

Improvement Of Anisomycin Production Through Mutation And Medium Optimization For *Streptomyces Griseolus*

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Abstract: Aims: To improve the production of anisomycin through the strain development and medium optimization for the developed strain. Methods and Results: The induced mutant strain (M-107) was selected boicts anti-fungal activity. The production medium was optimized and the highest productivity (130.9 mg l⁻¹) was achieved. Conclusions: The mutant strain (M-107) was obtained with a production titer of anisomycin reaching (70.8 mg l⁻¹), which was increased by (116%) in comparison with that of the parent strain. The factors affecting anisomycin production were studied including environmental conditions and nutritional requirements. The antibiotic yield of the mutant strain (M-107) using optimized medium was (130.9 mg l⁻¹) which was improved by (83.6 %) in comparison with that produced in the basal medium. Significance and Impact of Study: the potency of *Streptomyces griseolus* was improved to produce four folds more anisomycin by the mutant strain (M-107). The results of this study can be applied for efficient production of anisomycin.

Key words: Improvement, Mutation, Optimization, Anisomycin, *Streptomyces griseolus*, Fermentation.

INTRODUCTION

Anisomycin is a pyrrolidine antibiotic. It was first isolated by Sobin and Tanner at Pfizer Inc, in 1954 (Sobin and Tanner 1954) from culture filtrates of two different species of *Streptomyces*, subsequently identified as *S. griseolus* and *S. roseochromogenes* (Tanner *et al.*, 1955). Anisomycin also has been isolated from the culture filtrate of *Streptomyces* sp. No.638 (Ishida *et al.*, 1974) and had been found to be produced by a previously transformed macrotetrolide-producing strain *Streptomyces werreaensis* 1365T (Rusanova *et al.*, 2000). This antibiotic possesses a strong and selective activity against pathogenic protozoa and fungi and has been used successfully in the clinics for treatment of *Trichomonas vaginitis* and amoebic dysentery (Grollman 1967, Jimenez and Vazquez 1979, Santander *et al.*, 1961). Anisomycin shows cytotoxicity against human tumor cell line in vitro (Junghae and Raynes 2002). It is an antibiotic which inhibits protein Synthesis, activates p54, MAP kinases and stress-activated protein kinases. Anisomycin is also an anti-fungal used in Martin-Lewis Medium to inhibit *C. albicans* and aid in identification of *N. gonorrhoeae* (Kardalinou *et al.*, 1994, Kardalinou *et al.*, 1997, Barros *et al.*, 1998). Beside all these medical applications, it has a herbicidal activity (Hunter *et al.*, 1961) and has been used as fungicides on pathogenic fungi in plants (Merck index. 12th ed.). The diverse biological activity of anisomycin is due to the presence of a chiral pyrrolidine skeleton.

Strain improvement is important in applied microbiological research, especially in the production of clinically important antibiotics as well as antibiotics important in veterinary medicine and agriculture. Nonetheless, it would seem that optimizing the medium constituents for production of anisomycin by a mutant strain in a submerged culture has not yet been carried out. Accordingly, the objectives of the present study were to isolate the *Streptomyces griseolus* mutant strain by UV irradiation that produces a higher titer of anisomycin than the parent strain followed by optimizing the culture and environmental conditions for achieving the maximal production.

MATERIALS AND METHODS

Anisomycin -Producing Strain and maintenance of Culture Media:

Streptomyces griseolus NRRL B-1062 provided by the ARS culture collection (NRRL), Northern Regional Research Laboratory, Peoria, Illinois, USA was the parent strain of the mutant strain described in this study. Slant medium consisted of the following (g l⁻¹): Soybean, 30; Dextrin, 15; CaCO₃, 10; MgSO₄.7H₂O, 1;

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Agar, 20; pH 7.0-7.2. Solid culture was incubated at 30°C for 10 days until spore developed then the slants were stored at 4°C.

Anisomycin Fermentation in Flasks:

The spores were scraped from the surface of heavily sporulated slants with nine ml isotonic saline solution (0.9% NaCl). The spore concentration was quantified in the suspension as (2×10^8 spores ml^{-1}) using a haemocytometer slide. Spore suspension from solid culture was used to inoculate 250 ml Erlenmeyer flasks with a working volume of 50ml of the optimized production medium consists of the following per liter: Soybean meal, 12; corn starch, 10; NaCl, 2.5. The inoculated flasks were shaken at 200 rpm on a rotary shaker (New Brunswick, Edison, NJ, U.S.A) with an eccentricity of 2.0 cm, and the cultivation temperature was 30° C for four days.

Isolation of The Mutant Strain:

A 10 ml sample of spore suspension from solid culture of *Streptomyces griseolus* NRRL B-1062 was transferred to an aseptic plate. The plate with cover removed was exposed to UV irradiation for different exposure times (30, 60, 90, 120, 150, 180, 210, and 240 seconds) at a distance of 20 cm from the UV lamp with wavelength of 254 nm and power of 30 W. Spore suspension with or without UV irradiation was also spread over the surface of (International *Streptomyces* Project 2 medium) ISP-2 medium for 10 days at 30° C. Colonies which survived at each dose of the UV irradiation were isolated on ISP-2 slants composed of the following (g l^{-1}): D(+) glucose, 4; malt extract, 10; yeast extract, 4 and agar, 18 for 10 days and after the flask fermentation anisomycin concentration was measured and compared (Zhang *et al.*, 1989, Gamal *et al.*, 2005).

Selection of The Most Potent Mutant Strain in Anisomycin Production:

The direct demonstration of anisomycin production by sub-culturing streaks of each mutant, 10 days old, on plates containing ISP-2 medium. After 10 days, the plates were cut off using a cork borer previously sterilized and the discs were transferred to Petri dishes containing antibiotic assay medium seeded with *Candida albicans*. The diameter of the resulting inhibition zones were then measured and considered to estimate the mutants' antimicrobial potentialities. Colonies with a diameter of inhibition zone greater than that of the parent strain *Streptomyces griseolus* were picked up for further selection in shake flasks.

Optimization of Fermentation Conditions:

For the optimization of anisomycin production, *Streptomyces griseolus* mutant strain (M-107) was cultivated in the following chemically defined culture media at 30°C and 200 rpm (amounts are listed in gram per liter):

- 1) Glucose Asparagine medium: Glucose, 10; Yeast extract, 2; L-asparagine, 1; K_2HPO_4 , 0.5; Agar, 15. (Atlas 1997).
- 2) Strepto-antibiotic agar: D-glucose, 15; Soybean meal, 15; NaCl, 5; Yeast extract, 1; CaCO_3 , 1; Glycerol, 2.5. (Atlas 1997).
- 3) Streptomyces Agar: Glucose, 10; Beef extract, 4; Peptone, 4; NaCl, 2.5; Yeast extract, 1; Agar, 20. (Atlas 1997).
- 4) Patent medium for anisomycin production: Glucose, 10; Corn starch, 10; Hydrolyzed casein (N-Z amine B), 5; Molasses, 5; Soya bean meal, 15; Sodium chloride, 1; CaCO_3 , 1 (Sobin and Tanner 1954).
- 5) Soya bean medium: Soybean, 30; Dextrin, 15; CaCO_3 , 10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1; Agar, 20
- 6) Semi synthetic medium (SSM): Glucose, 10; $(\text{NH}_4)_2\text{SO}_4$, 2; NaCl, 2; KH_2PO_4 , 1.0; K_2HPO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; CaCO_3 , 5.0; Yeast extract, 2.0 (Bouras *et al* 2005).
- 7) GYM *Streptomyces* medium: Glucose, 4.0; Yeast extract, 4.0; Malt extract, 10.0; CaCO_3 , 2.0; Agar, 18 (Pridham *et al* 1957).

Carbon and Nitrogen Sources:

To determine the best carbon and nitrogen source that provide the maximum yield of anisomycin by the mutant strain *Streptomyces griseolus* M-107, each of the following carbon sources : fructose; galactose; ribose; sucrose; maltose; lactose; starch; dextrin and molasses was added to the fermentation medium No.3 instead of glucose before inoculation. On the other hand, the following nitrogen sources (ammonium sulphate, ammonium chloride, sodium nitrate, urea, peptone, beef extract, yeast extract, soybean and casein hydrolysate) were investigated.

PH and Temperature:

After selection of the best medium for anisomycin production, the pH of this medium was adjusted to the values of 4.5, 5.5, 6, 6.5, 7, 7.5, 8 and 9 by using pH meter that was calibrated each time by 1M HCl and 1M NaOH. pH 7.2 was used as a control.

The optimal temperature for production was tested by growing it in submerged cultures in 250 ml flasks containing 50 ml of the broth culture media at different temperature 20, 25, 28, 30 and 35°C. The 30 °C was used as control.

Determination of The Cell Dry Weight:

Fermentation culture samples were filtered through a pre-weighed filter paper (Whatman1) and washed four times with 20ml distilled water. Wet filter papers were dried overnight at 80°C and then weighed after cooling in a desiccator. The dry weight in grams per liter of the fermentative culture was determined.

Glucose Determination:

The amount of residual glucose present in the fermentation broth after the completion of fermentation period was determined enzymatically by glucose kit (Biocon, Cat. No. BD-461100-1, Germany) (Trinder 1969).

Biological Determination of Anisomycin:

Candida albicans ATCC 90028 obtained from the American Type Culture Collection was maintained on Sabouraud's agar medium. The experimental isolate was tested for its antibiotic activity against the fungal pathogen *Candida albicans* by the agar diffusion method. Inhibition zones diameter were visualized and recorded comparing with standard sample.

Extraction of Anisomycin:

After the fermentation, the cultures were filtered and the filtrate was adjusted to pH 9 with diluted sodium hydroxide and twice extracted with chloroform. The combined chloroform extract was concentrated and extracted with acidified water to pH 2 with diluted sulfuric acid. Thereafter, the acidified aqueous solution was concentrated and readjusted to pH 9 with further sodium hydroxide and re-extracted with chloroform again. The chloroform extract was evaporated until dry and the residue was dissolved in 1ml of chloroform (Tanner *et al.*, 1955).

Preparative Thin Layer Chromatography:

Thin layer chromatography (TLC) was used for purification of the previously obtained extract in chloroform. A 20×20 cm of aluminum silica gel sheet was used for purification of anisomycin. Repeatedly small drops extract and one drop of the standard antibiotic were placed over the marked area on the sheet (stationary phase) by a capillary glass tube. Then the sheet was placed in a glass jar in chloroform - methanol-conc.NH₄OH (4: 1: 0.1 v/v/v) solvent mixture and incubated at room temperature for 10 min. The developed spots were detected by UV light ($\lambda = 254$ nm) and their R_f values were recorded. The bands which have the same R_f value (0.6) as that of the authentic were scraped off and the silica gel was soaked in methanol. After filtration and evaporation of methanol, the residue was re-dissolved in methanol and injected into HPLC for anisomycin determination and comparison between the mutant strains and the parent (Hosoya *et al.*, 1993).

Determination of Anisomycin By High Performance Liquid Chromatography:

Anisomycin was determined after extraction and purification according to the following method (Kirchmeyer and Upton 1978). Mobile phase was prepared by mixing 125 ml of acetonitrile and 875 ml of pH 6, 0.05 M potassium di-hydrogen phosphate buffer solution in a 1-L vacuum flask. Determination was carried out using SYKIM HPLC (Germany) a reversed phase column (Polaris C-18, 5 μ m, 250×4.6 nm) at 30°C. The peaks were detected at 225 nm wave length using a variable wave length photos array detector model (Jasco – UV – 2070 Plus, Intelkigent UV/Visible detector, Japan). Elution was at flow rate of 1 ml/min. for 15 min. Injection was carried out manually using a micro-syringe (25 μ l) and the injection volume was (20 μ l). Autochro 3000 software was the data acquisition system. Calibration curve for anisomycin determination was made using different concentrations of anisomycin authentic sample (Sigma) (1, 0.5, 0.25, 0.125 mg ml⁻¹ methanol). The results obtained from HPLC analysis of the samples were calculated using the above mentioned calibration curve.

UV, IR and Mass Spectrum Analysis:

The UV spectrum analysis was carried out by using: T80+UV/VIS Spectrometer, PG Instrument Ltd. Range: 190-1000 nm. The IR spectrum analysis was carried out using: FTIR, Jasco 6100, Model Japan, Resolution: 4 cm⁻¹. The mass spectrum analysis was carried out using: JEOL-JMS-AX500, Model Japan. Mass spectrometer.

RESULTS AND DISCUSSION

Strain Improvement:

Because UV light is a fairly potent mutagen, *S. griseolus* NRRL B-1062 was treated with UV light to improve the production of anisomycin. Clones of mutants grown on ISP-2 were obtained after 10 days of incubation.

In this study it was found that the use of UV radiation has a strong mutagenic effect on *Streptomyces griseolus* NRRL B-1062 since considerable variability was observed between the survived isolates from each UV dose. After UV radiation for four min., only 0.01% of the mutants survived. Also the mutation frequency of the induced mutants ranged from 6.45×10^{-6} at 30 sec. of exposure to 5×10^{-2} after 240 sec.

Isolation of The Mutant *Streptomyces Griseolus* Strain:

After UV irradiation, it was found that some of the mutant strains produced a higher level of anisomycin than the parent strain. Among them, the mutant No. M-107 showed the highest level of anisomycin production reaching 70.8 mg l^{-1} in comparison with the wild strain 32.8 mg l^{-1} and the other mutant strains as showed in figure (1). This result has been confirmed by HPLC determination of anisomycin concentration in the fermentation broth extracts of the cultivated mutants table 1. Consequently, the mutant strain M-107 was selected as the improved strain for medium and fermentation condition optimization.

Table 1: Collective table of the HPLC chromatograms of the wild strain *Streptomyces griseolus* NRRL B-1062 and the best induced mutant strains as reported by the data acquisition software

Sample	Mutant No.	RT[min]	Ar ea [Mv *s]	Area%	Height[mV]	Height%	Amount ($\mu \text{ gm}$)
1	M 31	4.9833	791.6599	96.66	26.3682	95.90	5.1807
2	M 33	4.9500	885.8587	97.05	30.4789	95.91	5.7972
3	M 44	4.9333	931.1453	92.47	32.7728	91.86	6.0935
4	M 47	4.9000	887.4922	96.66	30.3310	95.71	5.8079
5	M 49	4.9333	1054.6294	96.75	36.2064	95.83	6.9016
6	M 50	4.9167	1151.7454	96.28	38.9124	95.15	7.5372
7	M 61	4.9333	941.5623	96.06	31.0557	95.09	6.1617
8	M 94	4.7833	1143.2333	96.22	36.8150	95.00	7.4815
9	M 107	4.9167	1776.5063	95.95	58.0250	94.54	11.6257
10	Wild type	4.9333	760.6160	95.76	24.7300	94.77	4.9776
Sum			10324.4502	959.85	345.6954	949.75	67.5645
Average		4.9183	1032.4451	95.98	34.5695	94.97	6.7564
Variance		0.0025	77351.4688	1.52	79.1092	1.30	3.3126
SD		0.0497	278.1213	1.23	8.8943	1.14	1.8201
%RSD		1.01	26.94	1.28	25.73	1.20	26.94

Selection of Fermentation Medium:

Cultivations were carried out in the seven different production media listed in material and methods. Results presented in figure (2) showed that medium No. 3 was the best medium for anisomycin production and gave $(70.80) \text{ mg l}^{-1}$ anisomycin. Media No. 2, 4, 5 and 7 produced lower amounts of anisomycin of 22.35; 17.74; 32.82 and 48.21 mg l^{-1} , respectively. The other media (1 and 6) did not support anisomycin production. The results also indicated that there is no clear correlation was observed between anisomycin produced in the culture medium and cell growth. The levels of anisomycin were greatly dependent on the composition of the medium.

Effect of Carbon Sources:

Carbon and nitrogen sources were optimized in the selected medium. Replacing glucose with ribose has enhanced anisomycin biosynthesis, followed by starch and glucose which gave the following yields; 112.30, 96.3 and 60.7 mg l^{-1} respectively figure (3). When corn starch was tried at different concentrations, it was found that the optimum concentrations of corn starch was 10 g l^{-1} gave production of 96.3 mg l^{-1} . Results in figure (4) showed that anisomycin yield increased with the increase of glucose concentration reaching its maximum value of 112.3 mg l^{-1} at 8 g l^{-1} glucose when corn starch concentration was fixed at 10 g l^{-1} which exceeded anisomycin production yield upon using the two carbon sources separately. Further increase in glucose concentration resulted in a gradual decrease in anisomycin yield.

Effect of Nitrogen Sources:

It was observed that soybean meal showed its superiority for anisomycin biosynthesis over the other investigated nitrogen sources or the combination between (peptone, beef extract and yeast extract) in medium No. 3 which has been used as a control figure (5). On the other hand, increase of soybean concentration reaching production maximum value of 152.7 mg l^{-1} at 12 g l^{-1} soybean meal. Further increase in soybean meal concentration resulted in a gradual decrease in anisomycin yield figure (6).

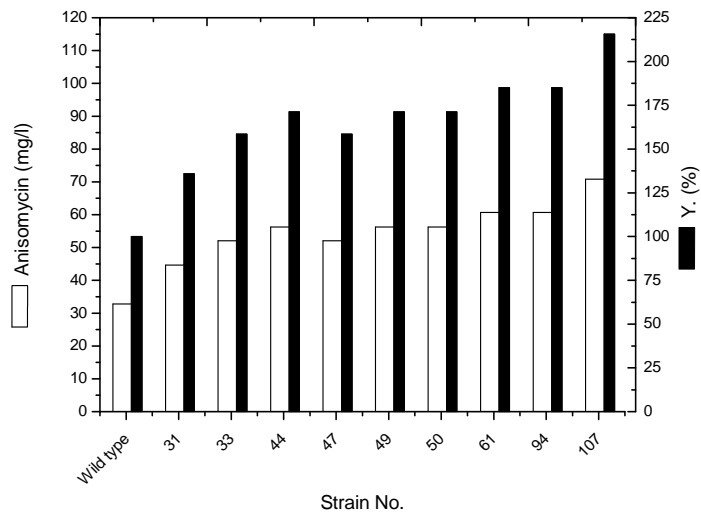




Fig. 1: Anisomycin production () & (Y %) yield improvement () by the wild type and the induced mutants which resulted from UV irradiation of *Streptomyces griseolus* NRRL B-1062.

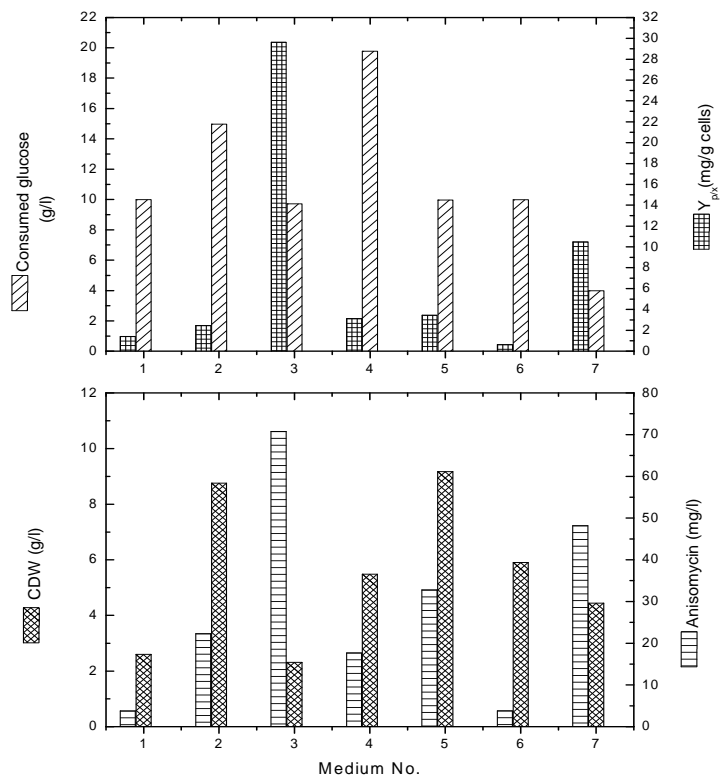






Fig. 2: Anisomycin production () & CDW () & Y_{p/x} () & Consumed glucose () by the mutant strain (M-107) on different media.

Effect of Phosphate Salts Concentration:

The modified fermentation medium was supplemented with different combinations of di-potassium hydrogen phosphate and potassium di-hydrogen phosphate at different concentration and was also examined for their effective anisomycin production but negative effects were observed (data not shown).

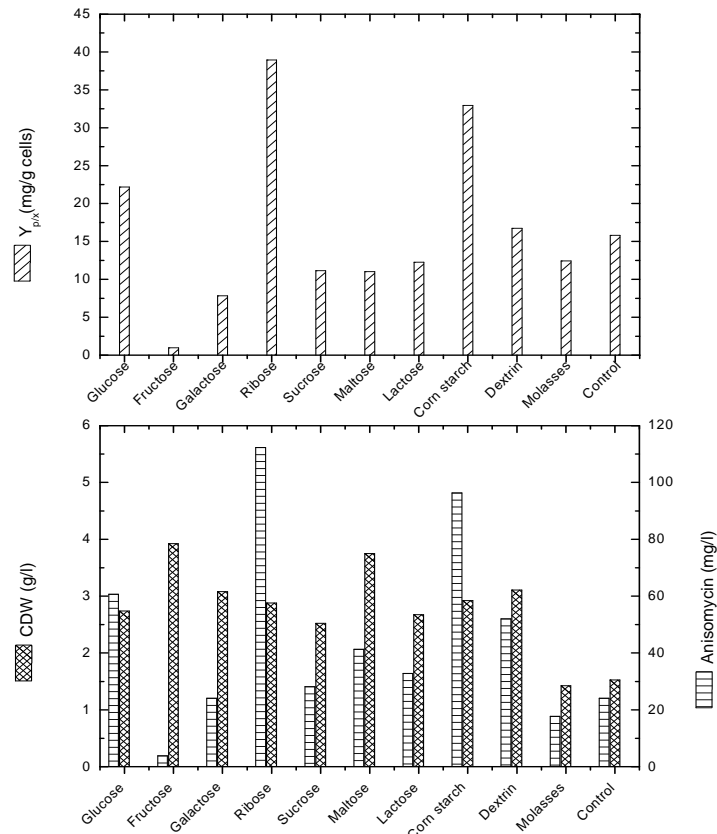


Fig. 3: (▨) Anisomycin production & (▩) CDW & (▧) $Y_{p/x}$ by the mutant strain (M-107) using different carbon sources.

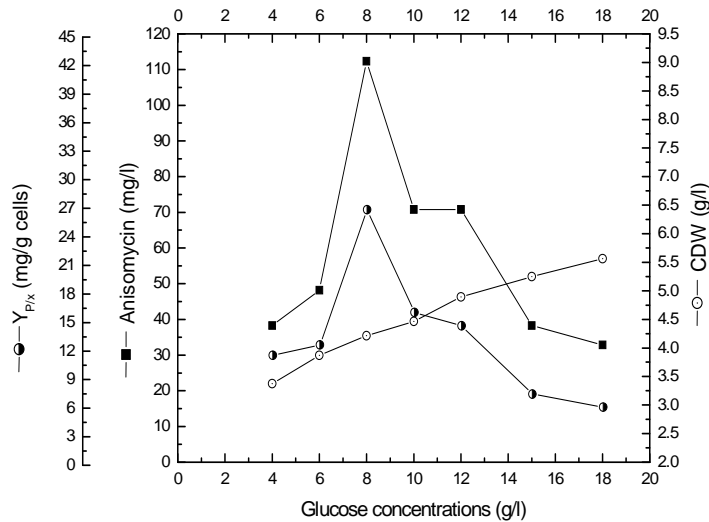


Fig. 4: (—■—) Anisomycin production & (—○—) CDW & (—●—) $Y_{p/x}$ by the mutant strain (M-107) using different glucose concentrations in the presence of 10 g l^{-1} corn starch.

Effect of pH and temperature:

The maximum level of anisomycin was obtained with initial culture pH value ranged between 7-7.5. figure (7). Above or below this range, a decrease in the level of the antibiotic was observed. Results also showed that

anisomycin production strongly affected by the change in incubation temperature because anisomycin yield increased with the increase in temperature reaching its maximum value; 152.7 mg l⁻¹ at 30°C. Further increase in temperature resulted in a gradual decrease in anisomycin yield as seen in figure (8).

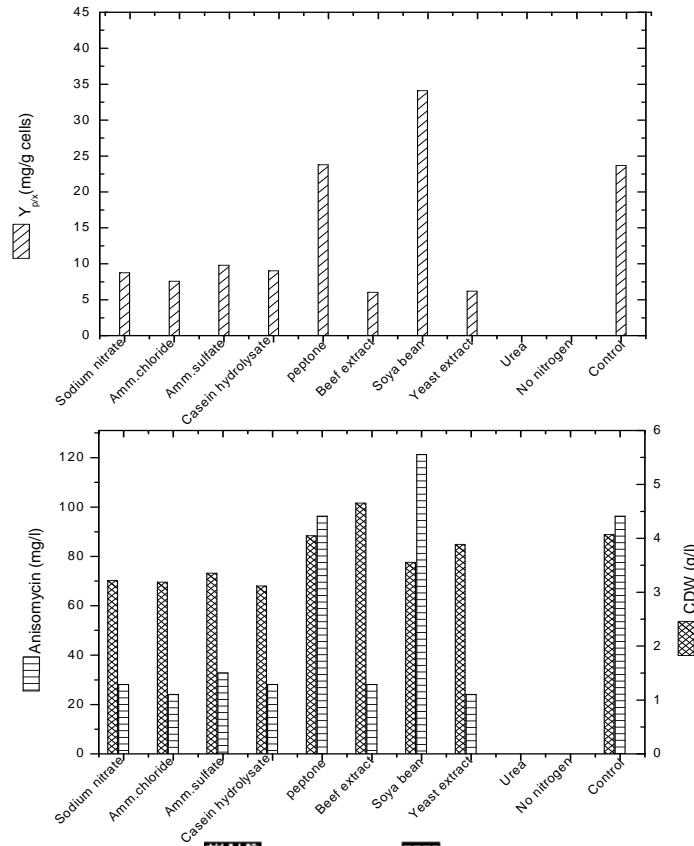


Fig. 5: (■) Anisomycin production & (□) CDW & (▨) Y_{p/x} by the mutant strain (M-107) using different nitrogen sources.

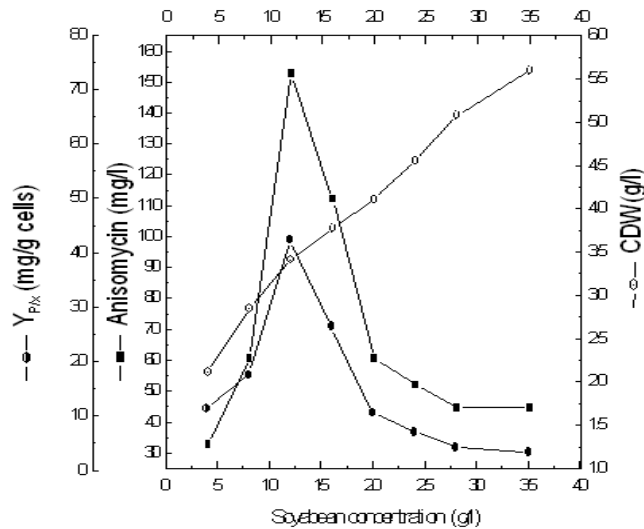


Fig. 6: (—■—) Anisomycin production & (—○—) CDW & (—●—) Y_{p/x} by the mutant strain (M-107) using different concentrations of soybean.

Effect Of Different Agitation Speeds On Cell Growth And Anisomycin Production:

In general, it was found that at low rpm (80 rpm) there was no anisomycin production and as the agitation speed increased the amount of produced anisomycin increased reaching its maximum value 130.9 mg l⁻¹ at 200

rpm but as the agitation speed increased to 250 rpm the amount of anisomycin decreased to become 60.7 g l^{-1} . Moreover, the cell dry weight decreased, as the agitation speed increased, reaching a lower value 2.84 g l^{-1} at 250 rpm. The yield coefficient ($Y_{p/x}$) was maximum at 200 rpm where it reached 42.045 mg/g cell .

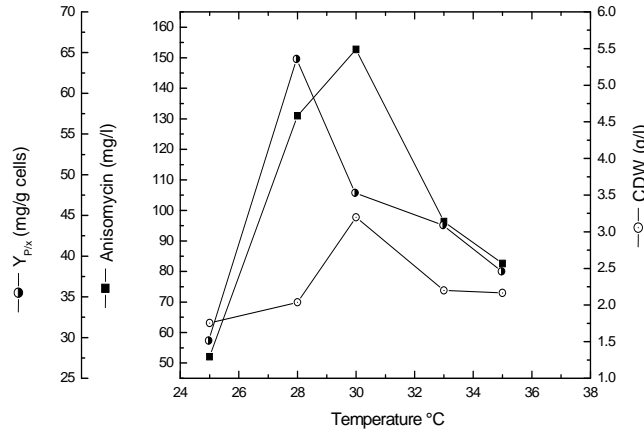


Fig. 7: (—■—) Anisomycin production & (—○—) CDW & (—●—) $Y_{p/x}$ by the mutant strain (M-107) using different temperatures.

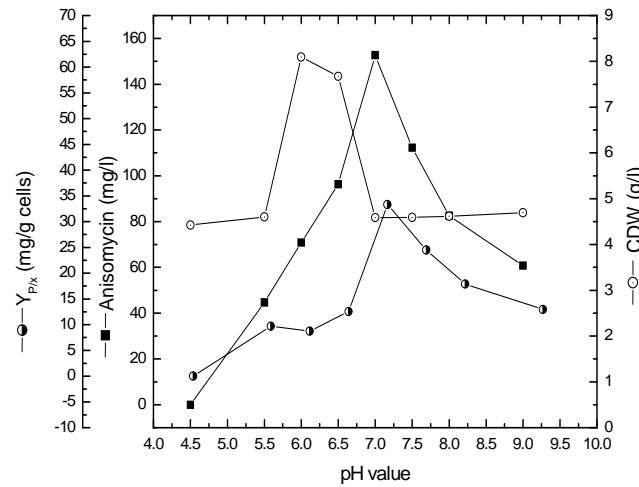


Fig. 8: (—■—) Anisomycin (mg l^{-1}) & (—○—) CDW (g l^{-1}) & (—●—) $Y_{p/x}$ (mg/g cells) by the mutant strain (M-107) using different pH values.

Effect of Inoculum Type:

The type of inoculum has a significant effect on anisomycin production because the highest anisomycin level of anisomycin (131.00 mg l^{-1}) was produced when the inoculum was in the form of spores. On using vegetative cells, the microorganism was able to produce appreciable levels of anisomycin.

Effect of Different Incubation Period on Cell Growth and Anisomycin Production:

The production of anisomycin was increased linearly up to 96 h giving the maximum yield of anisomycin (152.7 mg l^{-1}) and the yield coefficient ($Y_{p/x}$) revealed that it was maximum of $27.021 \text{ mg g}^{-1} \text{ cells}$ at 96 h incubation time. Above this phase of growth, a decline in the antibiotic production was recorded as the incubation time increased until a depletion of the antibiotic was noticed at 168 h figure (10), results also indicated that the cell growth increased as the incubation time increased reaching its maximum value; 6.77 g l^{-1} at 168 h on the other hand, the final pH was shifted towards alkalinity during the fermentation period reaching a pH value of 8.25 in the optimum time, a further increase in pH was noticed reaching its highest value at 168h which in turn indicated that there is a correlation between cell growth and shifting toward alkaline side.

Spectroscopic Analysis Of The Isolated Bioactive Compound Which Has Suggested To Be Anisomysin:

The IR spectrum of the isolated compound showed the following absorption beaks 3434 cm^{-1} , 2924 cm^{-1} , 1731 cm^{-1} , 1630 cm^{-1} , 1377 cm^{-1} , 1026 cm^{-1} . The UV spectra of the isolated compound showed λ_{max} at 224 and 276. The mass spectrum of the isolated compound also showed a molecular ion peak M^+ at m/z 266, 144, 121, 91, 84.

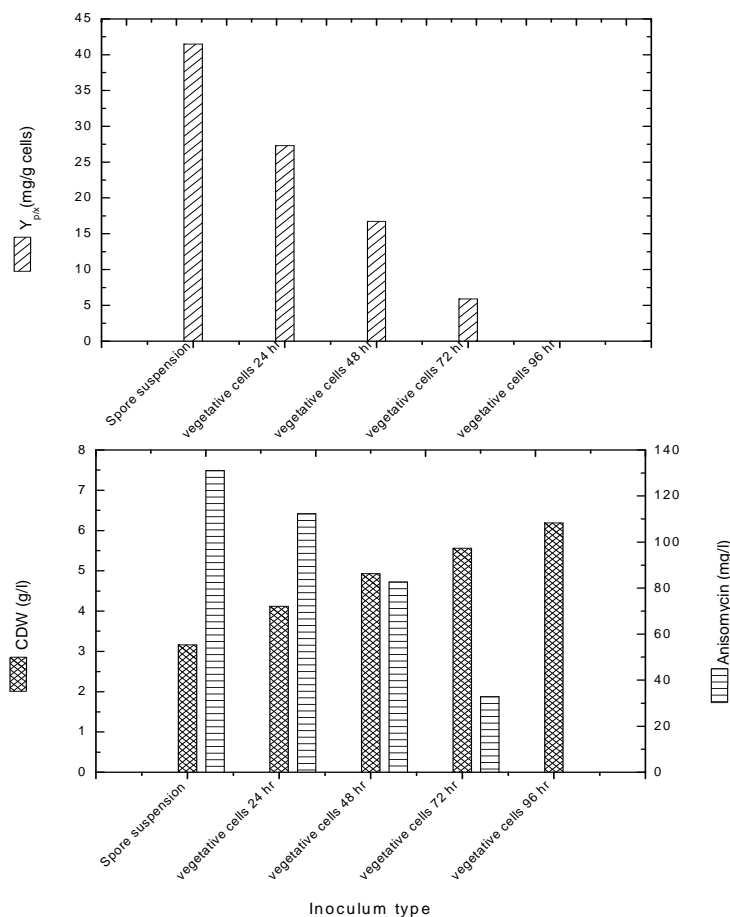


Fig. 9: () Anisomycin production & () CDW & () $Y_{p/x}$ by the mutant strain (M-107) using different inoculum type.

Discussion:

Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all industrial commercial fermentation processes (Parekh *et al.*, 2000). At present, strain improvement has been achieved chiefly through classical strain improvement technology and modern genetic engineering technology. To increase the titer of anisomycin, the wild-type strain was subjected to UV irradiation for strain improvement according to literature protocols (Kieser *et al.*, 2000). In this study it was found that the use of UV irradiation has a strong mutagenic effect on *Streptomyces griseolus* NRRL B-1062 since considerable variability was observed between the survived isolates from each UV dose. After irradiation for 4 min., only 0.01% of the mutants survived. Also the mutation frequency of the induced mutants ranged from 6.45×10^{-6} at 30 sec. of exposure to 5×10^{-2} after 240 sec which is lower than that reported in a study on oxytetracycline production by *Streptomyces rimosus* IMRU 3558 which ranged from 6.6×10^{-6} after 15 sec. to 0.01 after 90 sec. of exposure. The selected mutant showed an increased anisomycin yield, which is about two fold higher than that with the original strain.

Many previous studies proved that antibiotic biosynthesis in Streptomyces appeared to be regulated in response to nutritional status (Walker and Walker 1971). Accordingly; the production of anisomycin was examined in various complex media because there is no generalized medium applicable to all organisms. Paul and Banerjee, (1983.). Therefore, seven different media were tried for the fermentation process. The high antibiotic titer in the selected medium may be due to its content glucose, peptone, beef extract and yeast extract respectively, which were found to be preferred by the organism than other carbon and nitrogen sources. The

highest antibiotic titer recorded 70.8 mg l⁻¹ may possibly be due to the presence of soluble amino acids and peptide in them.

Since a better carbon source may improve the production of anisomycin, the effect of different carbon source was investigated and the results indicated that ribose and corn starch followed by glucose supported the growth of the microorganism as well as anisomycin production. To reduce the capital cost of the fermentation medium, we tried to use an alternative carbon source other than ribose. Different concentrations of glucose in combination with 10 g l⁻¹ of corn starch were investigated and optimum combination 8 g l⁻¹ glucose with 10 g l⁻¹ corn starch supported a comparable level of anisomycin titer upon using ribose alone instead of glucose. Hence, this result implies that the corn starch and glucose have been used as the principal substrates for anisomycin biosynthesis in the *Streptomyces griseolus* M-107.

The selected production medium contains peptone, beef extract and yeast extract which are relatively expensive for industrial application, and therefore the use of an alternative commercial and cheap source to these expensive nitrogen sources for anisomycin production is desirable from the economical view point. It is found that soybean showed its superiority for anisomycin biosynthesis over the other investigated nitrogen sources or the combination (peptone, beef extract and yeast extract). This result comes in agreement with that reported that the soybean meal is the preferable nitrogen source and this because soybean meal contains tyrosine which is the major precursor for anisomycin biosynthesis (Butler 1966). Moreover, the optimum concentration of soybean meal was 12 g l⁻¹ giving the highest level of anisomycin production (130.9 mg l⁻¹) and further increase of soybean meal concentration resulted in a gradual decrease in anisomycin yield.

Medium optimization studies revealed that anisomycin production by *Streptomyces griseolus* M-107 was suppressed by phosphate where as negative regulation by phosphate is often seen in secondary metabolites (Liras *et al.*, 1977, Zhang *et al.*, 1989). The optimum pH of 7-7.5 for growth indicates that the organism belongs to the neutrophilic actinomycete group (Williams *et al.*, 1971).

Oxygen supply in the culture medium during the growth phase was essential for achieving a better production performance. Similarly, Chen and his coworkers also demonstrated that oxygen participates in the regulation of key biosynthetic enzyme and thus in the final yield of the antibiotics tylosin and macrocin by *Streptomyces fradiae* (Chen and Wilde 1990). Thus, it seems reasonable to suppose that higher oxygen concentrations in *Streptomyces griseolus* M-107 fermentations increases the synthesis of enzyme involved in anisomycin production. Reports discussed that this may be attributed to the fact that increasing of the agitation speed tend to increase the amount of dissolved oxygen available to the culture the evidence presented by many investigators who reported that a mixing speed of 300 rpm required to supply enough dissolved oxygen to a culture of *Streptomyces noursei* producing nystatin (Loptanev *et al.*, 1973). Lower mixing speed resulted in depletion of dissolved oxygen and reduction in the formation of erythromycin. Also, it has been reported that an agitation speed of 300 rpm was required to keep the dissolved oxygen level from falling below 20% of saturation in candidin and candihexin fermentation (Martin and Mc Daniel 1974). These results were in correlation with those reported when adequate aeration was not provided, the dissolved oxygen level will fall below the critical level (the level above which the rate of oxygen uptake is independent of oxygen concentration). In such case, candidin production was reduced (Martin and McDaniel, 1977). These results demonstrate the importance of studying DOC profiles in fermentations and how advantageous oxygen control can be.

The performance of a microbial culture can be strongly influenced by the choice of the inoculum. The type, age and size of the inoculum should then be optimized to improve the performance of the biological process (Elibol *et al.*, 1995). In all experiments reported here, we used fresh spore inocula to minimize the lag phase in batch culture. All inocula were derived from a single spore stock. Investigation of the effects of spore and vegetative inocula showed that the inoculum in the form of spore 2×10^8 spores ml⁻¹ gave maximal cell productivity based on biomass [$Y_{p/x}$] of about 41.499 mg g⁻¹ cells whereas in case of inoculation by 48 h old vegetative cells, a lower value of 16.742 mg g⁻¹ cells was obtained. A proportionality to biomass yield was noticed when vegetative inoculum age was increased from 24h till 96h conversely to the production of anisomycin. The relationship between growth and anisomycin production was studied in batch cultures grown in the optimized medium and this study revealed that the production of anisomycin paralleled the accumulation of biomass in the cultures, indicating that the production is growth-associated in addition a rapid rise in pH corresponds closely to the period of rapid increase in anisomycin potency.

The spectral data obtained from the different analysis showed that the extracted compound was typical to authentic anisomycin as:

The UV spectrum of the extracted compound in chloroform had a maximum at 224 and 276 nm which was in agreement with that carried out by (Tanner *et al.*, 1954; Rusanova *et al.*, 2000) which proved that UV spectrum of anisomycin in chloroform had a maximum at 223-276 nm. Also, IR spectrum of the this compound contained the following peaks: 3434, 2924, 2856, 1731, 1630, 1517, 1377, 1247, 1176 cm⁻¹ which were going with the presented IR spectral data of anisomycin by (Lynch *et al.* 1954) who displayed the IR spectrum of

anisomycin to be: 2930, 2830, 1720, 1605, 1500, 1370, 1240, 1170, and 1025 cm^{-1} and (Rusanova *et al.*, 2000) who displayed the IR spectrum of anisomycin to be: 3650, 2925, 2830, 1720, 1600, 1515, 1440, 1275.

Moreover, the mass spectrum of the isolated compound showed m/z: 266, 204, 187, 144, 126, 121, 91, 84, 78, 69, 62 which was in agreement with that obtained by Lynch *et al.* 1954 who indicated that anisomycin had m/z at :266, 264, 222, 162, 164, 144, 126, 122, 121, 84. So it can be concluded from these spectral data that the extracted compound is anisomycin.

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