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Seed Stage Manipulation as a Tool for Improving Rapamycin Production by *Streptomyces hygroscopicus* ATCC 29253

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Abstract: Adopting standard fermentation process, the effect of different seed culture preparations on rapamycin production by *Streptomyces hygroscopicus* ATCC 29253 was realized. Concerning the type of cultivation medium, starch casein broth adjusted at pH 7 or 8 afforded the best formulation for seed culture. Rising pH to 9 was accompanied with sharp depletion in rapamycin titer. It was also found that 5 days old inoculum was the best suited. Four-fold increase in rapamycin yield was attained by rising cell count in seed culture from $25.8x10^2$ to $25.8x10^6$ c.f.u./flask. The study proposed growth morphology, instead of growth quantity, to be the proper approach for understanding the role of cell count in controlling microbial activity of seed culture. Growth morphology at different cell counts was depicted and its correlation to microbial activity was postulated. Effect of inoculum size has been also clarified where the highest rapamycin yield was attained at inoculum size of 6%. A typical fermentation with the developed optimal seed culture revealed that rapamycin yield was accelerated from 39.53 to 89.20 mg/l together with reduction of the incubation period to five days.

Key words: Rapamycin, *Streptomyces hygroscopicus*, Seed culture parameters, Growth morphology

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). 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). While the vast majority of published work concentrated on clinical activities of Rap, limited number of investigations studied the production of Rap (Kojima *et al*. 1995; Lee *et al*. 1997). Also, still fewer are the investigations concerning the role of seed culture manipulation in improving production of secondary metabolites.

In a previous communication, the authors optimized the fermentation conditions of Rap production (Sallam *et al*. 2010). In the current study, it was intended to proceed work aiming at improving Rap productivity focusing on seed culture manipulation.

MATERIALS AND METHODS

Microorganism:

The used organism, *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. The spores were collected by addition of 4 ml of 10% (v/v) glycerol to each slant. Thereafter, spore suspensions were pooled together to get a suspension of 25.8×10^6 c.f.u./ml that was then dispensed in cryopreservation vials each contained 1 ml and stored at -20 ºC to be the source of the experimental organism throughout the present study.

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Inoculum:

Inoculum culture was prepared by inoculating 1 ml of thawed spore suspension into 50 ml seed culture broth placed in 250-ml Erlenmeyer flask. The flask was then incubated at 28±2 ºC and 150 rpm. The type of seed culture medium, time of incubation, microbial cell count in the culture and the volume to be used in inoculating fermentation flasks, each of these parameters was individually studied, one-at-a-time, to get its optimum specification that has been used thereafter. The composition of the different tested media was as follow (g/l):

Medium I (modified SYPC medium by Xu *et al*. 2005)

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 (Xu *et al*. 2005)

Oat meal (20 gm) dissolved in 1 l tap water; pH 6.5.

Medium III (Sallam *et al.* 2010)

Soy meal, 20; D (+) mannose, 20; KH_2PO_4, 5. The components were dissolved in tap water and pH was adjusted at 6.5

Medium IV

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 6.5

Fermentation:

Fermentation was carried out in duplicate 250-ml Erlenmeyer flasks, each contained 50 ml production medium that composed of: soy meal, 20 g; D (+) mannose, 20 g; KH_2PO_4, 5 g; tap water 1 l; pH 6 (Sallam *et al.* 2010). Each flask was inoculated with 2 ml of seed culture, till mention otherwise, and incubated at 25 ºC ±2 for 7 days at 150 rpm.

Estimation of the Microbial Growth:

Microbial dry cell weight was determined by placing a 10-ml sample of culture medium into a pre-weighed 15-ml tube and centrifuging at 3500 rev/min for 5 minutes. The supernatant was decanted off and the microbial residue was dried at 80ºC for two days. The tubes were then reweighed to determine the growth yield.

Extraction of Rap:

At the end of fermentation, aliquots of 3 ml were taken where microbial growth was separated by centrifugation at 3500 rev/min for 5 minutes and extracted twice by shaking with 3 ml methanol for 30 minutes. Then the obtained extracts were pooled to be assayed for Rap concentration.

Estimation of Rap:

Bioassay determination of Rap was achieved by paper-disc agar diffusion method 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, the diameter of inhibition zone (mm) around each disc was recorded. Similarly, the diameters of inhibition zones around standard concentrations of Rap were recorded. Plotting the relation between logarithms of Rap concentration against inhibition zone diameter showed straight line whose linear equation was used to get Rap concentrations from inhibition zone diameter readings. It was referred to milligrams of Rap produced in 1 liter of fermentation media as "volumetric Rap titer".

RESULTS AND DISCUSSION

1. Cultivation Medium as a Determinative Parameter for Inoculum Quality in Rap Production:

Inocula were developed in four different media and were then used individually to inoculate the fermentation medium. Fermentation process was monitored through records of Rap volumetric titer and pH at the end of fermentation. The results presented in Table 1 showed that inocula prepared in different media exhibited different volumetric Rap titers. The optimal medium for seed culture preparation was starch casein broth (medium IV). Comparable Rap yield was obtained when propagating inoculum in SYPC medium.

Interestingly, poor Rap titer was yielded with inoculum cultivated in medium III which had been selected by Sallam *et al*. (2010) as the optimal for fermentation stage in Rap production. Accordingly, it was postulated that the lowest productivity in case of seed culture grown in medium III was attributed to high Rap concentration in this culture which proposed to cause a negative control on its biosynthesis in the latter fermentation stage. Several antibiotics have long been recognized to inhibit their own biosynthesis through feedback regulation mechanisms (Martin and Demain 1980). Examples include chloramphenicol, aurodox, cycloheximide, staphylomycin, ristomycin, puromycin, fungicidin, candihexin, mycophenolic acid and penicillin (Martin and Demain 1980). In addition, Warr *et al*. (1996) studied the effect of different seed media of different carbohydrates level on the production of milbemycin by *Streptomyces hygroscopicus* and they found variation in milbemycin titer with reducing carbohydrate levels.

2. Role of Initial pH Value of Seed Culture:

Starch casein media of different initial pHs were used for the preparation of inoculum cultures as described in materials and methods. Before being used in inoculating fermentation medium, the inoculum cultures grown at different pHs underwent analysis to find out specification of each culture concerning final pH and growth (g/l) as indicated in Table 2. It was found that seed cultures formed at different initial pHs had different profiles. Growth yield has been duplicated with rise in initial pH from 5 to 6 whereas it showed slight variations against more rise in pH (Table 2). The final medium pH varied between different cultures and it tended to increase than initial values. The different seed cultures have been used in inoculating fermentation media where productivity of Rap was followed out as indicated in Table 3. It was clearly shown that seed cultures prepared at different initial pHs had different potentialities in Rap production. The seed cultures prepared at pH 7 and 8 had the highest potentiality in Rap production. At pH 9, the inoculum gave the highest growth yield (Table 2), however it showed poor potentiality in Rap production that was half of that obtained at pH 7 (Table 3). As such, it was ascertained that seed culture media is not merely growth yielding medium i.e., it is not the ultimate role of seed culture to develop microbial culture of the highest yield but it is to yield microbial cells having the highest potentiality in product formation and that potentiality is substantially affected by pH of cultivation medium.

3. Effect of Inoculum Age:

As indicated in Table 4, seed cultures of different ages produced different Rap outputs. Two fold increase in volumetric Rap titer was achieved with increase in inoculum age from zero to 5 days. Growth profile of seed culture (Table 5) showed that seed culture within the first five days was under active growth phase (logarithmic growth phase) where there was gradual increase in biomass with time till reaching the maximum yield at the fifth day. At the seventh day, seed culture had entered the decline growth phase where growth yield began to fall down. Even though, at the seventh day the seed culture produced Rap with the same highest concentration recorded at the fifth day i.e., there was no reduction in volumetric Rap titer with increasing age of seed culture from five to seven days (Table 4) although decline in growth yield of the culture was observed (Table 5).

4. Effect of Microbial Cell Count:

Different seed cultures were prepared by inoculating 250-ml Erlenmeyer flasks each containing 50 ml starch casein medium with spore suspensions (1 ml/flask) of *Streptomyces hygroscopicus* having different microbial cell counts that were expressed as colony-forming unites/flask (c.f.u./flask). The results presented in Table 6 showed an obvious role of number of microbial cells seeded in inoculum culture in controlling the microbial activity during subsequent fermentation stage. Four-fold increase in Rap yield was attained by increasing cell count in seed culture from $25.8x10^2$ to $25.8x10^6$ c.f.u./flask. Growth profile of inoculum culture at different cell counts (Table 7) could not reveal any significant relation between quantitative specification of growth of inoculum culture and the activity of that culture in rap production. Growth yield (Table 7) was nearly the same in the two inoculum cultures of the lowest and the highest volumetric Rap titers. As such, it is not the way to interpret difference in activities of inocula depending on quantitative specification of growth in inoculum cultures. On the other hand, some significant features could be revealed from morphological pattern of growth in inoculum cultures.

As it has been illustrated in Fig. 1, several morphological changes have been discerned with change in cell count of inoculum culture. Significant gradual increase in pellet size was observed with reducing cell count in the seed culture. The pellets formed from 25.8×10^6 c.f.u./flask had the smallest size and consisted from less confluent aggregation of hyphae and were surrounded with out extending layer of free hyphae (Fig. 1.a).

In case of cell count of 25.8 X 10^5, the pellet turned to be of greater size and constituted of more confluent biomass with absence of outermost layer of freely extended hyphae (Fig. 1.b). With diluting cell count in inoculum culture down to 25.8 X 10^4, the pellets had an obvious greater size and tended to be distinguishable into two layers, the internal compact core and the outer enclosing layer (Fig. 1.c). Seed cultures of more diluted cell counts showed pellets of enormous size with central black spots of dense solid masses (Fig. 1.d&e).

From the preceding growth morphology profile, it was fully understood that smallest pellet size, obtained by increasing cell count up to 25.8 X 10^6, was the most potent with respect to Rap production due to feasibility of the whole pellet biomass to be incorporated in metabolic activities of Rap production. Decreasing cell count led to formation of larger pellet size with more compact mycelial aggregations having limitations in oxygen and nutrient diffusion. More increase in pellet size, conserved more part of the pellet out of Rap production process. Impact of spore count on the structure and size of the pellet was reported in elsewhere (Tucker and Thomas 1992; Znidarsic *et al.* 2000). The size of *Rhizopus nigricans* pellets was mainly influenced by the inoculum concentration (Znidarsic and Pavko 2001). Also, pellet size and structure have a critical effect on biochemical activity of the pellet (Znidarsic and Pavko 2001). Papagianni (2004) stated that small pellets as opposed to large ones would generally be considered desirable in developing filamentous fermentations due to early findings of Philips (1966) and Elmayergi *et al*. (1973) who found that the central region of larger pellets undergoes autolysis as a result of nutrient limitation. This autolysis has a significant effect on both cellular metabolism and product synthesis. In addition, Znidarsic and Pavko (2001) reported three examples on the strong correlation between maximum microbial activity and specific pellet size. The first concerned the highest production of aflatoxin which was achieved by compact, smooth pellets of *Aspergillus flavus* and *A. parasiticus* of 0.6 mm in diameter. In the second, optimum production of Mn (II) peroxidase by *Phanerochaete chrysosporium* was attained with intermediate pellet size (2-3 mm), while larger and smaller pellets exhibited lower protein excretion capacity. In the third example, pellets of 1–1.5 mm diameter of *Rhizopus oryzae* were the most potent in fumaric acid production. As such, the potent microbial culture is attained by combination of detailed knowledge about physiology as well as the growth morphological characteristics. And so, it is recommended to follow up change in growth characteristics while investigating physiology of the microbial strain to realize the interaction of growth morphology with culture physiology and to adjust the best combination for the maximum microbial performance.

5. Effect of Inoculum Size:

In this experiment, fermentation flasks were inoculated with different volumes of seed culture that contained cell count of 25.8 X 10^6 c.f.u./flask and allowed to grow for five days at 28±2 ºC and 150 rpm. The results given in Table 8 showed gradual increase in volumetric Rap titer as the inoculum size increased till getting the highest Rap yield at size of 6%. Elevation in inoculum size from 1% to 6% was accompanied with two fold increase in Rap titer with very conservative change in fermentation medium final pH. When feeding fermentation medium with more microbial cells than 6% inoculum size, fermentation medium pH shifted up and it is predicted for that shift to increase to be of remarkable suppressive effect on Rap production when inoculum size exceeds 10%.

6. Typical Fermentation Process with the Developed Optimal Seed Culture:

During the preceding investigations, the specifications of the optimal seed culture were realized. Therefore, it was intended to follow up a typical fermentation process using the best seed culture. The used seed culture was developed in starch casein medium adjusted at initial pH of 7 and incubated at 28±2 ºC and 150 rpm for five days. It contained 25.8 X 10^6 c.f.u./flask and inoculated into fermentation flasks in size of 6%. The results recorded in Table 9 indicated that Rap production initiated at the third day of fermentation with a concentration of 33.43 mg/l. The volumetric titer has duplicated in the fourth day, and at the fifth day it raised to the highest value (89.20 mg/l) which was nearly three folds as that at the third day. After the fifth day, Rap titer began to decrease indicating five-day fermentation as the optimal for its production.

Table 1: Effect of inoculum preparation medium on rapamycin production by *Streptomyces hygroscopicus* ATCC 29253

Inoculum preparation medium	Final pH of fermentation medium	Volumetric rapamycin titer (mg/l)
Medium I (SYPC medium)	6.17	37.89
Medium II (Oat meal based medium)	6.10	24.75
Medium III (Fermentation medium)	6.24	16.17
Medium IV (Starch casein broth)	6.21	39.53

Table 2: Final pH and growth profiles of inocula prepared at different initial pH values

Inoculum initial pH	Specification	
	Final pH	Growth (g/l)
5.00	5.60	1.83
6.00	7.80	3.47
7.00	8.38	3.59
8.00	8.58	3.97
9.00	8.66	4.00

Table 3: Production of rapamycin by *Streptomyces hygroscopicus* ATCC 29253 using inocula prepared in starch casein media at different initial pH values

Inoculum preparation pH	Final pH of fermentation medium	Volumetric rapamycin titer (mg/l)
5.00	6.28	15.47
6.00	6.23	21.73
7.00	6.24	42.87
8.00	6.24	40.06
9.00	6.26	21.73

Table 4: Production of rapamycin by *Streptomyces hygroscopicus* ATCC 29253 using inocula of different ages

Inoculum Age (day)	Final pH of fermentation medium	Volumetric rapamycin titer (mg/l)
Zero	6.24	21.73
1.0	6.24	30.52
3.0	6.24	30.52
5.0	6.20	42.87
7.0	6.24	42.87

Table 5: Growth profile of inocula prepared at different ages

Inoculum Age (day)	Growth (g/l)
Zero	0.00
1.0	1.67
3.0	2.60
5.0	3.51
7.0	3.07

Table 6: Production of rapamycin by *Streptomyces hygroscopicus* ATCC 29253 using inocula of different microbial cell counts

Microbial cell count in inoculum culture (c.f.u./flask)	Final pH of fermentation medium	Volumetric rapamycin titer (mg/l)
$25.8x10^2$	6.11	12.88
$25.8x10^3$	6.33	22.29
$25.8x10^4$	6.40	22.29
$25.8x10^5$	6.53	38.57
$25.8x10^6$	6.43	50.74

Table 7: Growth profile of inocula prepared from different cell counts of *Streptomyces hygroscopicus* ATCC 29253

Cell count in inoculum culture(c.f.u./flask)	Growth (g/l)
25.8×10^2	3.72
25.8×10^3	4.48
25.8×10^4	4.57
25.8×10^5	4.92
25.8×10^6	3.40

Table 8: Production of rapamycin by *Streptomyces hygroscopicus* ATCC 29253 using different inoculum sizes

% to volume of fermentation medium	Final pH of fermentation medium	Volumetric rapamycin titer (mg/l)
0.25%	6.30	22.29
0.5%	6.35	22.29
1%	6.44	29.32
2%	6.40	33.63
4%	6.43	50.74
6%	6.44	58.19
8%	6.59	50.74
10%	6.70	50.74

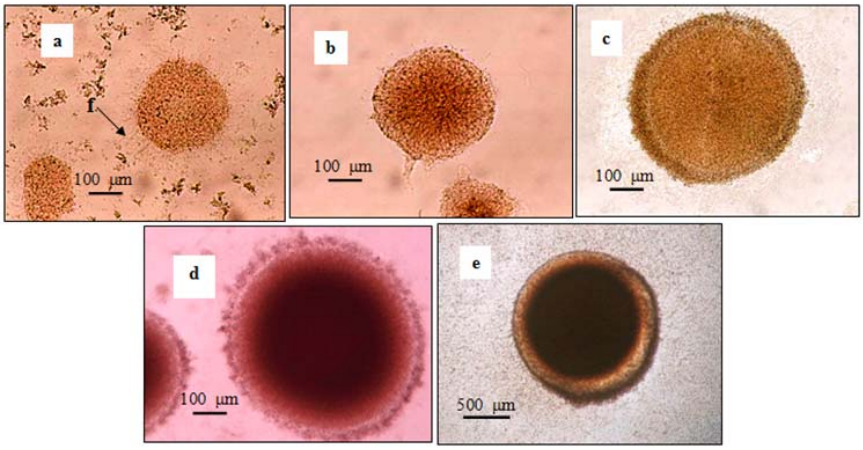

Fig. 1: Photomicrograph of characteristic growth morphology of *Streptomyces hygroscopicus* ATCC 29253 in inoculum cultures seeded with different spore counts; a) 25.8 X 10^6 c.f.u./flask (100 X), f: freely extended hyphae; b) 25.8 X 10^5 c.f.u./flask (100 X); c) 25.8 X 10^4 c.f.u./flask (100 X); d) 25.8 X 10^3 c.f.u./flask (100 X); e) 25.8 X 10^2 c.f.u./flask (20 X).

Table 9: A typical fermentation by *Streptomyces hygroscopicus* ATCC 29253 using optimal preparation of inoculum

Time (day)	Final pH of fermentation medium	Volumetric rapamycin titer (mg/l)
1	6.12	0.00
2	6.07	0.00
3	6.39	33.43
4	6.36	61.30
5	6.30	89.20
6	6.32	61.30
7	6.45	54.09

REFERENCES

Atlas, R.M., 1997. Handbook of microbiological media. Boca Raton, Fla. CRC Press.

Baker, H., A. Sidorowicz, S.N. Sehgal and C. Venzina, 1978. Rapamycin (AY-22989) a new antifungal antibiotic III In vitro and in vivo evaluation. J Antibiot, 31: 539-545.

Cruz, M.C., A.L. Goldstein, J. Blankenship, M. Del Poeta, J.R. Perfect, J.H. McCusker, Y.L. Bennani, M.E. Cardenas and J. Heitman, 2001. Rapamycin and less immunosuppressive analogs are toxic to *Candida albicans* and *Cryptococcus neoformans* via FKBP12-dependent inhibition of TOR. Antimicrob Agents and Chemother, 45: 3162-3170.

Douros, J. and M. Suffness, 1981. New antitumor substances of natural origin. Cancer Treat Rev., 8: 63-87.

Elmayergi, H., J.M. Scharer and M**.** Moo-Young, 1973. Effects of polymer additives on fermentation parameters in a culture of *Aspergillus niger*. Biotechnol Bioeng, 25: 845-859.

Kojima, I., Y.R. Cheng, V. Mohan and A.L. Demain, 1995. Carbon source nutrition of rapamycin biosynthesis by *Streptomyces hygroscopicus*. J Ind Microbiol., 14: 436-439.

Lee, M.S., I. Kojima and A.L. Demain, 1997. Effect of nitrogen source on biosynthesis of rapamycin by *Streptomyces hygroscopicus*. J Ind Microbiol Biotechnol., 19: 83-86.

Martel, R.R., J. Klicius and S. Galet, 1977. Inhibition of the immune response by rapamycin a new antifungal antibiotic. Can J Physiol Pharmacol., 55: 48-51.

Martin. J.F. and A.L. Demain, 1980. Control of Antibiotic Biosynthesis. Microbiological Reviews, 44: 230-251.

Marx, S.O. and A.R. Marks, 2001. The development of rapamycin and its application to stent restenosis. Circulation, 104: 852-855.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv., 22: 189-259.

Philips, D.H., 1966. Oxygen transfer into mycelial pellets. Biotechnol Bioeng, 8: 456-460.

Sallam, L.A.R., A.F. El-Refai, M.E. Osman, A.A. Hamdy, E.M. Ahmed and M.A. Mohamed, 2010. Some physiological factors affecting rapamycin production by *Streptomyces hygroscopicus* ATCC 29253. Journal of American Science, 6: 188-194.

Tsang, C.K., H. Qi, L.F. Liu and X.F. Stevan Zheng, 2007. Targeting mammalian target of rapamycin (mTOR) for health and diseases. Drug Discovery Today, 12: 112-124.

Tucker, K.G. and C.R. Thomas, 1992. Mycelial morphology: the effect of spore inoculum level. Biotechnol Lett., 14: 1071-1074.

Vezina, C., A. Kudelski and S.N. Sehgal, 1975. Rapamycin (AY-22989) a new antifungal antibiotic I. Taxonomy of the producing Streptomycete and isolation of the active principle. J Antibiot, 28: 721-726.

Warr, S.R.C., C.J.L. Gershater and S.J. Box, 1996. Seed stage development for improved fermentation performance: increased milbemycin production by *Streptomyces hygroscopicus*. J Ind Microbiol Biotechnol., 16: 295-300.

Xu, Z.N., W.H. Shen, X.Y. Chen, J.P. Lin and P.L. Cen, 2005. A high-throughput method for screening of rapamycin-producing strains of *Streptomyces hygroscopicus* by cultivation in 96-well microtitre plates. Biotechnol Lett., 27: 1135-1140.

Znidarsic, P. and A. Pavko, 2001. The morphology of filamentous fungi in submerged cultivations as a bioprocess parameter. Food Technol Biotechnol., 39: 237-252.

Znidarsic, P., R. Komel and A. Pavko, 2000. Influence of some environmental factors on *Rhizopus nigricans* submerged growth in the form of pellets. World J Microbiol Biotechnol., 16: 589-593.