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Enrichment of Soymeal Medium to Increase the Rapamycin Production by *Streptomyces hygroscopicus*

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Abstract. Research was carried out to study the improved and increased production of rapamycin by *Streptomyces hygroscopicus* with soymeal enriched media. Media containing soymeal produced rapamycin upto 82.89 mg/L. The medium was enriched with different additives that can interfere with biosynthesis process. L-tyrosine supplementations led to noticeable increase in the rapamycin production to 112 mg/L. However, the progress was achieved upon addition of the shikimic acid (precursor rapamycin moiety), where, it reached 160 mg/L. The greatest increase was recorded after addition of calcium superphosphate (CaP) and the production achieved 176 mg/L. Other substances like vitamins and trace elements had either no or negative effects on the biosynthesis of rapamycin. The study also showed the ability of low concentrations of calcium phosphate to replace the expensive large amount of shikimic acid.

Keywords: *Streptomyces hygroscopicus,* rapamycin, immunosuppressants, soymeal

Introduction

Rapamycin is a potent immunosuppressant, anticancer and antifungal medicine. Rapamycin was early discovered in 1975 as an antifungal agent produced by *Streptomyces hygroscopicus* and has a great potency against *Candida albicans* (Vezina *et al.,* 1975). Up to date, rapamycin was approved twice from American FDA, the first one in 1999 for its ability to prevent host rejection in kidney transplanting and the second was in 2003 for its use in drug eluting stent to prevent restenosis of coronary arteries following angioplasty (Vezina *et al.,*1975).

In the recent years extensive research aiming at exploring more rapamycin activities ascertained that it represents an unreal tapped resource of clinical activities and can afford a lot of in drug realm as multifunction medication. It is anti-inflammatory and reduces the expression of several genes related to inflammation. In addition, it has antiangionenic, antiplorefiration and antifibrotic (Nuhrenberg *et al.,* 2005; Morris, 1992). Rapamycin protects against hypoxic damage in primary heart culture *via* Na/Ca exchange activation (El-Ani *et al.,* 2011). Also rapamycin found to play role in regulation of gastric hormones (Xu *et al.,* 2010). Furthermore, rapamycin does not affect post-absorptive protein metabolism in human skeletal muscle (Dickinson *et al.,* 2013).

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There are some trials to use rapamycin in treatment of acute myeloid leukemia, retinal and choriodal vascular diseases (Recher *et al.,* 2005). With the tremendous nature of rapamycin activities the demands on this drug would be increased in future. Therefore, research work is focussed to improve its productivity and to reduce the production costs. The cuurent study aims increament of the rapamycin production by *S. hygroscopicus* through enrichment of natural media containing soybean meal. The media was selected after comparison with other synthetic and natural media. Additives that could interfere with the rapamycin biosynthesis supplied and tested for their effects.

This is the first time for such results to be recorded and present findings may be helpful for economic production of Rapamycin.

Materials and Methods

Microorganisms. The strain *Streptomyces hygroscopicus* ATCC 29253, was the experimental organism throughout the current study. *Candida albicans* ATCC 10231 was used in bioassay.

The Rap producer, *S. hygroscopicus* ATCC 29253, was grown on slants of oat meal medium (contained oat meal, 20 g/L; agar, 20 g/L and 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 × 10^6 c.f.u./mL that was then dispensed in cryopreservation vials, each contained 1 mL, and stored at -20 ºC.

Culture media. A medium composed of soybean meal, 30 g; glucose, 20 g; $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 5 g (Sehgal *et al.,* 1975), was selected after comparison with other seven synthetic and natural media of different compositions. The components dissolved in tap water of pH 6 was used.

Fermentation. The culture was initiated by propagating 1 mL of thawed spore suspension containing 25.8×10^6 c.f.u. in 50 mL medium contained in 250 mL Erlenmeyer flask. Then each Erlenmeyer flasks (250 mL) containing 50 mL fermentation medium inoculated with 3 mL of inoculum. The inoculated flasks were incubated at 25 ºC ±2 and 150 rpm for 7 days. The fifth day was the optimal, where, after all fermentations were allowed to run for only 5 days.

Growth estimation. The dry cell weight was determined by placing a 10 mL sample from the whole fermentation medium into a pre-weighed 15 mL tube and centrifuged at 3500 rpm for 5 min. The microbial residue in the tube was dried at 80 ºC for 2 days and then weighed. The growth yield expressed as gram dry weight per liter fermentation medium. Packed cell volume percentage was determined by placing a 3 mL sample of whole fermentation medium into 10 mL tube and centrifuged at 3500 rpm for 5 min. The percentage of packed cell volume to the total volume of the whole fermentation medium sample was the desired estimate of growth.

Analysis. Aliquots of 3 mL were taken and the microbial growth was separated by centrifuging at 3500 rpm for 5 min and extracted twice by shaking with 3 mL methanol for 30 min. The two extracts were pooled to be assayed for Rap concentration. Bioassay determination of Rap was achieved by paper-disc agar diffusion method as described by Sallam *et al.* (2010) and Kojima and Demain (1998). A sample of 20 µL was analysed with HPLC (SYKAM apparatus equipped with Injector, S5111; Pumps (two pumps), S1122; Column Thermo controller, S4011; Degaser, S7505; Column, Phenomenex Gemini C18 (250x4.6 mm, 5 µ); Detector, Jasco UV-2070Plus; Software, Autochro-3000). The sample injected and eluant of acetonitrile: water 9:1 was pumped at the rate of 1 mL/min in column controlled at 55 ºC. Rap was assessed by absorbance at λ 277 nm.

Results and Discussion

Amino acids addition. Different amino acids were added individually in the concentration of 5 g/L. The results presented in Fig. 1 show that, addition of L-tyrosine accelerated the production to be 48% greater than in control sample, whereas, L-lysine neither enhanced nor suppressed the production. All the other added amino acids suppressed Rap biosynthesis by different degrees and the complete cessation was in case of L-cysteine. Similar results were obtained by L-histidine, DL-aspartic acid, L-arginine, L-glutamic acid, glycin, L-asparagine and L-cystine, while, sharp deleterious effect was observed in case of L-tryptophan, L-alanine and DL-methionine. Also, with addition of L-valine and L-proline, Rap titre was dropped down to be around the third of that in control sample.

Growth measurement revealed some significant features. There was some degree of correlation between growth of the producing strain and Rap biosynthesis. In most cases, amino acids negatively affected the growth as well as Rap biosynthesis. In addition, L-tyrosine, that has potentiated Rap biosynthesis, has also favoured the growth. With addition of L-proline, fermentation process showed the same profile like in control sample, the same growth and final pH of fermentation medium, yet it had remarkable decrease in Rap titre.

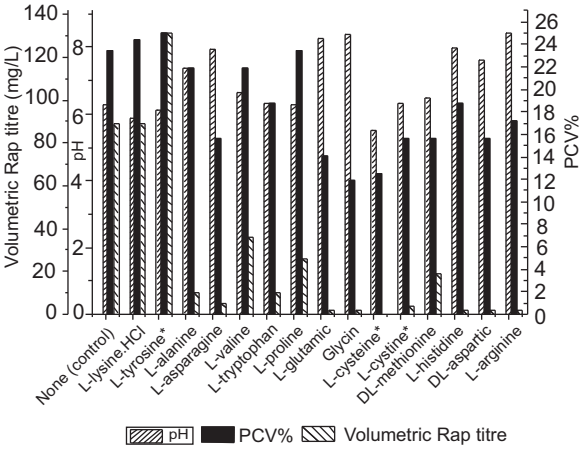

Fig. 1. Role of amino acids in biosynthesis of Rap by *S. hygroscopicus* ATCC 29253.

Biosynthesis of Rap in different augments of L-tyrosine. L-tyrosine was added to fermentation medium in different supplements namely; 1, 2, 5, 7.5, 10, 15, 20 and 30 g/L. The results that are represented in Fig. 2, show that decreasing L-tyrosine quantities to 2 g/L can successfully afford the same promotion in

Rap biosynthesis that was achieved at 5 g/L, and it yielded the best growth. More increase in L-tyrosine (more than 5 g/L) caused gradual depletion in Rap without effect on the microbial growth. The pH values remained constant at 6.

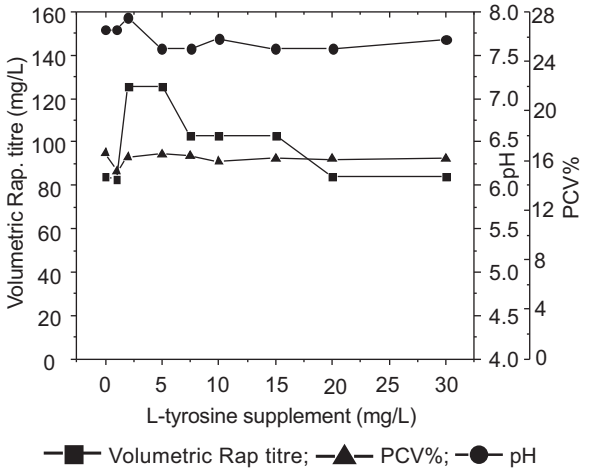

Fig. 2. Biosynthesis of Rap by *S. hygroscopicus* ATCC 29253 in different augments of L-tyrosine. PCV = packed cell volume.

Effect of time of L-tyrosine addition on Rap biosynthesis. According to the preceding investigations, L-tyrosine may play an obvious role in promoting Rap biosynthesis. In the current experiment, L-tyrosine (2 g/L) was added at different times during fermentation. Time of L-tyrosine addition has a critical role in its promoting action (Table 1). The results revealed that L-tyrosine should be added at the start of fermentation and not after more than 1 day of fermentation. The effect of L-tyrosine could be inversed to remarkable suppression to the Rap biosynthesis as well as the growth, if it was added at the late stages of fermentation or at least after two days.

Effect of trace elements on Rap biosynthesis. With a production medium containing L-tyrosine (2 g/L), trace elements were added, individually and in mixture, in concentrations like that were used by Lee *et al*. (1997). The data in Fig. 3 revealed that Rap biosynthesis was not favoured by addition of any of the studied trace elements. All of them have retarded Rap biosynthesis with only one exception in case of zinc salt, where, the results remained the same as that of control treatment. Copper ions (in very low concentration i.e., 1.3 mg/L) exerted reduction in Rap to less than half of that in control sample. However, copper ion increased the

Table 1. Effect of time of L-tyrosine addition on Rap biosynthesis by *Streptomyces hygroscopicus* ATCC 29253

Time of L-tyrosine addition (days)	Final pH of fermentation medium	Packed cell volume (PCV%)	Volumetric Rap titre (mg/L)
Control*	6.30	25.94	82.87
0	6.21	28.13	120.53
1	6.23	28.13	120.53
2	6.23	21.88	51.88
3	6.23	22.18	37.97
4	6.34	21.56	27.79

* = no L-tyrosine addition.

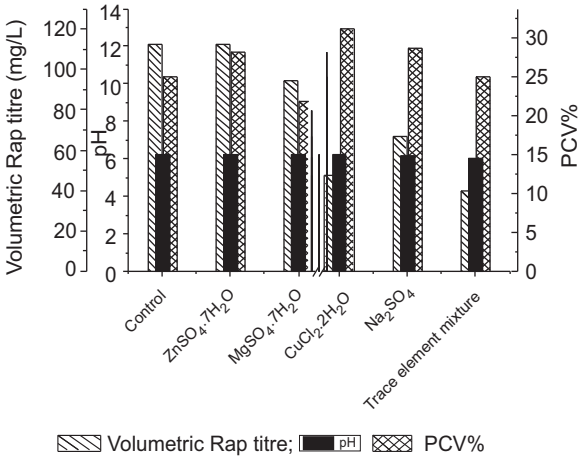

Fig. 3. Effect of trace elements on Rap biosynthesis by *S. hygroscopicus* ATCC 29253. PCV = packed cell volume.

growth from 25 to 31% PCV. The same effect was also observed in case of boron, molybdenum and sodium formulated as $Na_2B_4O_7$, $(NH_4)_6MoO_{24}.4H_2O$ and anhydrous Na_2SO_4, respectively. Mixture of trace elements resulted in negative effect on Rap production higher than that with all individual trace elements. With all studied elements, limited variation in pH of fermentation medium was observed.

Effect of addition of some vitamins on Rap biosynthesis. Different vitamins and vitamin precursors were added individually to the fermentation medium. Five milligrams of each vitamin were dissolved in 5 mL distilled water and the solution was then filtered and sterilised using syringe filter (of 0.45 µm pore diameter) to be added under aseptic conditions to 250 mL Erlenmeyer flask containing 45 mL sterile medium. From the results in Fig. 4, it was obviously

noted that Rap biosynthesis needs no medium enrichment with any of studied vitamins or vitamin precursors because none of them could increase Rap biosynthesis. On the contrary, only riboflavin had not hindered Rap biosynthesis, while, all the others exhibited inhibitory effect in different degrees. *p*-amino benzoic acid favoured the microbial growth. Remarkable suppression in Rap biosynthesis was observed with addition of biotin, pyridoxine and cyanocobalamine. The highest inhibition was in case of thiamine-HCl, nicotinic acid and L (+) ascorbic acid.

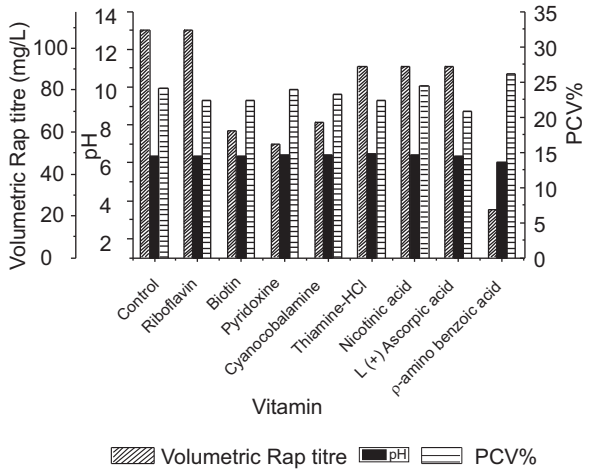

Fig. 4. Rap biosynthesis by *S. hygroscopicus* ATCC 29253 in media supplemented with different vitamins. PCV = packed cell volume.

Rap biosynthesis in media enriched with precursors and inducers. Knowing the building units and precursors involved in Rap biosynthesis justified the need to clarify the ability of these compounds to accelerate its production. The current experiment was developed to study the effect of adding acetate (building unit), shikimic acid (precursor of cyclohexane moiety in Rap) and calcium superphosphate (CaP) on biosynthetic rate of Rap. Graphically reflected results demonstrated strong promotion in Rap production under the effect of shikimic acid and CaP addition (Fig. 5). More than 42% achievement in Rap productivity was attained by addition of shikimic acid, which raised the titre from 112 to 160 mg/L. More close to that achievement was the increase from 112 to nearly 150 mg/L that had been attained with CaP. Beside the strong promoting action of shikimic acid and CaP in Rap biosynthesis, they showed slight decrease in growth yield comparing with control sample. On the other side, sodium acetate afforded complete suppression in Rap biosynthesis associated with very low PCV%.

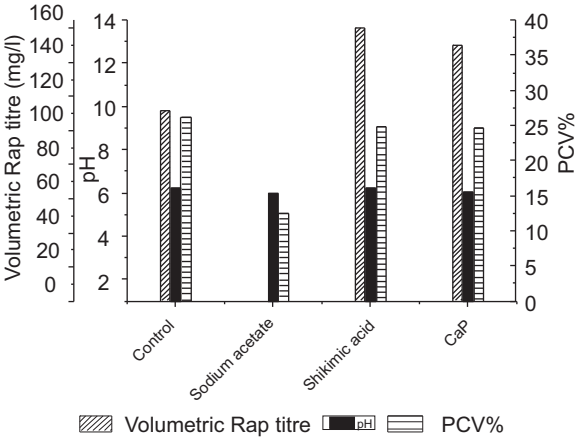

Fig. 5. Effect of medium enrichment with precursors and inducers on Rap biosynthesis by *S. hygroscopicus* ATCC 29253. CaP = calcium superphosphate; PCV = packed cell volume.

Rap biosynthesis in media enriched with different concentrations of shikimic acid and CaP. This experiment was directed to follow up the Rap biosynthesis in presence of different concentrations of shikimic acid and CaP which applied in supplements ranging from 0.1 to 5 g/L. As indicated in Fig. 6, promotion in Rap biosynthesis attained by 5 g/L shikimic acid could be achieved with lower concentration of 2 g/L. Shikimic acid was able to accelerate Rap production even at low concentration as 0.1 g/L. However, increasing its concentration to 0.7 g/L was associated with non-significant increase in Rap titre. With little increase in its concentration from 0.7 to 1 g/L, shikimic acid exhibited considerable advance in Rap productivity. On the other side, the results in Fig. 7 revealed that CaP was also strong Rap stimulator when supplied in special amount. Interestingly, it was found that promoting action of 0.7 g/L CaP that was recorded in the former experiment could be intensified by lowering its concentration to 0.4 g/L. Comparing with control culture, Rap titre achieved more than 53% increase in cultures amended with 0.4 g/L CaP. Concentration of 0.4 g/L was the best for maximum acceleration in Rap biosynthesis and minor change upward or downward caused a drop in Rap production. When CaP concentration exceeded 1 g/L, final pH of fermentation began to move

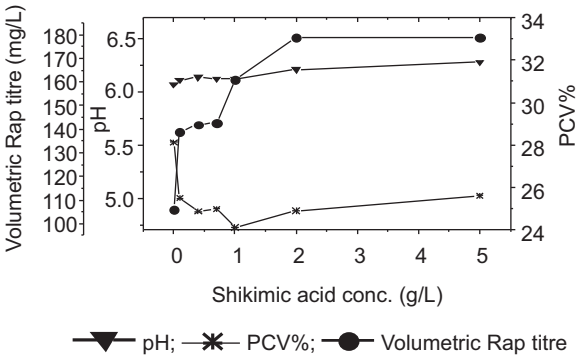

Fig. 6. Rap biosynthesis by *S. hygroscopicus* ATCC 29253 in media of different shikimic acid concentrations.

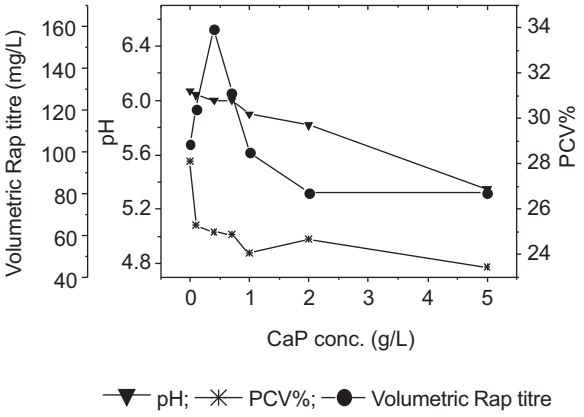

Fig. 7. Rap biosynthesis by *S. hygroscopicus* ATCC 29253 in media of different CaP concentrations.

belw 6 and Rap biosynthesis considerably decreased (Fig.7). The critical role of CaP in Rap biosynthesis, if well modulated, could be used to replace the promoting action mediated by high priced shikimic acid. In addition to its very low price comparing to shikimic acid, CaP works effectively in minor concentrations, which affords an extra advantage. The results indicated low growth yield in cultures amended with shikimic acid or CaP and this provides an industrial economic input.

Applying L-tyrosine at different amounts showed that each of 2 or 5 g/L supplements could afford the same promoting action in Rap biosynthesis though 2 g/L was the optimal for growth. Promoting action of L-tyrosine was attained only, when it was added at start of fermentation or at most after one day lapse. At the second day of fermentation, addition of L-tyrosine gave rise to severe drop in Rap titre (51.88 mg/L), which was lower than that in tyrosine free culture (82.87 mg/L). It was proposed that incorporating tyrosine after time lapse of fermentation stimulates the metabolism to direct towards L-tyrosine, which may interrupt already working metabolic activities, and cells would spend some lagging period before being adapted to metabolise tyrosine. The lost time is reflected as a reduction in growth and Rap titer as it has shown in the current results.

Various metals affect the production of antibiotics (Iwai and Omura, 1982). The results showed that Rap biosynthesis was not favoured by any of studied trace elements. Copper ions (in very low concentration of 1.3 mg/L) exerted reduction in Rap titre to less than half of that in control sample together with increasing growth from 25 to 31% PCV. With all studied trace elements, limited variation in pH of fermentation medium was shown indicating that variation in outputs of Rap and growth was not attributed to shift in medium pH.

Enriching fermentation medium with different vitamins and vitamin precursors elucidated the retarding effect. ρ-amino benzoic acid caused the greatest drop in Rap titre and in the same time it had favoured the microbial growth which reflected the difference between requirements needed for growth and Rap biosynthesis. The role of vitamins in Rap biosynthesis may better be investigated in chemically defined medium. It is important to note that soy meal, a medium component, contains self constituents of vitamins (USDA National Nutrient database). The negative action in the obtained results may be attributed to rise in vitamin concentration after exogenous vitamin supplementations.

Shikimic acid and acetate are known precursors in Rap biosynthesis (Reynolds and Demain, 1997). The results of the current work showed strong promotion in Rap biosynthesis under the effect of shikimic acid addition. Also, calcium superphosphate (CaP) has potentiated comparable promotion. Beside the strong promoting action of shikimic acid and CaP in Rap biosynthesis, they showed slight decrease in growth yield comparing with control sample. These findings may point to a specific positive interaction of shikimic acid and CaP with metabolic activities incorporated in Rap biosynthesis. Decrease in growth yield may be result of

stimulatory effect of these precursors that initiate Rap biosynthesis earlier after short time of growth proliferation. Low growth yield may provide an advantage due to conserved downstream processing and extracting Rap from less biomass yield. Although Fang and Demain (1995) have elucidated the stimulatory action of shikimic acid on Rap production in chemically defined medium, they failed to record its promotion in complex medium. As such, stimulatory action of shikimic acid in a complex medium was reported here for the first time. Relatively low concentration (0.7 g/L) could markedly induce Rap biosynthesis greater than 5 g/L shikimic acid. Calcium superphosphate may need extensive future studies to resolve such role. Omura *et al.* (1980) reported the tremendous promotion, 2-15 times, in production of spiramycin, a macrolide antibiotic, by *Streptomyces ainbofaciens* ATCC 23877 after CaP addition. Thus, the inductive action of CaP in biosynthesis of Rap, as one of the most valuable recent drugs, was recorded here for the first time. Complete suppression of Rap biosynthesis, very low PCV% value and non changed medium pH, in case of fermentation amended with sodium acetate makes the suggestion that sodium acetate has strong toxic effect on the experimental organism. It was found that shikimic acid concentration of 2 g/L has the same effect of 5 g/L, where about 176 mg/L of Rap was obtained and the effect of CaP was found to be intensified by 53% by lowering its concentration from 0.7 to 0.4 g/L. Another feature in CaP stimulatory function; when CaP concentration exceeded 1 g/L, the final pH was decreased and Rap biosynthesis was delayed. It may be assumed that relatively higher CaP concentrations (nearly more than 1 g/L) alter the fermentation medium pH, which decreases the Rap production. Care should be taken to select the suitable buffer of non-interference with metabolic activities of the experimental organism. Thus, replacement of 2 g/L shikimic acids which is expensive substance, with 0.4 g/L of cheap calcium superphosphate will be of economic value.

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