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Optimization of the Biosynthesis of the Macrolide Antibiotic WA52 Produced by the Alkaliphilic Isolate *Nocardiopsis dassonvillei* WA52

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The effects of some physical and nutritional parameters were studied for the optimum production of a macrolide antibiotic, designated WA52-A, that was produced in the fermentation culture of the alkaliphilic strain *Nocardiopsis dassonvillei* WA52. The effects of incubation period, initial pH and incubation temperature were evaluated. The results revealed that the maximum antibiotic production was achieved after 5 days incubation at 30ºC with an initial pH of 9. Subsequently, the effects of the nutritional requirements, carbon source, nitrogen source and mineral salts, were investigated. The results indicated that the maximum antibacterial activity was obtained using a fermentation medium with the following composition (g/100ml): Glucose, 2.5; $NaNO_3$, 0.2; K_2HPO_4, 0.12; $MgSO_4$, 0.05; KCl, 0.06; and $FeSO_4$, 0.008.

Key words: Optimization, antibiotic, alkaliphilic, *Nocardiopsis dassonvillei* WA52.

Secondary metabolites production in microorganisms is strongly influenced by nutritional factors and growth conditions [1]. Even small changes in the culture medium may not only impact the quantity of certain compounds but also the general metabolic profile of microorganisms [2]. Therefore, in the field of antibiotics, much effort was directed toward optimizing production rates and directing the product spectrum [3].

Imura and Tanaka[4] suggested the following conditions for the production of antibiotics and other secondary metabolites: (1) antibiotic production is strain specific; (2) it is unstable, and productivity tends to disappear on successive transfer of the producing organism and by mutational treatments; (3) antibiotic production follows growth associated kinetics in one medium, but is not growth associated in other media; (4) active antibiotic production often occurs in association with sporulation of the producing organism, which begins with nutritional limitation (such as carbon or nitrogen sources and inorganic phosphate) in cultivation media; and (5) they are produced as members of one or more families with many homologs, and distribution of the components is easily varied by a minor modification of the cultivation conditions.

Although the detailed mechanisms by which antibiotic biosynthesis is initiated and regulated are not well understood, many factors are suggested to influence antibiotic production. These factors are categorized into two types: biochemical (extracellular or nutritional and intracellular) and physicochemical (or operational)[5].

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The nutritional requirements for antibiotic biosynthesis include the carbon source, the nitrogen source, inorganic phosphate, inorganic salts, trace metals and others. The physicochemical factors that affect the biosynthesis of any antibiotic are pH, temperature, oxygen tension and others [6].

On this view point, attempts have been made to optimize the conditions controlling the biosynthesis of the macrolide antibiotic WA52-A. The alkaliphilic actinomycete strain *Nocardiopsis dassonvillei* WA52 was isolated from a desert soil sample collected at Wadi Araba, the Eastern Desert of Egypt. It was identified based on morphological, physiological and biochemical characteristics [7]. The extraction, purification and chemical characterization of antibiotic WA52-A was reported [8]. But the suitable fermentation conditions have not been reported yet which is the objective of this study.

MATERIALS AND METHODS

Effect of the incubation period on the biosynthesis of WA52-A

The spores and/or mycelia of the alkaliphilic actinomycete isolate *Nocardiopsis dassonvillei* WA52 were allowed to grow on a modified starch-nitrate medium[9] having the following composition: soluble starch, 20 g; $NaNO_3$, 2 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.5H_2O$, 0.01 g; Distilled water, 1000ml. The initial pH was adjusted at 10 after sterilization by 10% solution of Na_2CO_3. The medium was allotted among Erlenmeyer conical flasks of 250ml capacity. Fifty milliliters of the medium were dispensed in each flask. A triplicate set of flasks was used for each particular incubation time. The flasks were sterilized, inoculated and incubated on a rotary shaker of 200rpm at 30ºC. Cultures were removed after various intervals of incubation periods and tested for antibiotic biosynthesis. The potency of the antimicrobial activity was determined after neutralization of the broth using dilute HCl solution on *Bacillus subtilis* NCTC10400.

Effect of the pH value on the biosynthesis of WA52-A

The experiment was conducted as mentioned above except that the initial pH value of the media was adjusted to cover the range from 6 to 12 after sterilization. A triplicate set of flasks was always used for each particular pH value. The experiment was terminated after 5 days. The broth was filtered and neutralized using either dilute HCl or NaOH solutions. Finally, the potency of the antimicrobial activity was determined.

Effect of the incubation temperature on the biosynthesis of WA52-A

The experiment was conducted as previously described except that the initial pH of the medium was adjusted to 9.0 and the flasks were incubated at different temperatures covering the range from 10- 45ºC. A triplicate set of flasks was always used for each particular temperature. After 5 days incubation, the antimicrobial activity was assessed at each temperature.

Effect of different carbon sources on the biosynthesis of WA52-A

The basal modified starch-nitrate medium lacking the carbon source was fortified by various carbon sources. These carbon sources were added in equimolecular amounts to permit the presence of equal weights of carbon. The following carbon sources were added: glucose, fructose, mannitol, mannose, sucrose, lactose and soluble starch. The sugars under test were separately sterilized by the addition of diethyl ether, left to dry and then added separately to the constituents of the basal medium. The initial pH of the various media was adjusted at 9.0 after sterilization. The rest of the experimental steps were carried out as described before.

Effect of different glucose concentrations on the biosynthesis of WA52-A

In this experiment the following concentrations of glucose were used (gm/100ml): 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0. The rest of the experimental steps were carried out as described before.

Effect of different nitrogen sources on the biosynthesis of WA52-A

The following nitrogenous compounds were supplemented to the basal modified starch-nitrate medium in presence of 2.5% glucose: $NaNO_3$, $(NH_4)_2SO_4$, asparagine, peptone, yeast extract and beef extract. The nitrogen sources were added at an equimolecular amount of nitrogen found in $NaNO_3$ except peptone, yeast extract and beef extract which were used at a concentration of 0.2% for each. The rest of the experimental steps were carried out as previously described.

Effect of different $NaNO_3$ concentrations on the biosynthesis of WA52-A

Sodium nitrate was supplemented to the basal medium in presence of 2.5% glucose in the following concentrations (gm/100ml): 0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4.

Influence of K_2HPO_4 concentrations on the biosynthesis of WA52-A

K_2HPO_4 was supplemented to the basal medium in presence of 2.5% glucose and 0.2% $NaNO_3$ in the following proportions (gm/100ml): 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16 and 0.18. The rest of the experimental procedure was carried out as previously described.

Influence of $MgSO_4.7H_2O$ concentrations on the biosynthesis of WA52-A

The experiment was carried out as previously described after the addition of 0.12% (w/v) K_2HPO_4, but $MgSO_4.7H_2O$ was supplemented in the following proportions (gm/100ml): 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08 and 0.09.

Influence of KCl concentrations on the biosynthesis of WA52-A

After the addition of 0.12% (w/v) K_2HPO_4 and 0.05% (w/v) $MgSO_4.7H_2O$, KCl was supplemented to the basal medium in the following proportions (gm/100ml): 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08 and 0.1. The rest of the experimental steps were conducted as described before.

Influence of $FeSO_4.5H_2O$ concentrations on the biosynthesis of WA52-A

The same steps were carried out as in the above experiment except that KCl was added to the medium in a concentration of 0.06 %. Ferrous sulfate was supplemented to the basal nutrient medium in the following proportions (gm/100ml): 0.0004, 0.0006, 0.0008, 0.001, 0.0012, 0.0014, 0.0016 and 0.0018.

RESULTS AND DISCUSSION

It has been known that controlling cultivation parameters and nutritional requirements are critical to the secondary metabolites produced by microorganisms [3]. From the environmental conditions (conditions of cultivation) incubation period, pH and temperature were tested.

The effect of incubation period on the production of antibiotics is quite important, since it relates productivity to the time of incubation, which may be of economic value. Time required for high yield of antibiotic varied greatly from one microorganism to another. Komaki *et al.* [10] reported that the production of brasilicardin A, a terpenoid antibiotic from *Nocardia brasiliensis*, reached maximum after 70 to 80 hours incubation. Good result was obtained with incubation time from 2 to 4 days for the production of a clavam antibiotic synthesized by *Streptomyces clavuligerus* [11]. While, the production of nocardicyclin A, an anthracycline antibiotic produced by *Nocardia pseudobrasiliensis*, reached a maximum on day 6[12]. On the other hand, a maximum production of PC-766B, a macrolide antibiotic produced by *Nocardia brasiliensis*, was achieved at day 10 after inoculation [13]. Therefore, detection of the most

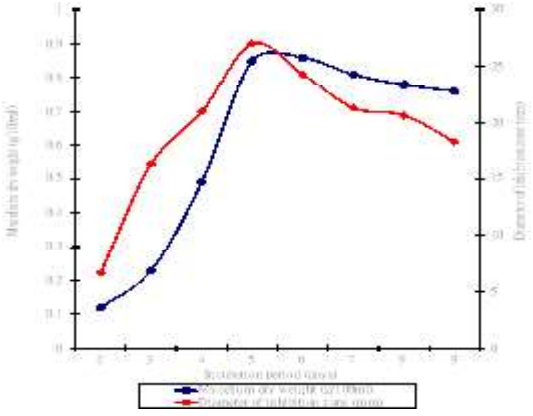

Fig. 1. Effect of different incubation periods on growth and biosynthesis of the antibiotic WA52-A produced by the alkaliphilic isolate *Nocardiopsis dassonvillei* WA52

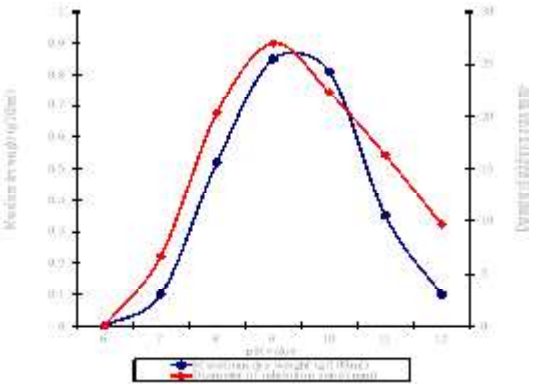

Fig. 2. Effect of different pH values on growth and biosynthesis of the antibiotic WA52-A produced by the alkaliphilic isolate *Nocardiopsis dassonvillei* WA52

suitable incubation period is of great economic value.

The results showed that the maximum biosynthesis of the antibiotic WA52-A was achieved after 5 days incubation (Fig. 1). Similar results were obtained by other authors for production of gentamicin and formamicin [14,15].

Production of antibiotics from actinomycetes in all types of media, acidic, neutral and alkaline is recorded now. Most antibiotic producing actinomycetes grow well at pH values 6.7 to 7.8 with optimal pH for production at 7. Igarashi *et al*. [16] reported that pH 7.4 was optimum for production of gremimycin, a 19-membered macrocyclic lactam antibiotic, by *Streptomyces* sp. However, acidophilic actinomycetes have been isolated and the optimum pH being from 3.5 to 6.5 [17]. On the other hand, the production of antimycin A by an alkaliphilic *Streptomyces* sp. at pH 9 was reported [18].

The obtained results (Fig. 2) revealed that the optimal pH value for maximum biosynthesis of WA52-A produced by the alkaliphilic isolate *Nocardiopsis dassonvillei* WA52 was 9 and that the antibacterial activity was restricted to the alkaline side only and no growth or activity was observed below pH 7. Similar results were obtained for the production of M119 complex by the alkalophilic actinomycete *Nocardiopsis* sp. M119, 16-membered macrolide antibiotics, produced by an alkaliphilic actinomycete, *Nocardiopsis* sp. M119 [19].

It was reported that the optimum temperature differs from one organism to another and most antibiotic producing actinomycetes are mesophilic with optimum temperature for growth and antibiotic production somewhere in the range 26- 30ºC [20]. It is obvious from the results (Fig. 3) that the alkaliphilic organism *Nocardiopsis dassonvillei* WA52 failed to grow well at both low and high temperature degrees (10ºC and 45ºC). The growth of the organism and the activity were detected at temperature range from 15ºC to 40ºC with maximum activity and growth at 30ºC. Similar results were reported for the production of erythromycin and gremimycin [21, 16].

The effect of certain nutritional factors (constituents of the medium) on the biosynthesis of the antibiotic WA52-A was also studied. These factors greatly affect the growth and production of antibiotics. The most important factors among them are the most suitable carbon source, the most suitable nitrogen source and the inorganic salts.

The results revealed that glucose is the best carbon source for the activity of WA52-A (Fig. 4). The effect of different carbon sources on the antibiotic biosynthesis was studied by many authors. Kralovcova and Vanek [22] stated that glucose is the most suitable carbon source for fermentation process. On the other hand, biosynthesis of some antibiotics are inhibited by addition of glucose as a carbon source such as actinomycin produced by *Streptomyces*

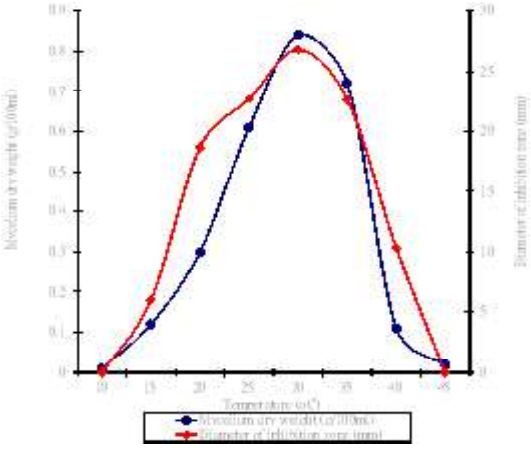

Fig. 3. Effect of different incubation temperatures (°C) on growth and biosynthesis of the antibiotic WA52-A produced by the alkaliphilic isolate *Nocardiopsis dassonvillei* WA52

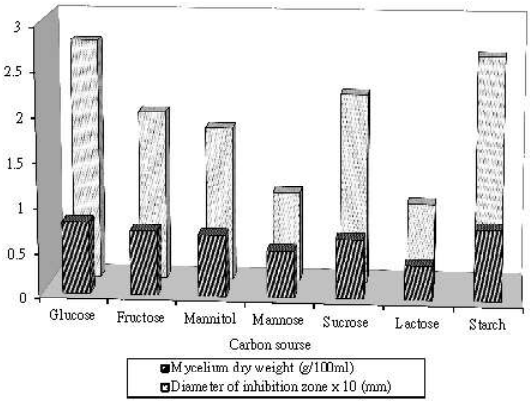

Fig. 4. Effect of different carbon sources on growth and biosynthesis of the antibiotic WA52-A produced by the alkaliphilic isolate *Nocardiopsis dassonvillei* WA52

antibioticus [23] and mitomycin produced by *Streptomyces verticillatus* [24]. However, in penicillin fermentation, Johnson [25] found that its production is better in the presence of lactose, while, Ketaki and Majumdar [26] reported that galactose is the most suitable carbon source for production of kanamicin.

It was found that 2.5% glucose was the best concentration for the biosynthesis of the antibiotic under study (Fig. 5). Similar results were recorded by some investigators as for erythromycin and bacteriocin biosynthesis [21, 22]. It is obvious that the higher concentrations greatly reduced the antibiotic production as well as growth. In the production of anticapsin by *Stereptomyces griseoplanus,* it was found that its maximum accumulation was obtained in the presence of 10% glucose [27], but this is an exceptional case.

It is well known that changes in the kind and concentration of the nitrogen source influence greatly antibiotic production [28]. It was found that $NaNO_3$ was the most favorable nitrogenous compound for the growth and the production of the antibiotic under investigation (Fig. 5). Other investigators found that ammonium sulfate and ammonium chloride containing reduced nitrogen had a favorable effect in subiromycin biosynthesis, while nitrates had a negative effect on antibiotic production [29]. While, soybean meal was reported to be the best nitrogen source for gentamicin production by *Micromonospora purpurae* [30] and malt extract was the best nitrogenous compound for the biosynthesis of arugomycin by *Streptomyces violaceochromogenes* [31]. The data obtained (Fig. 6) showed also that 0.2% sodium nitrate concentration was the best for the biosynthesis of WA52-A.

Macroelements (phosphorus, potassium, magnesium and sulfur) are of vital physiological importance. The role-played by different concentrations of the mineral salts for antibiotic biosynthesis has been studied by many authors. Temple [32] reported that magnesium and potassium salts exerted the most promising effects on both growth and antibiotic production. Iron, on the other hand, has a lesser effect on growth but was required for high streptomycin production.

The concentration of inorganic phosphate is very important for antibiotic production. Our results showed that 0.12% K_2HPO_4 gave maximum activity. Aharonwitz and Demain [33] reported that the production of cephalosporin by *Streptomyces clavuligerus* increased with increasing phosphate until the concentration reached 25mM. On the other hand, Kumagai *et al.*[13] reported that addition of 1mM KH_2PO_4 to the production medium decreased PC-766B, a macrolide antibiotic, productivity to 44% of control.

The magnesium ion is also important and

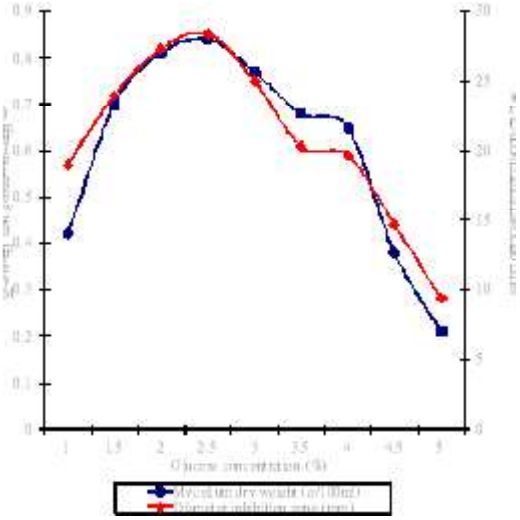

Fig. 5. Effect of different glucose concentrations on growth and biosynthesis of the antibiotic WA52-A produced by the alkaliphilic isolate *Nocardiopsis dassonvillei* WA52

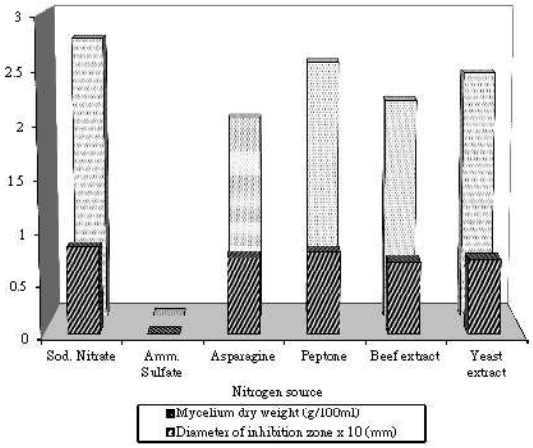

Fig. 6. Effect of different nitrogen sources on growth and biosynthesis of the antibiotic WA52-A produced by the alkaliphilic isolate *Nocardiopsis dassonvillei* WA52

the optimum concentration of magnesium depends on many factors specially, the concentration of the carbon source and the concentration of other ions, to many of which magnesium is the antagonist. The maximum production of WA52-A was obtained from production medium supplemented by 0.05% $MgSO_4$. Production of the antibiotic a-60 (a quinone) was accelerated by the addition of higher concentrations of $MgSO_4$ [34].

Mineral sulfates such as $MgSO_4$ and $FeSO_4$ are normally used as sources of sulfur. Based on our results, the higher concentrations of $MgSO_4$ and $FeSO_4$ decreased the production of the antibiotic under study significantly. This is may be due to the acidity of sulfate ions.

The obtained results in this study revealed that 0.06% of KCl gave maximum antibiotic production. This is closely similar to the results of Matsuoka *et al.* [35] who reported that soil isolates of *Streptomyces venezueleae* generally required 0.05% KCl to obtain the high yield of chloramphenicol, while, in the production of coumermycin, a typical fermentation medium contained 0.1% KCl for high yield production [36].

Iron ions are also important for antibiotic production. *Streptomyces subtropicus*, for example, forms the antibiotic albomycin when cultivated in a medium containing significant amount of iron. Iron is necessary for the production of chloramphenicol and other antibiotics like streptomycin [17]. In the production of erythromycin by *Streptomyces erythreus*, the suitable concentrations of ferrous sulfate for high yield production was 0.002g/100ml [21], while, 0.006g/100ml was the best level for chlorotetracycline production by *Streptomyces* sp. [37]. Similarly, the suitable concentration of $FeSO_4$ for maximum biosynthesis of the antimicrobial activity was 0.008g/100ml.

The above mentioned results revealed that the environmental parameters tested (incubation period, pH and incubation temperature) have the most significant effect on the production of the antibiotic WA52-A by alkaliphilic strain *Nocardiopsis dassonvillei* WA52.

The present study aimed to the optimization of the production of a macrolide antibiotic and stresses the importance of using the traditional medium and fermentation optimization which still effective in optimizing the secondary metabolites production.

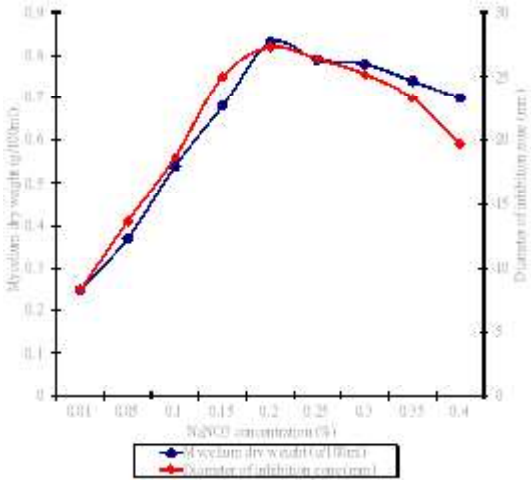

Fig. 7. Effect of different $NaNO_3$ concentrations on growth and biosynthesis of the antibiotic WA52-A produced by the alkaliphilic isolate *Nocardiopsis dassonvillei* WA52

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