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Production of rapamycin on agro-industrial substrates in benchtop bioreactor: Insights on process development and obstacles management

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Abstract

Rapamycin is a unique medication with endless clinical bioactivities and special potency. One of the drawbacks restricting its widespread use was its relatively high price. The current study presents a pioneering approach of rapamycin production using costless agro-industrial by-products in a stirred-tank bioreactor, which is assumed to usher a new era in rapamycin production, pricing, and distribution. A remarkable drop in rapamycin yield (≤0.81 mg/l) was recorded at the studied levels of agitation (100, 150 & 200 rpm). The clear signs of microbial growth and activity pointed to specific unfavourable conditions to rapamycin production. Cause analysis and basic differences between fermentations in a shaken-flask and fermentor were practically pursued, leading to valuable considerations about high working volume, oxygen availability, and shear stress in the fermentor. An interesting production of the drug (8.1 mg/l) after 72 hours was achieved at high agitation (600 rpm). Decreasing the aeration rate to 0.5v/v/m verified surprising daily productivity (4.3 mg/l/day), which was comparable to that in a flask (4.8 mg/l/day). The efficiency of different organic solvents in extracting rapamycin from cell debris and residues of natural substrates was clarified; ethanol for 60 minutes was the proper and potentiated more than 17% increase in the yield.

Keywords: Rapamycin, Stirred-tank bioreactor, Fermentation, Agro-industrial by-products

1. Introduction

Through a captivating story, rapamycin has evolved from an antifungal metabolite discovered in 1975 to become a billion-dollar drug with two approvals from the American FDA and a wide range of clinical activities with exceptional potency. Rapamycin was initially identified in 1975 as an antifungal agent [1] with no antibacterial activity [2]. Over the years, additional activities were discovered; it exhibited immunosuppressant properties [3] and demonstrated efficacy against mammary, colon, and brain tumor models [4]. Its immunosuppressant activity is remarkably potent, being 150 times more potent than cyclosporine A [5]. It received two approvals from the American FDA, the first in August 1999 for preventing host rejection in kidney transplants [6] and the second in 2003 for use in drug-eluting stents [7] to prevent restenosis of coronary arteries following angioplasty [8]. Other clinical activities of rapamycin included anti-HIV, antifibrotic, antileukemic, anti-inflammatory, antiangiogenic and antiproliferative actions [9-14].

The relatively high price of rapamycin is a remarkable drawback that limits its widespread use and considered a key challenge facing the researchers [15, 16]. Utilizing low-cost natural substrates in rapamycin production and implementing the fermentation process in benchtop bioreactors are crucial steps to reduce production costs and market prices, making the drug more affordable for patients and facilitating further clinical research. Developing a production medium primarily composed of affordable natural substrates has been successfully achieved in shaken-flask cultures [17]. However, these proposed media have not been optimized for larger scales. Scaling up microbial fermentation is a challenging process that faces many obstacles [18] due to significant differences in conditions between

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various scales, leading to changes in productivity, consistency, and stability [19, 20]. Therefore, the current study aims to address the challenge of implementing the natural substrate medium in a stirred-tank bioreactor.

2. Materials and methods

2.1. Microorganism

Streptomyces hygroscopicus ATCC 29253 was obtained from Cairo MIRCEN and stored as a frozen spore suspension at -20ºC [21].

2.2. Preparation of inoculum culture

Inoculum culture was developed from thawed spore suspension [22]. Three 500-ml Erlenmeyer flasks, each containing 100 ml starch-casein broth [23], were inoculated with 2 ml of thawed spore suspension per flask ($25.8x10^6$ spores/ml). After 5 days of incubation at 28±2ºC and 150 rpm, the contents of the three flasks (300 ml) were used to inoculate a 5-litre fermentor batch of fermentation medium (6% inoculum percentage). The same inoculum percentage was maintained when using shaken flask cultures.

2.3. Production

A bench-top fermentor (New Brunswick, BioFlo 310) with a total volume of 7.5 litres was filled with 5 litres of a natural substrate medium developed by Mohamed *et al*. [17]. The medium consisted of the following components gram per litre: soymeal, 10; acid treated wheat bran, 10; KH_2PO_4, 9; oat bran, 20; semolina, 10. The treated wheat bran was prepared by adding 50 ml of 0.2 N HCl to 10g of wheat bran, autoclaving the mixture at 120°C for 30 minutes, allowing it to cool, and adjusting the pH to 6 before adding it to the other medium components. Additionally, 10 ml of whey was added to the mixture, and the volume was adjusted to 1000 ml with tap water, with the initial pH set at 6. The fermentation process was carried out at 25°C with specific levels of agitation and aeration as specified for each experiment.

Production in shaken-flask culture was conducted in 250-ml conical flasks, each containing 50 ml production medium (till mention otherwise) of the composition described above, and were then incubated for 5 days at 150 rpm and 25°C.

2.4. Estimation of microbial growth

Microbial growth was estimated using the packed cell volume percentage (PCV%) method as described by Kaneko *et al*. [24]. By centrifuging 3 ml of the whole fermentation at 3500 rpm, the percent of the compact precipitate volume to the total volume was determined.

2.5. Extraction and quantification of rapamycin

As described by Sinha *et al*. [25], aliquots of 3 ml were taken where microbial growth was separated by centrifugation (3500 rev/min, 5 min) and extracted twice by shaking with 3 ml methanol for 30 minutes. The obtained extracts were then pooled together and centrifuged at 12000 for 20 minutes to be assayed by HPLC. Rapamycin was separated on a reversed-phase HPLC column at 40ºC and monitored by a UV detector at 277 nm. The chemicals used were of HPLC grade and filtered before being pumped to the column. A sample of 10 µl was injected, and the eluent of acetonitrile (95%) was pumped at a rate of 1 ml/min [26].

2.6 .Estimation of total carbohydrates

The total carbohydrates were estimated using the phenol-sulfuric acid method [27]. Briefly, to 1 ml of fermentation medium in a glass test tube (40x200mm), 1 ml. of 5% phenol in water was mixed and then 5 ml. of concentrated sulfuric acid was added. The tubes are allowed to stand for 10 minutes, then they are shaken and placed for 10 to 20 minutes in a water bath at 30ºC. A characteristic yellow-orange color has been developed. The color was permanent for several hours and its intensity was read at 490 nm. Total carbohydrates amount was calculated as glucose equivalent from the equation representing the relation between standard glucose concentrations versus absorbance.

3. Results and discussion

3.1. Selection of suitable antifoam

In majority of studies on rapamycin production, choose of antifoam agent was done without

comparative assessment of its role in rapamycin biosynthesis [28-30]. So, prior to being used in fermentor-scale production, different antifoams were tested for their toxic or inhibitory effects on rapamycin production. An amount of 0.5 ml of the tested antifoam was added to 50 ml of the production medium in a 250-ml flask. Due to its known inhibitory effect, Tween-80 was applied in a lower amount (0.05 ml). After a 5-day incubation period, the amount of rapamycin was estimated and expressed relative to the yield obtained in the control sample, which contained no antifoam. As shown in Fig. 1, castor oil was the most compatible for rapamycin production, with 97% of the rapamycin yield in the control sample obtained in the presence of castor oil. Sesame oil and silicon antifoams were also comparable to castor oil. Other antifoams interfered negatively to varying degrees with rapamycin production. Even with their functional role in controlling foam and their possible interference with rapamycin biosynthesis, studies on selection of the most proper antifoam were very scarce. Interference of antifoam with lincomycin production was reported by Lee *et al*. [31].

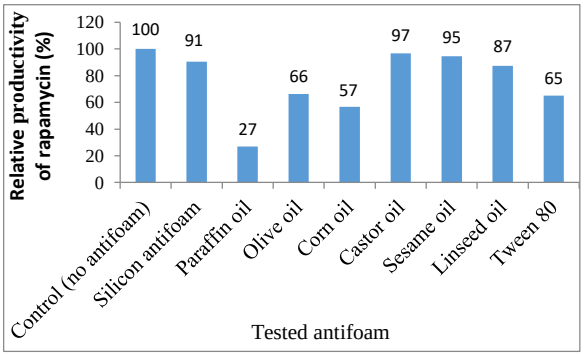


Fig. 1: Effect of different antifoams on rapamycin production

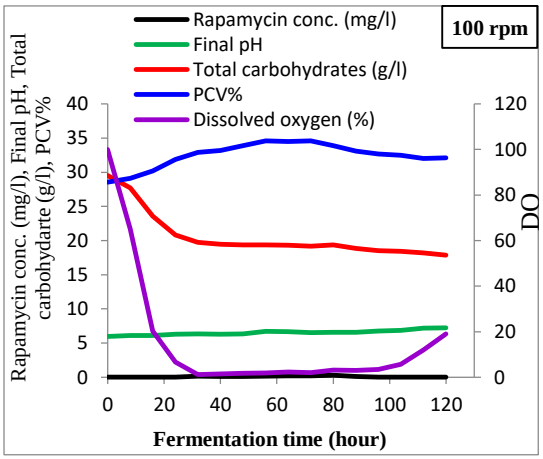
3.2. Implementing rapamycin production in fermentor-scale process

3.2.1. Conducting rapamycin production at different agitation speeds

Rapamycin production in was studied a fermentor at three different agitation levels: 100, 150, and 200 rpm. All trials were carried out at an aeration rate of 1 v/v/m and a temperature of 25^{0}C. The profiles of the three runs are shown in Fig. 2. It was observed that at all studied levels of agitation speeds, only insignificant amounts of rapamycin were detected (ranging between 0.0 and 0.81 mg/l). Variations in PCV% and percent of dissolved oxygen (DO) clearly indicated the vitality and activity of producing strain and pointed out to specific possible inhibition of rapamycin biosynthesis. Regarding agitation speeds used in rapamycin production, Yen and Li, [30] used variable agitation speeds of 100–300 rpm to produce rapamycin, while Yen and Hsiao [22] worked on the variable range of 50-500 rpm. So, the current range of agitation (100, 150 and 200) was reasonably chosen, and the obtained suppression of rapamycin production under such conditions may returned to inadequate agitation due to special content in production medium of natural non-soluble substrates. In addition, DO profile of the current runs (Fig. 2) showed dramatic depletion, to values tend to zero, that started after about 20 hours and continued for prolonged time, which pointed to critical need to oxygen. Availability of oxygen is critically affected by agitation speed [32]. It was so assumed that better production of drug may be attained at higher agitation speeds. This is may be supported by findings of Zhu *et al*. [29] who found that 400 rpm was the optimal for rapamycin production. As such, it was intended to investigate the drug production at 400 rpm. Also, the assumption that production of rapamycin may start after prolonged time justified to monitor the fermentation for longer time.

3.2.2. Production of rapamycin at 400 rpm

As depicted in **Fig. 3,** increasing the agitation speed to 400 rpm and observing the fermentation for 9 days did not reveal significant increase in rapamycin yield.



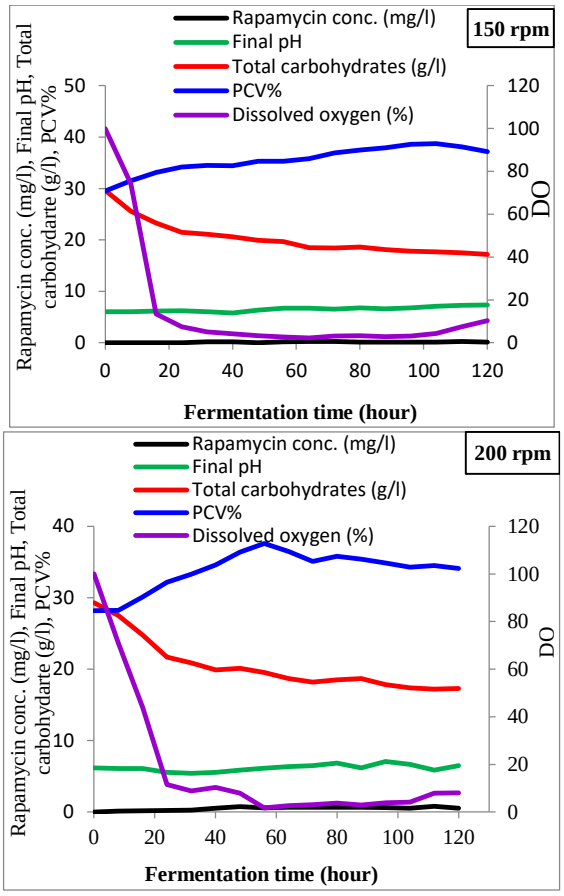


Fig. 2: The profile of fermentation conducted in 7.5-litre fermentor for rapamycin production at agitation speed of 100, 150 and 200 rpm.

As previously ascertained that productivity under shaken-flask cultures verified 24.03 mg/l, possible concerns about right preparation of production medium, activity and purity of inoculum culture and efficient inoculation of fermentor under sterile conditions should be pursued. So, tracking the accuracy and quality of different preparations and steps involved in fermentation process was conducted by tracking flask, which was conducted by transferring 50 ml from fermentation vessel, just after administration of inoculum culture, to 250-ml flask under sterile conditions to be incubated at 25^{0}C at 150 rpm for 5 days. The tracking flask demonstrated an impressive yield of 25.8 mg/l, which affirmed the effectiveness of different fermentation steps. Use of tracking flask in comparison with fermentor study was conducted previously by Zhu *et al.* [29] and ascertained the comparable yields between tracking flak and fermentor. The current observed difference between productivity under shaken-flask and stirred-tank fermentor was also reported previously by Yen and Hsiao [22] who found that rapamycin yield in stirred-tank fermentor, whether at low or high DO, was about one to two thirds of the yield obtained in flask. A possible reason was the shear force mediated by stirring in fermentor which affected the pellet morphology [22]. As a trial to elucidate the difference in pellet morphology between the two systems (shaken-flask and stirred-tank fermentor), successive imaging for microbial growth in fermentor was attained (**Fig. 4**). It was clearly noted that growth in fermentor appeared initially at 21 hours as small aggregates of hyphae which in turn gradually increased in size and consistency till reaching the mature pellet shape at 88 hours (confluent dark globose pellets). After that, gradual disintegration in size and consistency had been occurred. On the other side, the pellets obtained in tracking flask at the end of fermentation (120 hr) were of remarkable greater size and more condensed mass of growth. As shown by Yen and Hsiao [22], production of rapamycin was favoured under condensed mature pellets shape and markedly ceased under reduced pellet size mediated under shear stress in fermentor. The internal condensed masses inside large pellet contains higher content of degenerated hyphae due to high rate of programmed cell death which releases free radicals and oxidative stress capable of triggering the antioxidant metabolite "rapamycin" to high extent [33]. Programmed cell death plays a crucial role in the development of *Streptomyces* and production of secondary metabolites [34], and off course differs substantially with different morphological pattern in flask and fermentor. As such, it was realized that the whole fermentation profiles differed greatly between shaken-flask and stirred-tank fermentor due to the inherent technical differences between the two systems. To get closer to the cause for ceased productivity in fermentor, the intrinsic differences between shaken-flask and stirred-tank fermentor are theoretically reviewed (**Table 1**) and practically tested for its possible inhibitory effect on shaken-flask culture in subsequent investigations.

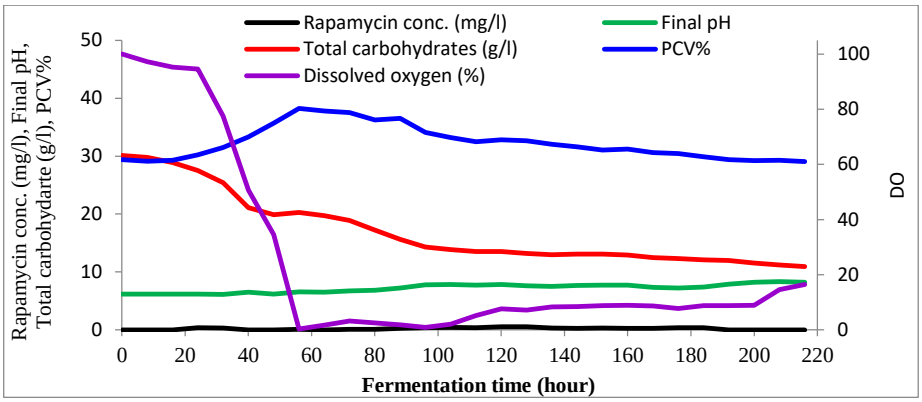


Fig. 3: The profile of fermentation conducted in 7.5-litre fermentor for rapamycin production at agitation speed of 400 rpm.

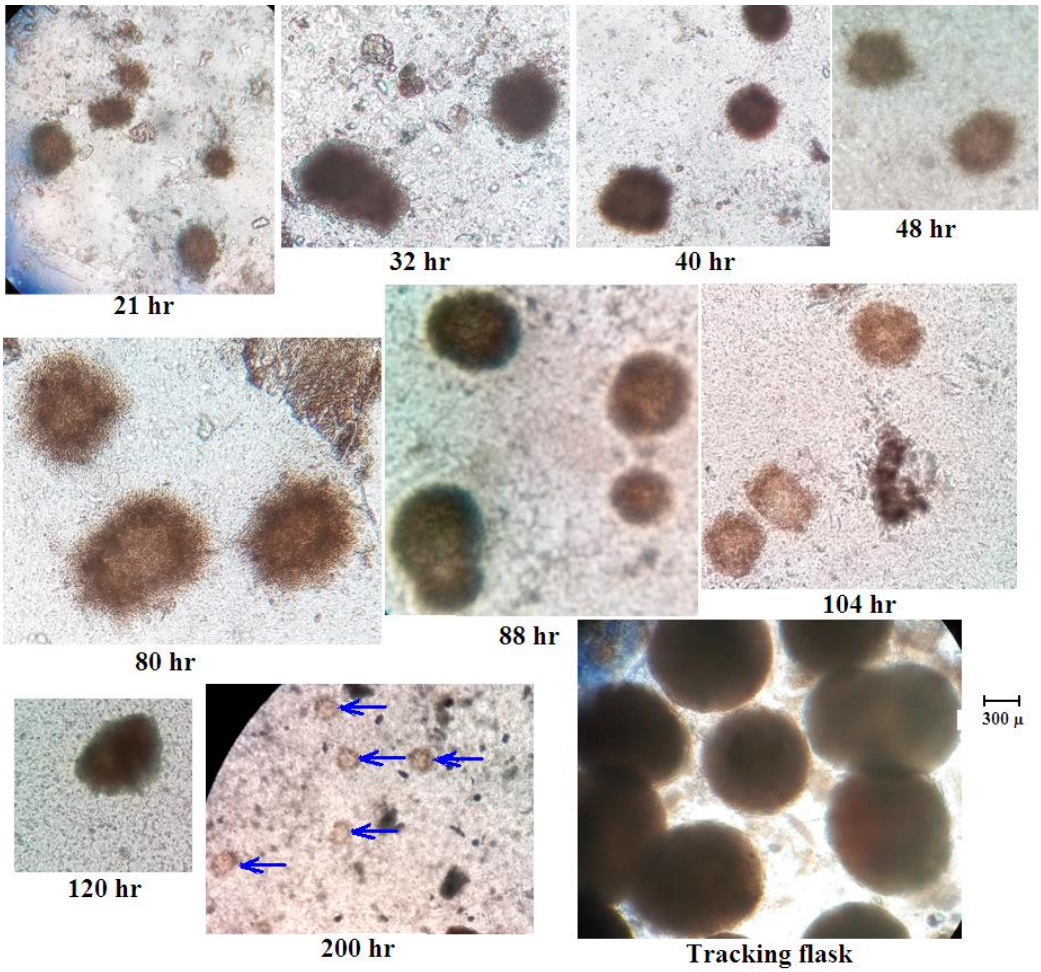


Fig. 4: Photomicroscopy of characteristic growth features of *Streptomyces hygroscopicus* ATCC 29253 at different times in 7.5-litre fermentor using agitation speed of 400 rpm. The blue arrows in the image captured at 200 hr pointed to the reduced disintegrated pellets at prolonged fermentation time. For comparative prospect, the image at the lowest right side showed the growth formed in tracking flask at the end of fermentation. All photos have been conducted by trinocular light microscope (OLYMPUS) using magnification power of 100X.

Table 1: Comparative prospect to main features of shaken-flask and stirred-tank fermentor conducted in preceding investigations

	Shaken-flask	Stirred-tank fermentor
Shape of fermentation space	Conical shape flask	Cylindrical shape vessel
Working volume	50 ml	5 litres
Working volume/total volume ratio	0.2 (50ml/250 ml)	0.67 (5 litres/7.5 litres)
Mixing and agitation	Depends on rotatory motion of flask which mediates smooth circulation of medium	Depends on "stirred tank" concept in which static vessel is stirred by rotating blades on axe extending inside the vessel in presence of baffles fixed on walls of the vessel. It mediates vigorous motion of medium in irregular directions.
Aeration	Slow and occur by mixing sterile air, which present above the liquid production medium, with the surface layer of medium	Depends on forced aeration which is mediated by pumping sterile air into bulk of liquid production medium

3.2.3. Shaken-flask investigations to evaluate probable inhibitory effect of some parameters

a) Effect of increasing working volume

In 250-ml conical flasks, rapamycin production was tested at different working volumes of 50, 100, 150, and 200 ml to maintain ratios of 0.2, 0.4, 0.6, and 0.8, respectively, for working volume/total volume. The results in **Table 2** indicated that increasing the working volume was accompanied with drastic decrease in rapamycin yield. When the volume was increased from 50 ml to 100 ml, the yield dropped from 24.57 mg/l to approximately one-third (8.75 mg/l), representing a loss of two-thirds of the yield (64.4% inhibition). A substantial decrease in yield to the lowest value of 4.29 mg/l (the highest inhibition percentage, 82.5%) was observed at a working volume of 150 ml, which is corresponding to a 0.6 ratio for working volume/total volume, the closer value to that used in the fermentor (0.67). Therefore, the negative impact of high working volume in the fermentor was reasonably explained. However, the extent of this negative impact in the fermentor should be less than that observed in the flask because aeration in the fermentor is mechanically induced by pumping air and is not as significantly affected by an increase in working volume. Little data was addressed about the effect of working volume ratio on fermentation performance, yet they had elucidated the better activities at low ratios [35, 36]. The current results clearly nominated, for the first time, one of most restricting factor in rapamycin fermentation, which should be considered prior to initiating scaling-up fermentation processes.

Table 2: Effect of increasing working volume on rapamycin production in 250-ml conical flask

Working volume	Working volume/total volume	Rapamycin yield (mg/l)	% inhibition*
50 ml (Control)	0.2	24.57	0.0%
100 ml	0.4	8.75	64.4%
150 ml	0.6	4.29	82.5%
200 ml	0.8	5.44	77.9%

* % inhibition= (rapamycin yield in control – rapamycin yield of the referred case) * 100 / rapamycin yield in control

b) Effect of enhancing the aeration by forced pumping of sterile air

In an attempt to enhance rapamycin production, a trial was conducted with a working volume/total volume ratio of 0.6. Sterilized air was pumped into a 2-litre flask at a rate of 1 v/v/min using a small aquarium air pump, containing 1.2 litres of medium. A control flask was also prepared without forced aeration. **Table 3** shows that forced aeration did not significantly improve rapamycin production, but it did result in the highest yield in a shorter time period

(3 days). The current results are significantly supported by the most recent investigation of Takahashi *et al.* [37] that realized the privilege of forced aerated submerged culture in achieving 35% reduction in fermentation time to reach the maximum bacterial concentration, and 1.25-fold increase in the growth.

Table 3: Effect of enhancing the aeration by forced pumping of sterile air

	Control (no forced aeration)	Forced aeration in rate of 1 v/v/min
Rapamycin yield after 3 days (mg/l)	2.2	5.0
Rapamycin yield after 6 days (mg/l)	4.8	1.8

c) Effect of flask shape (conical versus cylindrical)

The difference in the shape of the fermentation container, which is conical in the flask and cylindrical in the fermentor, may reasonably promote different degrees of mixing and mass transfer. To investigate this effect, fermentation was conducted in two flasks of the same capacity (2 litres) with the same amount of medium (1.2 litres) but different geometrical shapes, conical and cylindrical. In both cases, the ratio of working volume to total volume was 0.6, which closely resembled that in fermentor studies. The results presented in **Table 4** indicated that the conical-shaped flask had a higher rapamycin concentration in the early stages of fermentation (3 days). However, after 6 days of fermentation, the two shapes showed comparable yields. According to Munroe [38], vessel geometry plays an important role in fermentation, though it showed little effect when studied by Speers & Stokes [39].

d) Stability of rapamycin production against increased amounts of oxygen

High agitation and forced aeration in the fermentor can lead to the generation of a highly oxygenated medium. Therefore, the current study aimed to investigate the stability of the production process against elevated levels of oxygen under optimal shaken-flask conditions. Under sterile conditions, definite amounts of H_2O_2 were added to 50 ml production medium (in 250-ml conical fermentation flask) at start of fermentation or after 3 days to get the final concentrations of 10, 20, and 40 mM. As shown in **Fig. 5**, when H_2O_2 was added at the start, it significantly reduced rapamycin production, especially at higher concentrations (20 and 40 mM), indicating the high sensitivity of vegetative cells to oxidative stress during the initial growth stages. However, when H_2O_2 was supplemented after 3 days, a specific dose (10 mM) actually increased rapamycin production to 29.48 mg/l compared to the control sample with no supplements (24.17 mg/l). This suggests that microbial cells after 3 days of fermentation were more resilient to oxidative stress and could benefit from a moderate level of H_2O_2 to enhance rapamycin production, which has potent antioxidant properties to protect against oxidative stress. In conclusion, oxidative stress can have a detrimental effect on rapamycin production but can also be stimulatory if applied in a controlled manner. This in complete accordance with Wei *et al.* [40], who could activate validamycin production in *Streptomyces hygroscopicus* after manipulating H_2O_2 amount and addition time. Additionally, increased production of secondary metabolites under the effect of different stress factors was reported elsewhere [33, 41].

Table 4: Production of rapamycin in shaken-flask cultures of different shapes

	Conical flask	Cylindrical flask
Rapamycin yield after 3 days (mg/l)	2.4	1.3
Rapamycin yield after 6 days (mg/l)	4.5	4.2

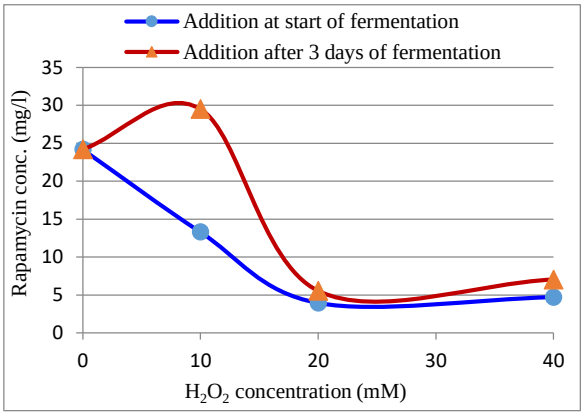


Fig. 5: Production of rapamycin at different supplements of H_2O_2 added at start or after 3 days of fermentation start.

After extensive investigatory endeavors in shaken-flask culture, it was ultimately concluded that there is a critical problematic issue concerning availability of oxygen in fermentations done previously on stirred-

tank fermentor, or at least more availability of oxygen could probably enhance rapamycin production. Interestingly, the impressive findings of Salazar-Magallon *et al.* [42] realized that agitation speed of 250 rpm under shaken-flask conditions had an oxygen transfer coefficient that is largely equivalent to that of 650 rpm in stirred-tank fermentor. Moreover, Zhou *et al.* [32] addressed the evidence that oxygen transfer coefficient is a critical determinant in scaling-up fermentation processes, and keeping it at comparable values among fermentations of different scales can allow productivities at comparable levels. As such, it has intended to investigate rapamycin production at high agitation speed as 600 rpm.

3.2.4. Back to fermentor to study rapamycin production at 600 rpm

The fermentation was conducted at an agitation speed of 600 rpm, aeration rate of 1 v/v/m, and a temperature of 25°C. To control foam formation, sterile castor oil (10 ml/litre) was added at the start of fermentation. Interestingly, significant amounts of rapamycin were detected **(Fig. 6).** Up to 8.1 mg/l of rapamycin was obtained after 72 hours. The yield was nearly one-third of that in shaken-flask (24.03 mg/l). However, considering that the yield in the flask was obtained after 5 days (i.e., 4.8 mg/l/day), it is nearly two times the yield obtained in the fermentor per day (2.7 mg/l/day). Regarding the previous works [22, 43] on rapamycin production in stirred-tank bioreactor by the typical producing strain used currently (*Streptomyces hygroscopicus* ATCC 29253), the agitation speed of 600 rpm, that applied currently, was markedly higher than all previously used values, which reflected the specific need of the currently used production medium to high agitation to get adequate mass transfer of oxygen, gases and nutrients through its special high content of natural substrates.

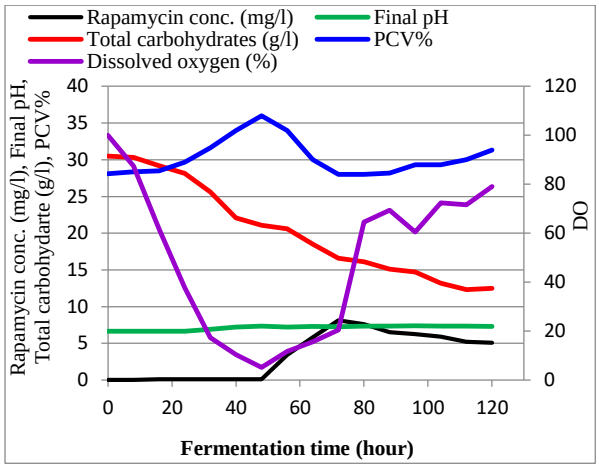


Fig. 6: The profile of fermentation conducted in 7.5-litre fermentor for rapamycin production at agitation speed of 600 rpm.

3.2.5. Optimization of the aeration rate

At an agitation speed of 600 rpm and a temperature of 25^{0}C, batches with aeration rates of 0.5 and 1.5 v/v/m were monitored (**Fig. 7**) and compared with the previous results at 1 v/v/m (Fig. 7). At an aeration rate of 0.5 v/v/m, the maximum yield of rapamycin was 8.6 mg/l, which was comparable to the yield obtained previously at 1 v/v/m (8.1 mg/l). However, in terms of productivity at aeration rates of 0.5 and 1 v/v/m, the time required to achieve the maximum yields of 8.6 and 8.1 mg/l was 48 and 72 hours, resulting in daily productivities of 4.3 and 2.7 mg/l/day, respectively. The daily productivity was significantly higher at an aeration rate of 0.5 v/v/m and surprisingly comparable to that obtained in a flask (4.8 mg/l/day). On the other hand, at 1.5 v/v/m, the maximum yield was only 2.9 mg/l after 72 hours, indicating a markedly lower productivity compared to the aeration rates of 0.5 and 1 v/v/m. Previous studies aimed at optimizing rapamycin production in bioreactor were not concerned in investigating the effect of aeration rate, and worked at 1 v/v/m with no prior optimization studies [21, 22, 43]. Work at variable aeration rates to modulate DO at controlled level was also conducted [28]. As such, the stimulatory role of the aeration rate of 0.5 v/v/m on rapamycin production, basing on comparative study, was reported currently for the first time to the best of authors' knowledge.

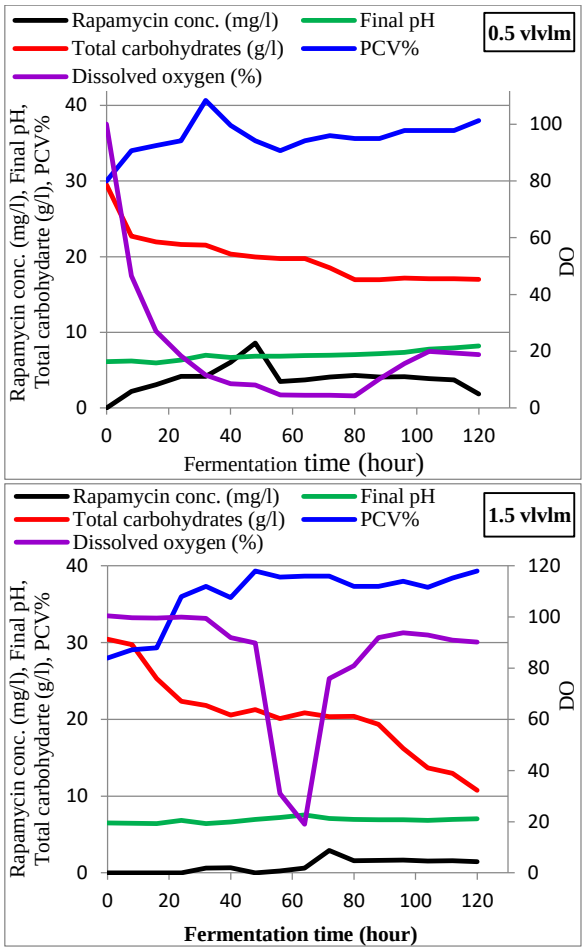


Fig. 7: The profile of fermentation conducted in 7.5-litre fermentor for rapamycin production at 600 rpm and aeration rates of 0.5 and 1.5 v/v/m.

3.3. Effectiveness of different organic solvents in extraction of rapamycin from microbial cells with natural substrate residues

The currently used production medium had special content of suspended solids of natural substrates, which may impede extraction process. So, the efficiency of extraction by the conventionally used solvent, methanol, was assessed and compared with other organic solvents of different polarities. For standardization, equal weighed amount (0.6 g) of wet solid suspensions (WSS) were re-suspended in 4 ml of assigned solvent and shaken at 150 rpm and 30°C for varying durations (30, 60, and 90 minutes). The extracted rapamycin was quantified and expressed as mg per kg of WSS **.** As shown in **Fig. 8**, all tested organic solvents, except for ethyl acetate, were more efficient than methanol. Ethanol was the most efficient after 60 minutes, extracted 272 mg/kg WSS, which represented a more than 17% increase compared to methanol after 90 minutes (232 mg/kg WSS). Rapamycin extraction by methanol was reported in elsewhere [21, 22, 25, 44], however, minor use of toluene was also reported [45]. As such, the superiority of ethanol over conventionally used solvent "methanol" in rapamycin extraction was one of interesting findings reported by the current investigation.

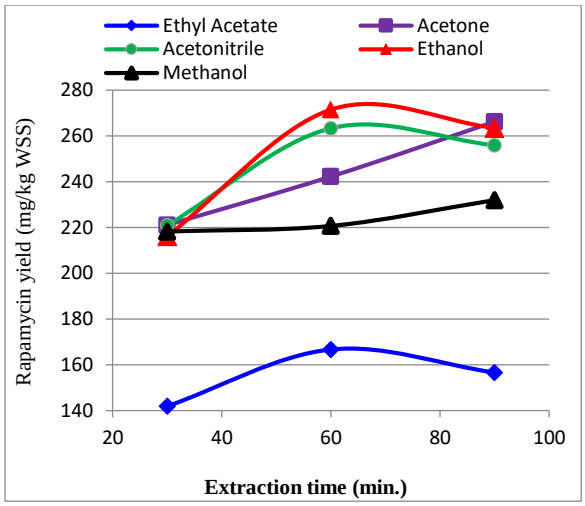


Fig. 8: Extraction of rapamycin from wet solid suspends (WSS) by different organic solvents applied for different times.

4. Conclusion

A pioneer work targeting production of rapamycin from costless agro-industrial substrates in stirred-tank bioreactor was conducted. A significant decrease in rapamycin yield was observed at agitation levels of 100, 150, and 200 rpm, with yields as low as 0.81 mg/l. Microbial growth and activity indicated unfavorable conditions for rapamycin production. Analysis revealed differences between fermentations in shaken flasks and fermentors, highlighting the importance of high working volume, oxygen availability, and shear stress. However, at high agitation (600 rpm), a production of 8.1 mg/l was achieved after 72 hours. Lowering the aeration rate to 0.5v/v/m resulted in a daily productivity of 4.3 mg/l/day, comparable to flask conditions (4.8 mg/l/day). Extraction of rapamycin by ethanol from residuals of production medium for 60 minutes potentiated over 17% increase in the yield.

5. Conflicts of interest

All the authors declare no conflict of interest.

6. Acknowledgments

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