respectively while the amylases production reduced using the other detergents. Surfactants such as SDS, cholic acid, Tween, etc. in the fermentation medium increased the secretion of proteins by increasing cell membrane permeability and thereby enhanced enzyme yield whereas addition of Tween-80 (1.3%) to the fermentation medium increased α-amyrase production by 2-fold in Thermomyces lanuginosus from the third day of culture\textsuperscript{27}. In addition, Tween-80 gave 2-fold enzyme yields by culturing of Geobacillus thermoleovorans at a concentration of 0.03% mass per volume ratio\textsuperscript{28}. On the other hand, the production of amylases rapidly reduced by DSM-strain using all organic solvents and detergent compounds. The protease production was enhanced compared to the previous phase and controlled by the two strains used. The protease production was enhanced by DSM-strain using Triton-X-100, SDS and bile salts with yields of 1.92, 1.87 and 1.4 U/mL, respectively. In addition, the protease production was increased by the culturing of NRRL-3754 strain using bile salts, Tween-80=Triton-X-100 as well as ethanol and methanol with yields of; 1.40, 0.95, 1.01 and 0.61 U/mL, respectively. While, the other solvents and detergents reduced the proteases production compared to the control by the above used strains.

With the continuous fermentation, at the decline phase, maximum production of amylases was obtained by the culturing of NRRL-3754 strain as compared to all the controls and other phases using benzene with yields of 18.86 and 10.66 U/mL for α- and β-amylases, respectively while the amylases production was reduced using n-butanol, cyclohexanone, ethanol and methanol but higher than its control and other solvents. However, the culture of DSM-strain has the maximum yield of amylases over its control, 16.66 and 9.10 U/mL for α- and β-amylases, respectively. The amylases production partially reduced using all solvents compared to their control but the continuous fermentation in the presence of organic solvents and detergent compounds obviously avoided this phenomenon of the strain. Although, Tween-80 enhanced the production of amylases by culturing of NRRL-strain in comparison to its control with yields of 4.33 and
2.12 U/mL for α- and β-amylases, respectively followed with CPC and bile salts but the other detergents reduced the production of amylases. Although, the production of proteases in control media decreased with the increase of fermentation period by both the strains. The maximum proteases production (1.36 U/mL) was obtained by culturing DSM-strain in the presence of benzene. So the proteases production was enhanced by NRRL-strain on medium that contained ethanol with yields of 1.06 U/mL. In addition, the proteases production was induced by culturing of S. griseus-strains except bile salts with the same values using Tween-80, CPC, Triton-X-100 and amylacetate, SDS enhanced the proteases production by culturing of both-strains with the same yields of 1.1, 1.06, 0.82, 0.74 and 0.68 U/mL, respectively. While the proteases were variously enhanced using the other solvents by culturing of the two strains under study. In extended fermentation period, the other solvents reduced the protease production compared to the idiophase, except n-butanol and n-hexane reduced it.

Effect of Ethyl Alcohol and Tween-80 Concentrations on the Production of SM and Hydrolytic Enzyme by NRRL-3754 Strain

The production of SM by culturing of NRRL-3754 strain was increased sharply to the maximum yields of 1128 mg/L with 174.5% increase using ethanol concentration 1.0% (v/v) at the idiophase and the pH value of 7.63, the SM production decreased gradually with increasing of ethyl alcohol concentrations (Fig. 5). This is one of the most important results where the pH at neutral range is the most favourable condition for the production of SM. After that, the pH value was increased to alkaline range using 2% ethanol concentration, which then gradually decreased with the increase of ethanol concentrations. The proteases production increased to 0.72 U/mL using 2% ethanol concentration, when compared to the control, then decreased with the increase of ethanol concentrations. Above this concentration the proteases production decreased. While production of amylases was decreased compared to the control medium using 2% ethanol then the secretion of amylases increased with

Fig. 5—Effect of different ethyl alcohol and Tween-80 concentrations on the production of SM and hydrolytic enzymes by S. griseus NRRL-3754 and DSM-40759 strains at optimum production phase (idiophase).
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the increase of ethanol concentrations up to a maximum ethanol yield of 9.10 and 5.21 U/mL for α- and β-amylases, respectively.

The previous results emphasized that Tween-80 was the best detergent compound to induce SM production especially at idiophase by NRRL-3754 strain. The results obtained in Fig. 5 showed that Tween-80 at concentration 0.1% (v/v) recommended for the maximum production of SM, 780 mg/L with 90% increase. More or less at this concentration the SM yields decreased. In addition, the maximum SM production characterized with pH value at neutral range and gradually decreased with increasing of Tween-80 concentrations. So production of α- and β-amylases with values 6.22 and 3.85 U/mL, respectively compared to the control. While, the maximum production of SM characterized with α-and β-amylases production almost as the same as the control then the enzyme activity reduced with increasing of Twee-80 concentrations. At the same time, the maximum SM production at optimum Tween-80 concentration (0.1%) had been characterized with the maximum proteases production, 0.96 U/mL/min. Increasing and decreasing of these concentrations, the production of proteases decreased.

Conclusions

This study showed that enhancement of streptomycin (SM) and production of hydrolytic enzymes depends on the specific strain of S. griseus, different types of organic solvents and detergent compounds at each specific growth phase where the addition of short chain alcohols such as ethanol at 1% (v/v) improved the production of SM to the maximum, 1128 mg/L with 174.5% increase by the culturing of NRRL-strain. This concentration results in the pH value of the fermented medium around the neutral values and recommended for the optimum SM production at idiophase by culturing of NRRL-3754 strain. However, ethanol was also used as a single carbon source for the production of pyoluteorin antibiotic with P. fluorescens S-27210, the ethanol affected only on the physiological properties of NRRL-strain when added at this low concentration specially at the beginning of the fermentation. Similarly, glycosyltransferase gene has a flexible role towards the alcohol substrate for high-yielding of antibiotics by actinomycetes16. The level of SM enhanced to 780 mg/L at the idiophase by the culturing of NRRL-strain using 0.1% of Tween-80, which controlled the pH value around the neutral range29. The activity of α- and β-amylases by culturing of the NRRL-3754 strain, was enhanced at the trophophase and idiophase with most of the organic solvents used. The highest protease production (1.92 U/mL min) obtained at the idiophase using DSM-strain, Triton-X-100 and SDS. In addition, the maximum production of streptomycin, by culturing of NRRL-strain, was characterized with α- and β-amylases production almost to be more than the control, as the protease production was peaked to the maximum but still less than DSM-strain. This is probably for the understanding of physiological effect of ethanol and Tween-80 on this organism30. Although, DSM-strain characterized with highest amylases production whereas the enhancement of proteases production was carried only by methanol at the trophophase and benzene at the death-phase as well as all the used detergents except bile salts reduced it. In this case, the organic solvents, at low concentration caused the physiological effect on microorganisms especially on membrane fluidity and the transport system causing increase in the secretion of hydrolytic enzymes especially amylases and when the glucose was increased in the medium the SM production repression occurred.

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