Some Physiological Factors Affecting Rapamycin Production by *Streptomyces hygroscopicus* ATCC 29253

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Abstract: The production of rapamycin, a potent antifungal, immunosuppressant and antitumor, by *Streptomyces hygroscopicus* ATCC 29253 has been studied in eight culture media. Rapamycin titer varied considerably in the tested media. The medium composed of soy meal, glucose, ammonium sulphate and KH_2PO_4 was the optimal for rapamycin production and so selected for further optimization. Studies for formulating the best carbon and nitrogen nutrition for rapamycin biosynthesis revealed that replacing glucose by D (+) mannose and excluding ammonium sulphate with decreasing soy meal concentration to 20 g/l led to four fold increase in rapamycin titer. Also, the effect of KH_2PO_4 concentration and medium initial pH were elucidated and the best requirements have been specified as 5 g/l KH_2PO_4 and pH 6. [Journal of American Science 2010;6(6):188-194]. (ISSN: 1545-1003).

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1. Introduction

Since it has been discovered, and along the last few decades, rapamycin (Rap) has showed a panel of interesting bioactivities which attracted many researchers overall the world and encouraged them to explore more of its activities and to expect a promising role waiting for this compound as a multi-function drug. Rap was firstly discovered in 1975 as an antifungal agent (Vezina *et al.*, 1975) having no any antibacterial activity (Baker *et al.*, 1978). Few years latter, other activities have been frequently discovered; it was shown to have an immunosuppressive activity (Martel *et al*., 1977) and it showed a good activity against mammary, colon and brain tumor model systems (Douros and Suffness, 1981). As an immunosuppressant, Rap acts via a mechanism that is completely different to that of cyclosporine A, and it has the fabulous advantage to be of greater activity which is 150 times as that of cyclosporine A with remarked lower toxicity (Kojima *et al*., 1995). Up to date, Rap has got two approvals from the American FDA, the first was in August 1999 for preventing host-rejection in kidney transplants (Cruz *et al.*, 2001) and the second was in 2003 for use in drug-eluting stent (Tsang *et al.*, 2007) to prevent restenosis of coronary arteries following angioplasty (Marx and Marks, 2001).

Considering the chemical structure of Rap, it is nitrogen containing macrolide of the molecular formula of $C_{51}H_{79}NO_{13}$ with a very large 31-membered lactone ring. It contains three conjugated double bonds and could be classified as a polyene compound.

The first Rap producing isolate was identified as *Streptomyces hygroscopicus* ATCC 29253 that has been isolated from soil sample collected from an island known as Rapa Nui (Vezina *et al.*, 1975).

While the vast majority of published works concentrated on clinical activities of Rap, limited number of investigations studied the production of Rap (Kojima *et al*. 1995; Lee *et al*. 1997). According to the available literatures, there are no published data about the optimum conditions of Rap production by the original strain *Streptomyces hygroscopicus* ATCC 29253. This justified the present efforts aiming to optimize the physiological conditions allowing maximum production of Rap by *Streptomyces hygroscopicus* ATCC 29253.

2. Material and Methods

Microorganism

The organism used in this investigation *Streptomyces hygroscopicus* ATCC 29253 was purchased from Microbiological Resources Centre in Cairo (Cairo MIRCEN), Egypt. It was grown on slants of oat meal medium (contained oat meal, 20 g/l; agar, 20 g/l; pH 7) for 10 days at 28 ºC after which spores were collected by addition of 4 ml of 10% (v/v) glycerol to each slant. Spore suspensions were then pooled together to get a suspension of 25.8 x 10^6 CFU/ml that was then dispersed in cryopreservation vials each contained 1 ml and stored at -20 ºC to be the source of organism during this study.

Inoculum

Inoculum culture was prepared by inoculating 1 ml of thawed spore suspension (25.8 x 10^6 CFU/ml) into 50 ml starch casein broth (contained in g/l: starch, 10; casein, 0.3; KNO_3, 2; NaCl, 2; K_2HPO_4 2; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01; pH 7) in 250-ml Erlenmeyer flask. The flask was then incubated at 28±2 ºC for 7 days at 150 rpm. Two milliliters of the grown culture were used to inoculate 50 ml of fermentation medium.

Fermentation

Fermentation was carried out in duplicate 250-ml Erlenmeyer flasks, each contained 50 ml production medium, and they have been incubated at 25 ºC ±2 for 7 days at 150 rpm. Production of Rap was initially tested in eight different media. The most suitable medium underwent further detailed studies for maximizing Rap production. The composition (g/l) of the eight tested media was as follow:

Medium I (modified Xu *et al*., 2005)

It contained: soluble starch, 10; yeast extract, 6; peptone, 6; N-Z amine type B, 1.5; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 1; pH 6.5.

Medium II (modified Sehgal *et al*., 1975)

It composed of: soybean meal, 30; glucose, 20; $(NH_4)_2SO_4$, 5; KH_2PO_4, 5. Components have been dissolved in tap water and pH was adjusted to 6.

Medium III

It is modified Bennette's agar medium (Atlas, 1997). It consisted of: glucose, 10: N-Z amine type B, 2; beef extract, 1; yeast extract, 1; pH 7.3.

Medium IV

It had the same composition of Krainsky's asparagine agar medium (Atlas, 1997) with elimination of agar. Its composition was: glucose, 10; L-asparagine, 0.5; K_2HPO_4, 0.5; pH 7.

Medium V

It had the same composition of starch casein agar (Atlas, 1997) with elimination of agar. It contained: soluble starch, 10; K_2HPO_4, 2; KNO_3, 2; NaCl, 2; casein 0.3; $MgSO_4.7H_2O$, 0.05; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 0.02; pH 7.

Medium VI (modified Starch-Casein medium)

It is modified than Starch-Casein medium mentioned before by replacing casein and nitrate with 2 g/l ammonium sulphate.

Medium VII

Its composition is the same like chitin agar medium (Hsu and Lockwood 1975) after elimination of agar. It contained: Colloidal chitin, 4; KH_2PO_4, 0.3; K_2HPO_4, 0.7; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.001; $MnCl_2.4H_2O$, 0.001; pH 8.

Medium VIII

It is modified Benedict's medium (Porter *et al*., 1960). It composed of the following: glycerol, 20; L-arginine, 2.5; NaCl, 1; $CaCO_3$, 0.1; $FeSO_4.7H_2O$, 0.1; $MgSO_4.7H_2O$, 0.1; pH 7.

Analysis

The data obtained throughout the present investigation were the average of triplicate treatments.

Estimation of the microbial growth

At the end of fermentation, the microbial growth under the submerged conditions appeared as spherical pellets. Microbial dry cell weight was determined by placing a 10-ml sample of whole fermentation medium into a pre-weighed 15-ml tube and centrifuging at 3500 rpm for 5 minutes. The supernatant was decanted and the microbial residue present in the tube was dried at 80ºC for two days. The tubes were placed in a desiccator before reweighing to determine the growth yield expressed as gram dry weight per liter fermentation medium.

Extraction of Rap

At the end of fermentation, aliquots of 3 ml were taken where microbial growth was separated as mentioned before and extracted twice by shaking with 3 ml methanol for 30 minutes. Then the two extracts were pooled to be assayed for Rap concentration.

Estimation of Rap

Bioassay determination of Rap was achieved by paper-disc agar diffusion method as described by Kojima *et al.* (1995). The assay was conducted in agar plates of assay medium seeded with *Candida albicans* ATCC 10231 as the test organism. Assay medium composed of (g/l): peptone, 2; glucose, 5; agar, 11; pH 6. Five µl of cells methanol extract have been loaded onto paper discs (Whatman no. 3) of 6 mm diameter. The discs were then carefully placed on the surface of bioassay medium seeded with test organism. After incubation for 20 hr at 37 ºC, inhibition zone around each disc was recorded. Similarly, inhibition zones around standard concentrations of Rap were recorded. Plotting the relation between logarithms of Rap concentration against inhibition zone showed straight line whose linear equation was used to get Rap concentrations from inhibition zone readings. It was referred to milligrams of Rap produced in 1 liter of fermentation media as "volumetric production" whereas milligrams Rap produced per gram dry cell weight has been referred as "specific production".

3. Results and Discussion

1. Suitability of the fermentation media

This experiment was directed to test the ability of eight different fermentation media to support the production of Rap. The results illustrated in Table (1) showed great variation in Rap titer in different fermentation media. Medium II had remarkable superiority over all other tested media and its volumetric Rap titer was approximately 30 times as that of medium III which gave the secondary highest Rap yield. Minor concentrations of Rap were produced in media I, V and VI which were still comparable to yield obtained in medium III. Out of the media under the study, media IV, VII and VIII showed inability to support Rap production. Medium II which produced the highest yield contained soy meal that represents a good source of proteins, amino acids and vitamins. It was slightly modified than that used by Sehgal *et al*. (1975) in production of Rap. Although Xu *et al*. (2005) produced high concentrations of Rap in medium closely related to medium I, it showed here poor yield that was nearly1/40 of that in medium II. Each of the media IV, VII and VIII failed to satisfy nutritional requirements for Rap biosynthesis. The current results profoundly clarify the impact of the composition of nutritional media on the production of secondary metabolites; the productivity may remarkably raise many folds or completely suppressed under the influence of the used medium.
Also, the results showed noticeable incoherence between volumetric and specific titers of Rap; the profile revealed from one of them differed completely from that of the other. The results here gave a true example for contradiction between specific and volumetric Rap yields; medium VI produced the lowest volumetric titer comparing with other media that could support Rap production, and in the same time it had the highest specific titer. This discrepancy could be easily solved when considering biomass yield in medium VI which was the lowest than that at all tested media. Considering the economics, the volumetric titer represents the actual quantity of Rap that could be gained from fermentation and thus it is the realistic quantity that is reliable to compare between different fermentation variants. As such, medium II was the best suited medium for Rap production and it was selected to be optimized for maximizing Rap yield. Because of interference from insoluble soy meal with growth measurements, there was no ability to determine specific Rap titer in medium II and it was fully satisfied to depend on volumetric titer in subsequent investigations.

Table 1: Production of Rap by *Streptomyces hygroscopicus* ATCC 29253 in different fermentation

Production medium	Final pH	Growth (g/l)	Volumetric Rap production (mg/l)	Specific Rap production (mg/g dry cell weight)
I	7.90	2.34	0.25	0.11
II	4.75	Nd*	10.66	Nd
III	5.09	1.24	0.36	0.29
IV	7.09	0.10	0.00	0.00
V	7.68	1.03	0.20	0.19
VI	5.80	0.01	0.10	10
VII	5.52	1.77	0.00	0.00
VIII	7.18	0.05	0.00	0.00

* Not determined

2. Role of carbon nutrition

Glucose in medium II was individually replaced by one of the tested carbon sources on basis of carbon equivalent. Initial pH was adjusted to 6 and after 7 days final pH and volumetric Rap titer were determined. The data in Table (2) showed that replacing glucose by D (+) mannose caused three times increase in Rap titer which supported the investigation of Kojima *et al*. (1995) where mannose was one of the best carbon sources for Rap production. Lactose monohydrate produced nearly the same yield as that of glucose. With other tested carbon sources, Rap was produced in very small amounts. The medium having no additions of carbon sources showed detectable concentrations of Rap which refers to the ability of that medium to completely sustain growth basing on the carbon constituents of the soy meal. On the other hand, fructose, sucrose, cellulose, sodium acetate and citric acid could not exert considerable change in Rap titer although observable change in pH has occurred.

Speculations within results obtained in case of D-glucose, D-fructose and sucrose reveal some features about physiology of Rap production. Sucrose is a disaccharide that consists of glucose and fructose and these two monosaccharides have different effects on Rap production; D-glucose is the second best carbon source to be added for Rap production and where it was supplemented with D-fructose in the form of sucrose there was sharp depletion in Rap titer from 9.41 mg/l to 0.55 mg/l. This may point out to carbon metabolite repression effect of fructose on Rap production. On the other hand, when glucose supplemented with galactose in the form of lactose,

the yield of Rap was 10.35 mg/l which is close to that obtained with glucose (9.41 mg/l) and thus repression attributed to fructose disappeared if it was replaced with galactose.

Table 2: Effect of different carbon sources on Rap production by *Streptomyces hygroscopicus* ATCC 29253.

Carbon Source	Final pH	volumetric Rap titer (mg/l)
None*	8.42	0.53
D-Glucose	4.72	9.41
D (-) Fructose	4.42	0.13
D (+) Mannose	6.47	30.71
Sucrose	7.86	0.55
Lactose Monohydrate	5.76	10.35
Maltose Monohydrate	7.46	2.53
Dextrin from potato**	5.56	2.05
Starch**	5.7	1.68
Cellulose**	7.7	0.80
Sodium Acetate	5.91	0.10
Citric Acid	5.88	0.11

None*: Medium II without glucose. It contained: soy meal, 30g; $(NH_4)_2SO_4$, 5g; KH_2PO_4, 5g; tap water 1L; pH 6

The work has extended to determine the optimum concentration of D (+) mannose, as the best carbon source for Rap production. The results depicted in Fig. (1) revealed that Rap yield increased linearly with increasing mannose concentration up to 20 g/l. Within that range Rap production was carbon source dependent due to availability of all nutrients other than carbon source. The balance between carbon source and other nutrients was obtained at 20 g/l mannose at which Rap produced in the highest yield. Further increase in mannose concentration to 30 g/l was not corresponded with increase in Rap titer. This is in agreement with results of Kojima *et al.* (1995) who found that the best concentration of carbon source was 20 g/l with no better yields at the higher concentrations. In addition, final medium pH decreased obviously with increasing mannose concentration and it was nearly around the initial value (pH 6) at 20 g/l mannose.

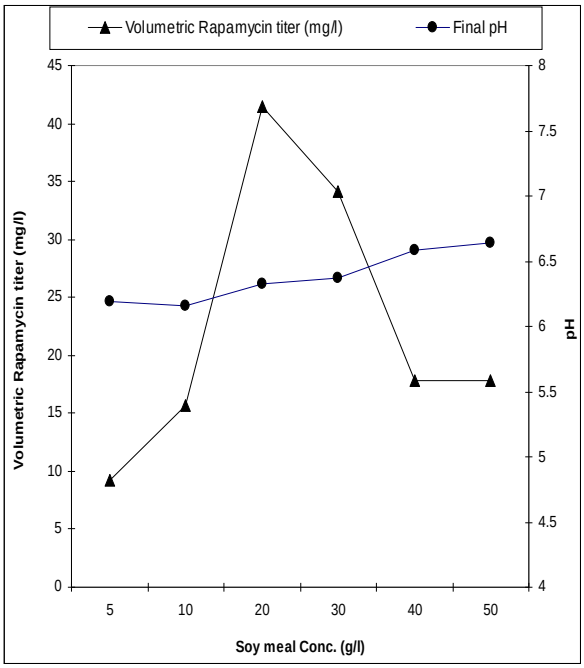

Fig. 1: Effect of different D (+) mannose concentrations on Rap production by *Streptomyces hygroscopicus* ATCC 29253.

3. Role of nitrogen nutrition

To medium II containing D (+) mannose (20 g/l) instead of glucose, different nitrogen equivalent sources were added, one-at-a-time, as replacements of ammonium sulphate. Also, medium without ammonium sulphate was studied. As illustrated in Table (3), the medium having no additional nitrogen source was fully satisfied for Rap production basing on soy meal as a rich source for nitrogenous compounds. Moreover, nearly with all tested nitrogen sources, Rap titer has clearly dropped. This may be explained as nitrogen metabolite repression caused by easier nitrogenous compounds provided by the tested nitrogen sources.

As previously mentioned, soy meal, with no additional nitrogen source, was a good and sufficient source for nitrogenous compounds. So, it was of an importance to study Rap production at different soy mal concentrations. The results shown in Table (4) revealed that soy meal in 20 g/l was the optimal for Rap production. Concentrations lower and higher than the optimal showed markedly retarded productivities. Medium final pH varied slightly with change in soy meal concentration.

Table 3: Effect of addition of different nitrogen sources on Rap production by *Streptomyces hygroscopicus* ATCC 29253.

Nitrogen Source	Final pH	volumetric Rap titer (mg/l)
None*	6.28	33.81
Ammonium sulphate	6.43	30.14
Ammonium Chloride	4.99	13.50
Diammonium Hydrogen Phosphate	6.03	19.62
Sodium Nitrate	7.23	7.27
Urea	7.11	9.02
Yeast Extract	6.63	3.80
Beef Extract	5.78	7.59
Peptone	7.31	11.58
Casein	6.35	6.38

None*: It contained: soy meal, 30g; D (+) mannose, 20 g; KH_2PO_4, 5g; tap water, 1L; pH 6

Table 4: Effect of different soy meal concentrations on Rap production by *Streptomyces hygroscopicus* ATCC 29253.

Soy meal conc. (g/l)	Final pH	volumetric Rap titer (mg/l)
5	6.19	9.18
10	6.16	15.63
20	6.33	41.46
30	6.37	34.15
40	6.58	17.85
50	6.64	17.85

4. Effect of different KH_2PO_4 concentrations

The results demonstrated in Table (5) showed that increasing the level of KH_2PO_4 from 0 to 5 g/l was associated with a drop in the medium final pH value from 8.36 to 6.43 and accompanied with an increase in Rap titer up to the highest value of 40.16 mg/l. Above 5 g/l, KH_2PO_4 negatively interfered with Rap production although medium pH was kept around the initial value, indicating a possible buffering effect of the added phosphate. Increase of phosphate salt to10 g/l obviously inhibited Rap production. Inhibition of secondary metabolites biosynthesis by high inorganic phosphate concentration was reported in elsewhere. Aharonowitz and Demain (1977) found that production of cephalosporin increased with increase in phosphate concentration up to 25 mM after which further addition of phosphate led to sharp decrease in the production of antibiotic. Iwai and Omura (1982) stated that addition of relatively high concentration of inorganic phosphate increased the consumption of carbon and nitrogen sources and respiration resulting in good growth with reduced antibiotics titer. Also, phosphate inhibition of macrolide synthesis in different strains of *Streptomyces hygroscopicus* has been reported by Gersch *et al*. (1979). With respect to Rap biosynthesis by *Streptomyces hygroscopicus*, Cheng *et al.* (1995) reported on specific negative control of its production by elevated phosphate concentrations. Moreover, recently Rouf *et al.* (2007) studied the stability of Rap in different media and found that Rap was very unstable in phosphate buffer saline where it degraded in faster rates with increase in temperature and at 37 ºC almost all drug was destroyed in 24 hours. These findings are surprisingly pointing out to another effect by which phosphate interferes specifically with Rap production in addition to conventional nutritional role of phosphate.

Table 5: Effect of different KH_2PO_4 concentration on Rap production by *Streptomyces hygroscopicus* ATCC 29253.

KH_2PO_4 Conc. (g/l)	Final pH	volumetric Rap titer (mg/l)
0.0	8.36	0.00
1.0	7.51	25.13
2.0	7.05	30.66
3.0	7.09	32.05
4.0	6.93	33.81
5.0	6.43	40.16
7.5	6.39	37.82
10.0	6.40	28.91

5. pH value relations

Production of Rap was affected by the initial pH value of the production medium. Referring to data presented in Table 6, highest Rap titer was obtained at initial medium pH of 6, and comparable yields have been produced around this pH i.e., pHs 5.5 and

6.5. Interestingly, Rap biosynthesis was very sensitive to rise in pH over 6.5; Rap titer at pH 7 was less than half of that recorded at pH 6.5. Further increase of pH was accompanied with remarkable decrease in Rap yield. Below the optimum pH, Rap production has also been suppressed and ultimately ceased at pH 4. These results are in agreement with many literatures reported on Rap production where it has been produced in media of pH 6 (Kojima *et al*., 1995; Cheng *et al*., 1995a&b; Fang and Demain, 1995; Lee *et al*., 1997). However, Xu *et al*. (2005) produced Rap in solid media of slightly increased pH (ranged from 6.3 to 6.8). Also, it is of an importance to refer to the advantageous choice of using pH 6 as the initial pH for Rap production at the present experimental conditions since the final medium pH value remained closely near the initial optimum value which maintains good Rap productivity with no need to use buffer solutions.

Table 6: Effect of initial pH of production medium on Rap production by *Streptomyces hygroscopicus* ATCC 29253.

Initial pH value	Final pH	volumetric Rap titer (mg/l)
4.0	4.28	0.00
5.0	6.19	23.27
5.5	6.22	30.75
6.0	6.30	38.14
6.5	6.91	34.06
7.0	7.39	15.34
7.5	8.11	6.35
8.0	8.20	2.35
9.0	8.20	2.86

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