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Production of antibacterial metabolites by strain no.10/2 (*S.albovinaceus*) and media optimization studies for the maximum metabolite production

Thaer Abdelghani*

Department of Medical Lab Sciences, Faculty of Allied Medical Sciences,
Arab American University, P.O. Box 240, Jenin, Palestine.

Corresponding Author: Thaer Abdelghani **Email address:** t.abdelghani@aauj.edu

ABSTRACT:

A new actinomycete strain no.10/2 (*S.albovinaceus*) with broad antibacterial and antifungal activities was isolated from soil sample of Palestine and characterized at our lab. Nutritional and cultural conditions for the production of antibiotic by this organism under shake-flask conditions have been optimized. The parameters resulted for maximum antibiotic production are 1.25 % mannitol as carbon source, 1 % malt extract as nitrogen source, initial pH of 7.2, 10 % level of inoculum, incubation period of 96 h, agitation in 250 ml bottom indented flask and medium to flask ratio of 1:10. The optimization studies resulted in the development of a modified production medium with enhanced yield of antibiotic formation compared with the basal fermentation medium**.** This is one of the first reports for the strain no.10/2 (*S.albovinaceus*) has been reported from Palestine soil with antibacterial and antifungal activities.

Key words: *Actinomycete, S. albovinaceus,* Antibiotics, nutrient effects, cultural conditions

1. INTRODUCTION

The discovery and use of naturally occurring products for the treatment of human diseases is prevalent throughout the human history. The discovery and the phenomenal success of penicillin had led to the search for other antibiotics from antibiotic producing microorganisms especially from soil environment. *Streptomyces* are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics[1]. About 75 % of the known commercially and medically useful antibiotics are produced by *Streptomyces*[2].

Waksman[3] has recognized some natural substrates as ideal sources for the isolation of actinomycetes, of which soil was found to be the richest source. Different soils all over the world had been exploited in search of bioactive actinomycetes.

So, in order to discover new antibiotics, our approach is to investigate unexplored regions of the world with the aim of isolating bioactive actinomycetes from these regions and from those organisms whose potential was neglected through out the history. The aim of our work was to conduct intensive screening programme on different soil samples of Palestine which is an unexplored territory and which is likely to yield purposeful results towards isolation of either new species of actinomycetes and/or new antibiotics. As such, it was felt that systematic screening of Palestine soil samples was very much necessary.

Various regulatory mechanisms exist in microorganisms that control the production of metabolites by fermentation[4]. The composition of the culture medium, closely connected with the metabolic capacities of the producing organism, greatly influences the biosynthesis of antibiotics[5,6]. Nature and concentration of some components in fermentation medium have a marked effect on antibiotic production[7]. Influence of particular nutrients on the antibiotic biosynthesis is determined by the chemical structures of antibiotic substances[8].

The paper reports here the antimicrobial activity profile of the isolated strain of *S.albovinaceus* (10/2) and the optimum fermentation conditions for antibiotic production.

2. MATERIALS AND METHODS

2.1 Isolation and maintenance:

The strain no.10/2 (*S.albovinaceus*) with a broad antibacterial and antifungal activities was isolated from soil sample of Palestine and characterized at our lab. It was isolated and grown on yeast extract malt extract (YEME) agar medium and incubating at 28 ºC. Cultures were stored at 4 ºC. For long storage, it was grown in YEME broth for 7 days. To it glycerol was added to a final concentration of 15% (v/v) and stored at –20 ºC [9].

2.2 Optimization of nutritional and cultural conditions:

Seed culture was prepared by inoculating one full slant culture into 50 ml of inoculum medium and incubated on rotary shaker (220 rpm) at 28 °C for 48 h. After 48 h 10 % level of inoculum was transferred to the selected production medium and incubated on rotary shaker (220 rpm) at 28 °C for 96 h. The inoculation as well as the production media were they are the same and selected from different media and contained % (w/v): meat extract 0.3, tryptone 0.5, yeast extract 0.5, dextrose 0.1, potato starch 2.4, calcium carbonate 0.2, pH was adjusted to 7.0 before sterilization.

The literature indicated that yield is depending on the nature of the strain, composition of medium and also on the cultural conditions[10]. As such it is decided to investigate the optimum cultural conditions for optimum antibiotic production. The following parameters were investigated for the optimum antibiotic production by our isolate no. 10/2: selection of a suitable inoculum medium, selection of a suitable production medium, effect of various carbon sources and its optimum concentration, effect of various nitrogen sources and its optimum concentration, effect of initial pH of the medium, effect of level of inoculum, effect of incubation period, effect of aeration, effect of agitation, and

production of antibiotic with the optimum conditions (using modified production medium and cultural conditions formulated based on the above observations).

2.3 Antimicrobial activity:

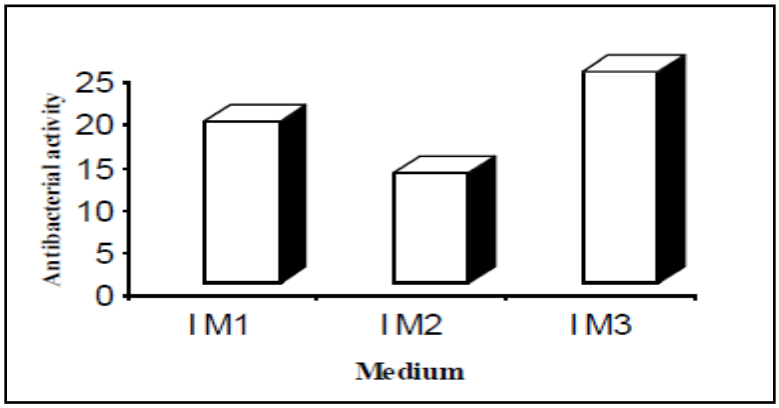
Antibacterial activity was performed by withdrawning samples at the end of the fermentation cycle (96 h), centrifuged, and the supernatant was assayed for extra-cellular antimicrobial activity by standard cup plate method using *Bacillus pumilus* as test organism.

3. RESULTS

Selection of a suitable inoculum medium for antibiotic production

In order to select the best inoculum medium for maximum antibiotic production, three different inoculum media IM_1, IM_2 and IM_3 were tried. The results are shown in Fig. 1. Inoculum medium III (IM_3) gave maximum antibiotic yield; hence it was used in further studies as the inoclum medium.

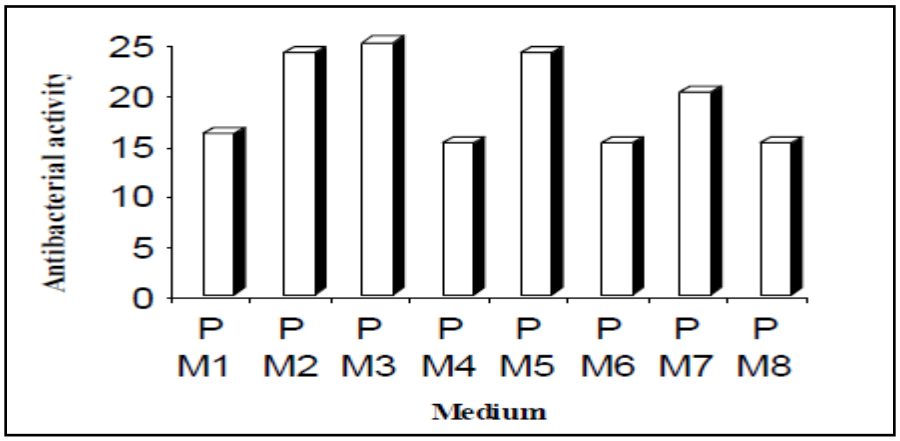
Figure 1: Selection of a suitable inoculum medium for maximum antibiotic production



Selection of a suitable production medium for antibiotic production

In order to select the best production medium for maximum antibiotic production 8 different media PM_1 to PM_8 were tried. The results are presented in Fig. 2. Production medium (PM_3) gave the maximum yield of the antibiotic in terms of inhibition zone. Hence it was selected for further optimization studies for antibiotic production. So, the inoculation as well as the production media used was the same and the composition is as given before in materials and methods.

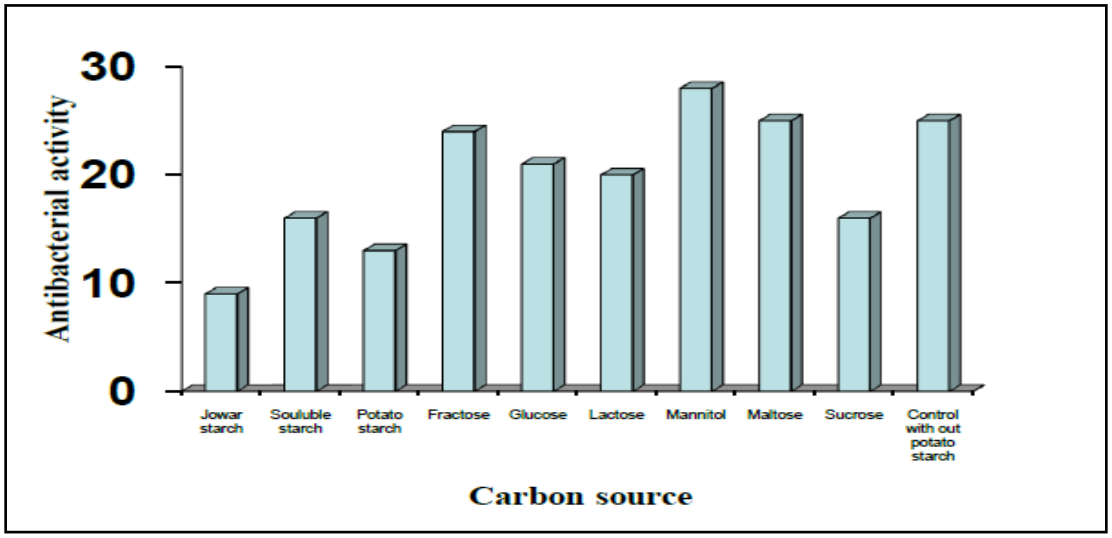
Figure 2: Selection of a suitable production medium for maximum antibiotic production



Effect of various carbon sources on antibiotic production

To study the effect of various carbon sources on antibiotic production, different carbon sources were tried at a concentration of 1 % in the basal production medium (without potato starch). The results were shown in Fig. 3. Among various carbon sources used mannitol was found to be the best carbon source for the maximum antibiotic production followed by maltose. So, mannitol was selected for further optimization studies for antibiotic production.

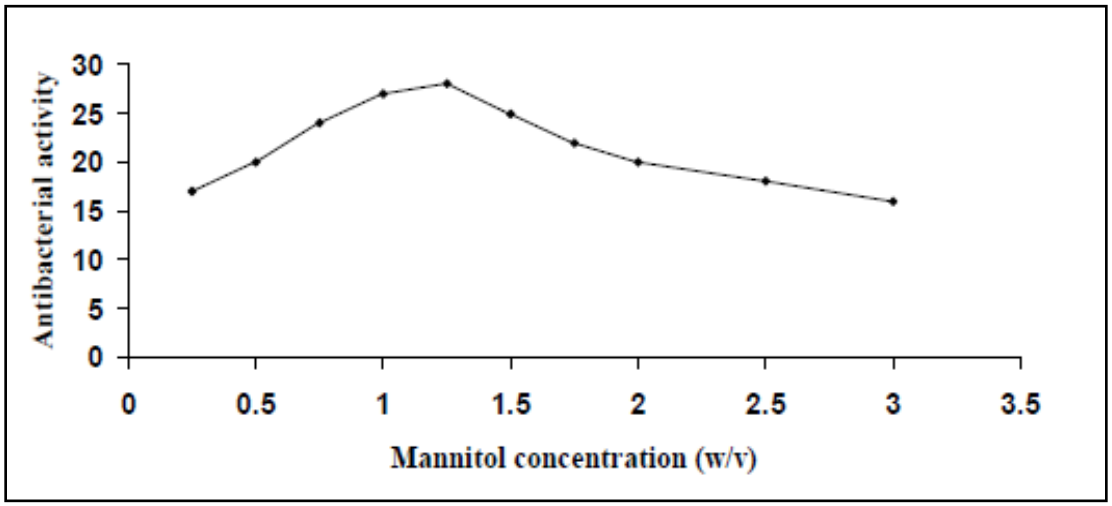
Figure 3: Effect of various carbon sources on antibiotic production



Determination of optimum concentration of mannitol for antibiotic activity

Various concentrations of mannitol were tried to study their effect on antibiotic production. The results are given in Fig. 4. Mannitol at a concentration of 1.25 % (w/v) gave maximum antibacterial activity.

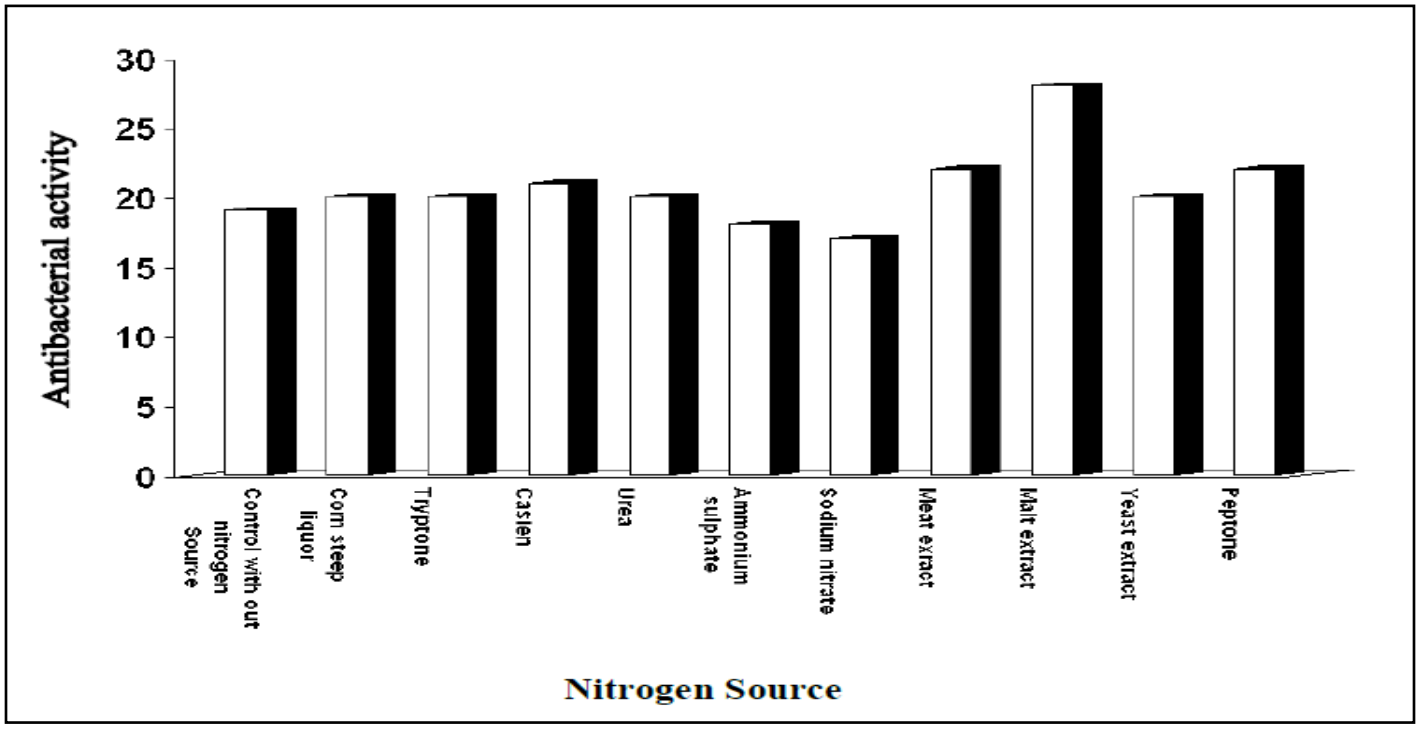
Figure 4: Determination of optimum concentration of mannitol for antibiotic production



Effect of various nitrogen sources on antibiotic production

Different nitorgen sources at a concentration of 1 % were tried to study their effect on antibiotic prduction. The results are shown in Fig. 5. Among the nitrogen sources employed, malt extract was found to be the best nitrogen source for antibiotic production.

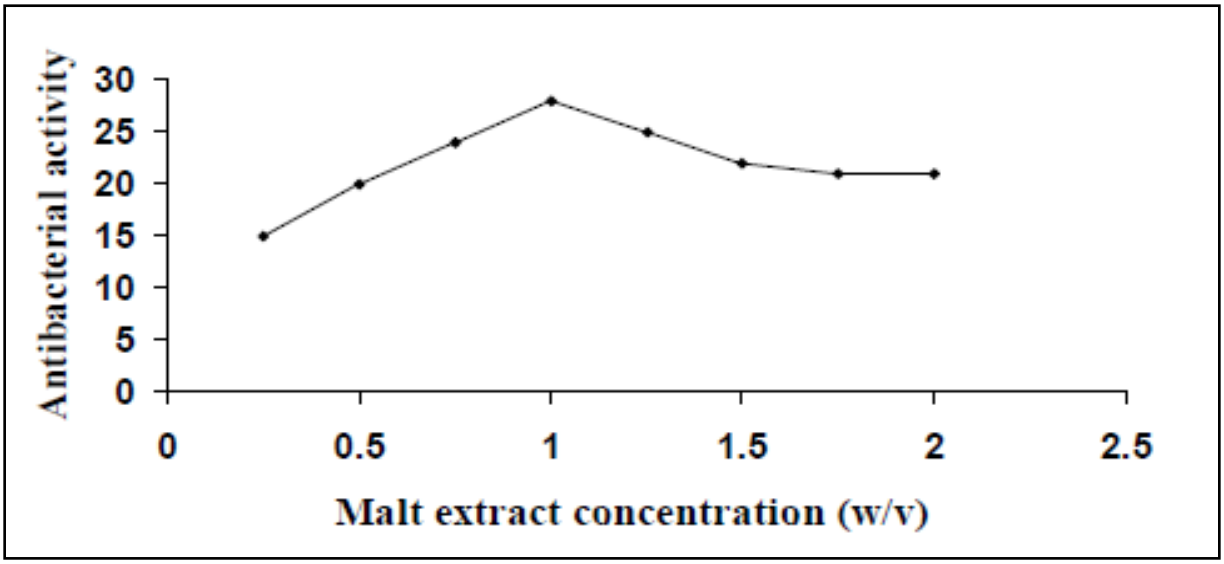
Figure 5: Effect of various nitrogen sources on antibiotic production



Determination of optimum concentration of malt extract for antibiotic production

Different concentrations of malt extract were tried to study their effect on antibiotic production. The results are shown in Fig. 6. Malt extract at a concentration of 1% gave maximum antibacterial activity.

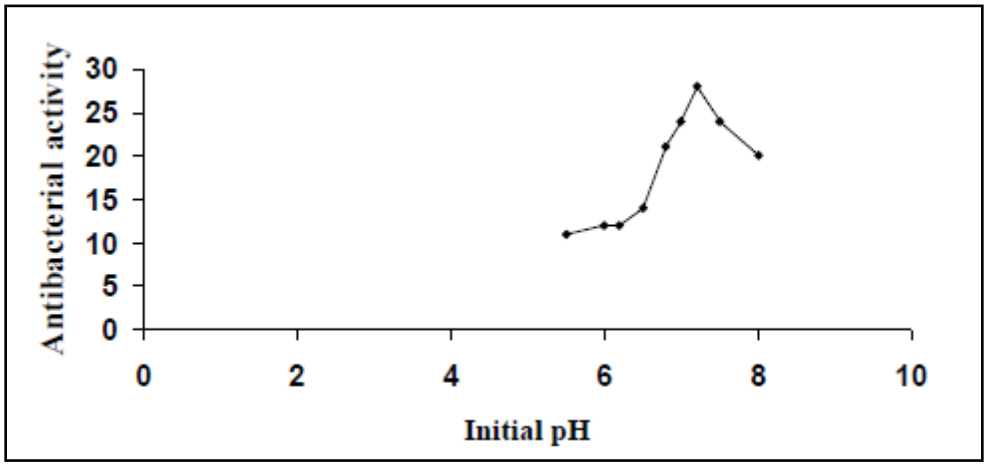
Figure 6: Determination of optimum concentration of malt extract for antibiotic production



Effect of initial pH on antibiotic production

In order to study the effect of pH on antibiotic production, production media with different initial pH were tried. The results are shown in Fig. 7. Medium with an initial pH 7.2 gave maximum antibacterial activity.

Figure 7: Effect of initial pH on antibiotic production



Effect of level of inoculium on antibiotic production

To study the effect of level of inoculum on antibiotic production, different levels of inocula were tried. The results are shown in Table 1. A 10 % inoculum level gave maximum antibacterial activity.

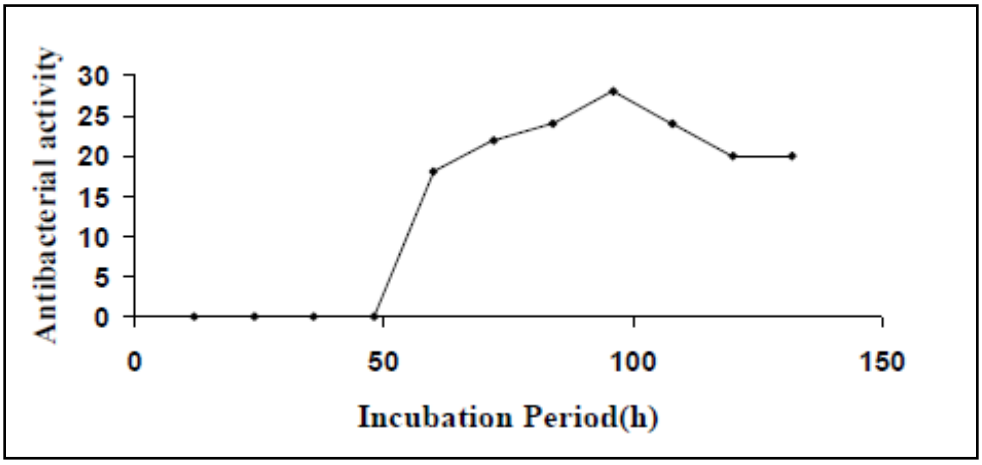
Table 1: Effect of level of inoculum on antibiotic production

Level of inoculum in % (v/v)	Antibacterial activity
	Inhibition zone diameter (mm)
2.5%	24
5.0%	25
10.0%	**29**
15.0%	28

Effect of incubation period on antibiotic production

To determine the effect of incubation period on antibiotic production, different incubation periods were tried. The results are shown in Fig. 8. An incubation period of 96 h was found to be optimal for maximum antibiotic production.

Figure 8: Effect of incubation period on antibiotic production



Effect of aeration on antibiotic production

To study the effect of aeration on antibiotic production, different volumes of medium were tried in 250 ml EM flasks. The results are shown in Table 2. A 25 ml production medium in 250 ml flask gave maximum inhibition zone for the antibacterial activity.

Table 2: Effect of aeration on antibiotic production

Volume of medium (ml)	Antibacterial activity
	Inhibition zone diameter (mm)
25	**30**
50	25
75	21
100	19

Effect of agitation on antibiotic production

In order to estimate the effect of agitation on antibiotic production, different types of flasks with different capacities were tried. The results are presented in Table 3. Fermentation when carried out in 250 ml bottom indented flask gave maximum antibacterial activity.

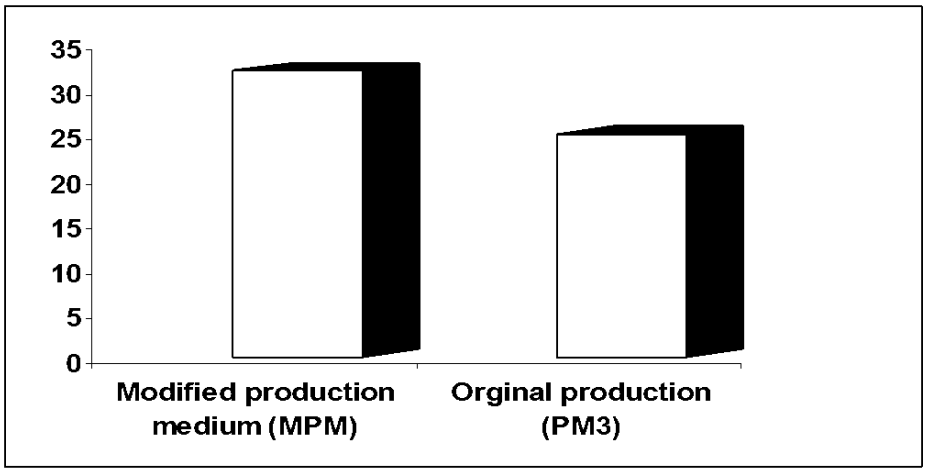
Table 3: Effect of agitation on antibiotic production

Type of flask	Antibacterial activity
	Inhibition zone diameter (mm)
Un-indented 250 ml 500 ml	25 12
Side indented 250 ml 500 ml	 29 19
Bottom indented 250 ml 500 ml	 30 20

Production of antibiotic with optimum conditions

Based on the results obtained with all optimum parameters, an attempt was made to evalute the extent of improvement in the modified formulated production medium. The results are shown in Fig. 9. Significant improvement in the antibiotic titre was observed with the modified production medium for antibacterial activity.

Figure 9: Production of antibiotic with optimum conditions



4. DISCUSION

Qualitative and quantitative aspects of antibiotic production by the microorganisms are dependent on the selective environmental pressure prevailing at its source of isolation and manipulation of growth and nutritional conditions during fermentation exerts substantial influence on the level of metabolite production[10]. Wild strains are very rich in chemical diversity and novel lead drug molecules[7]. Perlman et al.,[11] showed that antibiotic production was significantly reduced when the streptomycete was grown on a synthetic medium or when it was grown under conditions such that the pH of the medium rose above pH 8.

The biosynthesis of antibiotics is regulated by the type and concentration of different medium components such as carbon, nitrogen, phosphate, metal ions and other medium ingredients[12]. Many factors influence the extent of growth of microbial cells, chemical composition, and the nature and concentration of specific metabolic products produced[3]. There is considerable selectivity in the utilization of these substances by different kinds of actinomycetes[13,14]. For our isolate the inoculation as well as the production media were they are the same and selected from different media (Fig.1 and 2). The environmental factors like incubation temperature, pH and incubation time were also found to have profound influence on antibiotic production. In preliminary studies using a one factor-at-a-time approach, three independent variables, namely concentration of carbon source, nitrogen source and incubation temperature, were found to be the most important factors influencing production[15].

In our study, the use of this type of production medium gave the maximum antibiotic production. This may be because of the decrease of lag time due to the use of the same inoculum as well as production medium. Optimization of antibiotic production was carried out in batch culture. This strain was able to grow in all the tested carbon sources (Fig. 3). However, maximum antibiotic production was obtained in medium supplemented with mannitol as a sole carbon source followed by maltose. Sanchez and Demain[7] have listed several carbon sources which interfere with the antibiotic production. Sugar can affect the metabolism directly by decreasing the time for switching over to stationary phase[16]. Abbanat et al.,[17] have found that high glucose concentration (10 g/l) decrease the production of pyrroindomycin by *S. rugosporus.* In case of non-sulfated liposidomycin production, when glucose was replaced with D-xylose and sucrose, the antibiotic production was found to increase substantially[18]. The best carbon source for AK-111-81 synthesis was lactose[19]. Mohamed et al.,[20] showed that 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. This is in agreement with our results. Our experiment has shown that mannitol is the best carbon source for antibiotic production. It was thought, therefore, to test whether antibiotic production could be improved by varying mannitol concentration in the cultivation

medium. For this purpose mannitol was applied in different concentrations varied from 0.25 % to 3.0 %. The results in Fig. 4 showed that production of antibiotic continues to increase and reached a maximal value at 12.5 g/l. Above this concentration, the antibiotic production decreased. As mannitol concentration increased from 12.5 to 30 g/l the antibiotic production decreased. On the other hand, mannitol was completely consumed when used at a concentration of 12.5 g/l or lower. The increase in mannitol concentration above this level resulted in the accumulation of mannitol in the cultivation medium and the remained amount depended on the initial concentration. Therefore, mannitol in a concentration of 12.5 g/l was used in the subsequent experiments.

The level of antibiotic production may be greatly influenced by the nature; type and concentration of the nitrogen source supplied in the culture medium. Depending on the biosynthetic pathways involved, nitrogen sources may affect antibiotic formation[19]. Mohamed et al.,[20] showed that nitrogen source exhibited a significant effect on the natamycin production. Different nitrogen sources, inorganic and organic were tested. The results (Fig. 5) show that nitrogen source exhibited a significant effect on the antibiotic production. Among different nitrogen sources investigated, results revealed that inorganic nitrogen sources did not support antibiotic production. The best nitrogen source for supporting antibiotic production was malt extract followed by meat extract. Our results are in agreement with the results of Mohamed et al.,[20]. These results are in agreement with those observed by Gil et al.,[21] where they reported that the utilization of inorganic nitrogen source resulted in a poor production in case of polyene macrolid antibiotic. Gesheva et al.,[19] reported that incorporation of inorganic salts in the medium inhibited the antibiotic AK-111-81 by *Streptomyces hygroscopicus*. It was noted by Sanchez and Demain[7] that ammonium salts did not favour biosynthesis of novobiocin, actinomycin, neomycin, kanamycin and others, but for rapamycin ammonium sulfate was the best nitrogen source[22]. Our results also showed that the concentration of malt extract (Fig. 6) greatly influenced the production of the antibiotic with maximum antibiotic yield being obtained in cultures supplemented with 10 g/l of malt extract.

Also the environmental factors like pH, level of inoculum, and incubation period were also found to have profound influence on antibiotic production[2,23]. The environmental requirements and tolerance for growth and antibiotic production has been studied in detail.The maximum antibiotic activity was obtained at a pH of 7.2 (Fig. 7) suggesting its inclusion in the neutrophilic actinomycetes group. This is in agreement with the results obtained by Sujatha et al.,[2]. Borenstajn & Wolf [24] reported that for oxytetracycline production a pH value of 7.0 was suitable. Antibiotic production was significantly reduced when the streptomycete was grown under conditions such that the pH of the medium rose above pH 8[11]. The effect of inoculum level on the production of penicillin and griseofulvin was studied. It was reported that the biochemical factors such as the levels of the enzyme activity and effciency were at least as important as morphology in determining the yield of the antibiotic25. This is in agreement with our results (Table 1). The results also indicated that an incubation time of 96 h is optimal for maximum antibiotic production (Fig. 8). This is in agreement with the results obtained by Sujatha et al.[2].

Dissolved oxygen (DO) is known to be an important parameter in industrially important antibiotic fermentations[26]. Increased levels of dissolved oxygen have lead to enhanced antibiotics production[27,28]. Flickinger and Perlman[29] found a 2-3-fold increase in neomycin production by *Streptomyces fradiae* when DO was maintained above 5 kPa, by oxygen enrichment. Several techniques have been used to control DO in fermentations, the most common being the use of agitation speed and the aeration rate to the fermentor[26]. Malcolm et al.,[30] demonstrated that controlling dissolved oxygen levels close to saturation during periods of rapid growth markedly improves the efficiency and duration of cephamycin C biosynthesis in *S. clavuligerus*. This is in agreement with our results. A 25 ml production medium in 250 ml flask gave maximum inhibition zone for the antibacterial activity (Table 2). Also fermentation when carried out in 250 ml bottom indented flask gave maximum antibacterial activity (Table 3).

From the present investigations, it is clear that a novel strain *S. albovinaceus* (10/2) with excellent antibiotic production has been identified in our laboratory, and the medium and cultural conditions for maximum antibiotic production have been optimized. The optimization studies resulted in the development of a modified production

medium with enhanced yield (Fig. 9). The parameters resulted for maximum antibiotic production are 1.25 % mannitol as carbon source, 1 % malt extract as nitrogen source, initial pH of 7.2, 10 % level of inoculum, incubation period of 96 h, agitation in 250 ml bottom indented flask and medium to flask ratio of 1:10.

The antibiotic producing microorganism isolated and investigated in this study has shown broad spectrum antimicrobial activity. Preliminary chemical characterization (data not shown) of the isolated compounds has demonstrated that the organism produced antibacterial and antifungal compounds which is very advantageous as this organism produces two broad spectrum antimicrobial compounds simultaneously and their isolation is easy. Full chemical characterization of the compounds isolated and anticancer activity are in progress. Also strain improvement and gene manipulation studies, use of different inducers to improve the yield of the antibiotic, and whole cell immobilization techniques are in progress.

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