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Statistically Improved Formulation of Agro-Industrial Substrates for Economic Production of Rapamycin by *Streptomyces hygroscopicus* ATCC 29253 in Submerged Fermentation

Mohamed A.M. Abdalla[a]*, Abd El-Hamid A. Hamdy[a], Enas M. Mostafa[b]

[a]*Chemistry of Natural and Microbial Products Department, Pharmaceutical Industries Research Institution, National Research Centre (NRC), Dokki, P.O. 12622, Giza, Egypt.*

[b]*Chemical Engineering and Pilot Plant Department, Engineering Research Institution, National Research Centre (NRC), Dokki, P.O. 12622, Giza, Egypt.*

Abstract

Rapamycin is a multi-functional medication with an endless list of clinical activities and impressive potency. Regarding its remarkable high price, efforts intending rapamycin production from low-cost natural substrates are highly appreciated in the framework of its economic production. Ten natural additives were tested one-at-a-time in a basal medium of natural substrates to potentiate the overproduction of the drug. The results were analyzed by single-factor ANOVA (at a 95% confidence level) which proved a very significant variation in rapamycin concentration (p-value=8.592x10-8). A significant increase in rapamycin yield from a base of 11.2 mg/l in the control to 15.1 and 15.6 mg/l was recorded in the case of oat bran and semolina verifying more than 34% and 39% increase in the yield, respectively. Various supplements of different precursors, stimulators, and carbon sources were also tested, and markedly higher outputs were obtained with D-(+)-mannose, fructose, glycerol, L-tyrosine, and L-lysine. To get the optimal formulation of all-natural and additional substrates, two-step statistical designs were employed. Significant models of Plackett-Burman and Box-Behnken (p-value 0.0017 and 0.0003, respectively) revealed a formula that potentiated more than a 70% increase in drug production.

Keywords: Rapamycin, *Streptomyces hygroscopicus*, Agro-industrial substrates, Submerged fermentation

1. Introduction

Rapamycin (sirolimus) is a multifunction medication having long list of clinical bioactivities with interesting potency. It was early discovered in 1975 as an antifungal agent produced by a strain of *Streptomyces hygroscopicus* isolated from volcanic rock soil of Easter Island (known as Rapa Nui) in Chile [1]. Later after discovery, many other activities of rapamycin were disclosed which included immunosuppressive, anti-inflammatory, antiaging, neuroprotective and antitumor activities [2-4]. Its role in treatment of acute myeloid leukemia, retinal and choroidal vascular diseases and its activity against replication of human immunodeficiency virus type 1 has been well established [5-7]. Additionally, it attenuates atherosclerosis [8] beside its roles in age-related diseases [9] and the healing of bladder and abdominal wound [10].

Rapamycin is a polyketide consisting of a 31-membered lactone ring with characteristic α-ketonic group. The large ring (macrocyclic) is commonly known as a macrolide ring. Its biosynthesis starts with a cyclohexane moiety (derived from the shikimate pathway) to which seven acetate and seven propionate units participate to build up a polyketide backbone in a head-to-tail fashion. Finally, pipecolate (synthesized from L-lysine) attaches to the polyketide chain, followed by ring closure via lactone formation. Three methyl groups are transferred from methionine via S-adenolsyl methionine to form the three methoxy groups [11, 12].

Focusing on the physiological aspects of production, enormous number of studies had been addressed targeting cultural, nutritional, biochemical and molecular factors affecting its biosynthesis [2, 13, 14]. The studies were interested with increasing

*Corresponding author e-mail: mmabdelaziz1976@yahoo.com; (33 El Bohouth st., P.O. 12622, Mob. +201007613420).
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rapamycin yield via medium optimization and strain improvement, and completely neglected the cost of used production media. Considering the relatively high price of rapamycin, which restricting its widespread usage in research and clinical studies, there was a critical need to develop and improve rapamycin production process on affordable low-cost natural substrates in an attempt to achieve intrinsic reduction in its production cost and market price. As such, the current study was intended to introduce a pioneer endeavor for utilizing costless natural substrates in rapamycin production for in-future economic production of the drug.

2. Materials and methods

2.1. Microorganism

Streptomyces hygroscopicus ATCC 29253 was purchased from Cairo MIRCEN and was preserved as frozen spore suspension at -80ºC [15].

2.2. Preparation of inoculum culture

One milliliter of thawed spores' suspension ($25.8x10^6$ spore/ml) was inoculated into 50 ml starch-casein broth (Difco, NJ, USA) [16] in 250-ml Erlenmeyer flask. After incubation for 5 days at 28±2ºC and 150 rpm, 3ml of culture was used to inoculate production flask.

2.3. Production

Production was conducted in 250-ml conical flasks, each containing 50 ml of production medium, which were incubated for 5 days at 150 rpm and 25°C. Basal production medium consisted from soymeal (Local market) (20g), KH_2PO_4 (Sigma, St. Louis, USA) (5g.), treated wheat bran (20g), whey (Local market) (20ml), tap water (880ml) and pH 6 [17]. Treated wheat bran was prepared by adding 100 ml inorganic acid (0.2 N HCl, Thermo Fisher Scientific Inc. Waltham, Massachusetts, U.S) to 20g wheat bran (Local market) and autoclaving the mixture at 121ºC for 30 minutes, then the mixture was allowed to cool and pH was increased to 6 before being added to other medium components. To such basal production medium, different additives were supplemented separately and tested for nutritive role in rapamycin production.

2.4. Estimation of microbial growth

Packed cell volume percentage (PCV%) was employed [18]. The final estimate of growth was the ratio of the volume of packed cells to the volume of the entire fermentation media sample.

2.5. Extraction and quantification of rapamycin

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 [14]. Then the obtained extracts were pooled together and centrifuged at 12000 for 20 minutes to be assayed by HPLC (Agilent 1260 series). Rapamycin was separated on C18 reversed-phase HPLC column (250 mm) at 40ºC and monitored by UV detector at 277 nm. The used chemicals were HPLC grade and filtered before pumped to the column. A sample of 10µl was injected and the eluant of acetonitrile (Thermo Fisher Scientific Inc. Waltham, Massachusetts, U.S) (95%) was pumped at the rate of 1 ml/min. [19].

2.6. Statistical calculations

Calculations of standard deviation and single-factor analysis-of-variance (ANOVA) at 95% confidence level were attained by Microsoft Office Excel 2007. The standard error of the mean (SE) was calculated using the following equation:

$SE = SD / \sqrt{n}$

Where SD is the standard deviation and n is the sample size [20].

2.7. Statistical design and modeling

Statistical designs, modeling and surface plots were achieved by "Design Expert" software (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA) statistical package [20].

3. Results and discussion

3.1. Production of rapamycin in media augmented by different natural substrates

Ten natural additives were tested separately for the ability to potentiate over-production of rapamycin in basal medium (BM). Tested substrates included two types of fiber-rich substrates (oat bran and rice bran) and were supplemented in a concentration of 20 g/l which matched the wheat bran amount in BM. The other natural substrates were applied in a concentration of 10 g/l to avoid probable inhibitory effects appeared at higher concentrations. Profiles of microbial growth (estimated as packed cell volume percentage, PCV%) and final pH were recorded to follow microbial physiology during fermentation. The results were analyzed by single factor ANOVA (at 95% confidence level). According to the results depicted in **Fig. 1**, variation in rapamycin concentration was very significant (p-value=$8.592x10^{-8}$, with much greater F value of 51.77 than F critical of 2.85). Out of all tested supplements, remarkable increase in rapamycin yield from a base of 11.2 mg/l in BM medium to 15.1 and 15.6 mg/l in case of oat bran and semolina which verified more than 34% and 39% increase in the yield respectively. Oat bran and semolina are affordable natural substrates and because their surprising role in production of rapamycin has not ever been reported, it was thought that the current finding will launch rapamycin fermentations of exceptional economics. Other tested additives showed deterioration in rapamycin production by different degrees, probably due to their content of trace elements that negatively affect rapamycin production [14]. Complete halt in production was observed in case of carob grind which was attributed to sever drop in growth (PCV%= 13%). Change in pH of fermentation in all cases was conserved near the initial value of pH 6.

3.2. Enriching the medium with precursors, stimulators and others

Twelve chemical substances which showed previous roles in rapamycin production were tested. Different precursors (L-lysine and shikimic acid) and stimulators (L-tyrosine, $FeSO_4$, calcium superphosphate, $Ca(H_2PO_4)_2$ in addition to several carbon sources (mannose, fructose and glycerol) and amino acids (Merck & Co Inc, Rahway, NJ USA) were tested. They were applied in a concentration guided by Mohamed [21]. As inferred from the results in **Fig. 2**, significance of variation in rapamycin concentration under the effect of different additives was clearly reflected from ANOVA results inside frame. The results showed that several supplements could potentiate the production to markedly higher outputs. D-(+)-mannose, fructose, glycerol, L-tyrosine and L-lysine achieved the yields of 15.8 and 13.9, 14.4, 15.3 and 13.7mg/l from a base of 11.1 mg/l in PM, which realized a percent increase of 42, 25, 30, 38 and 23%, respectively. Role of mannose and fructose in production of high yields of rapamycin was reported in elsewhere [22-25]. Hamdy *et al*. [18] recorded 48% increase in rapamycin biosynthesis by the action of L-tyrosine on rapamycin biosynthesis. Glycerol and lysine were employed previously by Zhu *et al*. [26] in

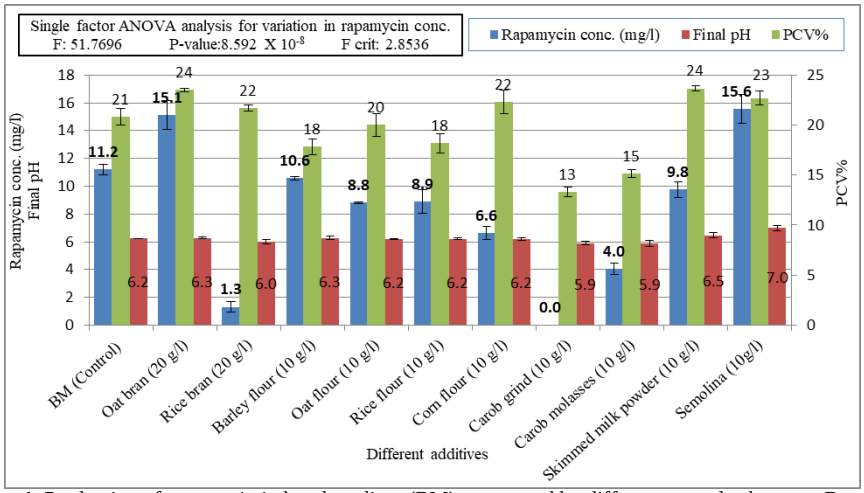

Fig. 1. Production of rapamycin in basal medium (BM) augmented by different natural substrates. Bars in the figure represent the standard error between replicates. ANOVA analysis of the variation in rapamycin concentration between different treatments was indicated inside the frame.

production of high yields of rapamycin. According to Sinha *et al*. [25], using l-lysine at concentration of 5g/l potentiated 4% increase in the yield. In addition, the findings of Kim *et al*. [27] supported the positive role of glycerol in production of rapamycin.

3.3. Plackett-Burman design (PBD) to detect the most affecting components

Throughout the preceding work, several additives (from natural or chemically purified resources) were proved to have positive effect on rapamycin production. Formulating such additives together with the original components of patented medium was followed through PBD (basing on 11 factors, two center points and 14 runs). **Table 1** showed the codes, levels and configuration of the design. The outputs of rapamycin production, microbial growth and final pH were presented in **Table 2**. ANOVA analysis of the design (**Table 3**) showed the high significance of the model and the effects of all tested variables. The Model p-value of 0.0017 and F-value of 202631.75 revealed that the model is significant at

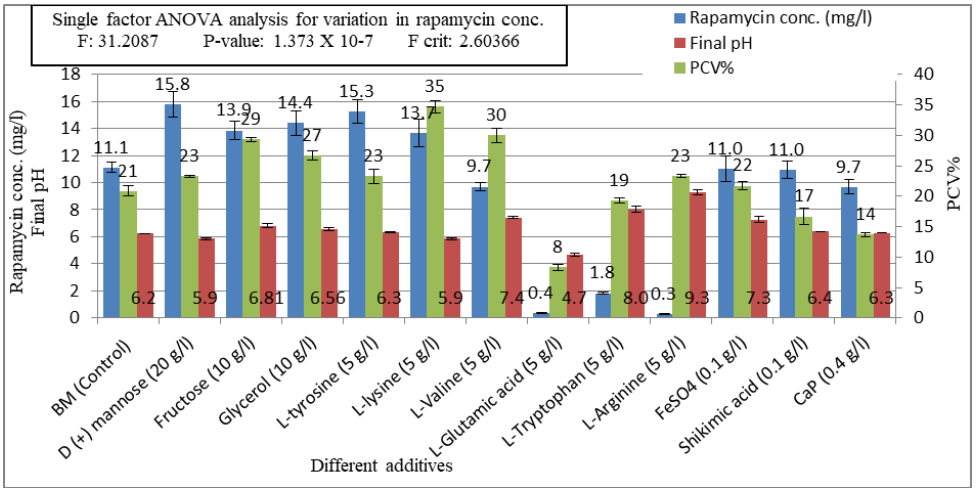

Fig. 2: Testing the proficiency of BM in presence of precursors, inducers and enhancers. Bars in the figure represent the standard error between replicates. ANOVA analysis of the variation in rapamycin concentration between different treatments was indicated inside the frame. CaP = $Ca(H_2PO_4)_2$

Table 1: Codes, levels and configuration of PBD.

	Factor **A**	Factor **B**	Factor **C**	Factor **D**	Factor **E**	Factor **F**	Factor **G**	Factor **H**	Factor **J**	Factor **K**	Factor **L**
Run	Treated wheat bran (g/l)	Soy meal (g/l)	KH_2PO_4 (g/l)	Whey (ml/l)	Oat bran (g/l)	Semolina (g/l)	Glycerol (g/l)	D-mannose (g/l)	Fructose (g/l)	L-tyrosine (g/l)	L-lysine (g/l)
1	10	30	7	10	30	20	20	0	0	0	10
2	20	20	5	20	20	10	10	10	10	5	5
3	30	10	7	30	30	0	0	0	20	0	10
4	30	10	7	30	10	20	20	20	0	0	0
5	10	10	7	10	30	20	0	20	20	10	0
6	20	20	5	20	20	10	10	10	10	5	5
7	30	30	7	10	10	0	20	0	20	10	0
8	10	10	3	30	10	20	20	0	20	10	10
9	10	30	3	30	30	0	20	20	20	0	0
10	30	30	3	10	10	20	0	20	20	0	10
11	30	30	3	30	30	20	0	0	0	10	0
12	30	10	3	10	30	0	20	20	0	10	10
13	10	30	7	30	10	0	0	20	0	10	10
14	10	10	3	10	10	0	0	0	0	0	0

confidence level of 99.83% and noise could largely interfere by only 0.17%.

The results depicted in **Fig. 3** showed the effects of the 11 factors. Three of the factors had a positive effect (KH_2PO_4, oat bran and semolina). However, the other factors showed negative effects which were presumed to be originated mainly from factor-factor-interaction. Percent contribution of the studied factor in total effect on rapamycin production (**Fig. 4**) showed that the most affecting factors were the treated wheat bran and whey (together had near two thirds of total effect). Employing PBD in screening for factors affecting rapamycin production was conducted previously by Sinha *et al.* [25], Kim *et al.* [27] and Abdel-Fattah [28]. Sinha *et al.* [25] studied the effect of four factors on rapamycin and the factors namely soy meal, mannose and lysine were assigned as significant by PBD, which was in complete accordance with the current results. Kim *et al.* [27] screened the effect of 14 factors on rapamycin biosynthesis by PBD and elucidated the significance of only 7 factors, of which soybean was recorded.

Table 2: Outputs of rapamycin production, final pH and microbial growth (PCV%) in PBD.

Run	Rapamycin conc. (mg/l)*	Final pH*	PCV%*
1	14.44 ± 0.48	5.91 ± 0.10	26.33 ± 3.67
2	0.17 ± 0.14	5.54 ± 0.01	21.00 ± 2.67
3	1.10 ± 0.00	5.32 ± 0.01	19.83 ± 0.17
4	0.97 ± 0.00	5.75 ± 0.00	20.17 ± 5.17
5	16.36 ± 14.86	5.74 ± 0.09	17.17 ± 7.83
6	0.19 ± 0.09	5.50 ± 0.01	21.00 ± 2.33
7	1.20 ± 0.00	5.74 ± 0.00	20.00 ± 0.67
8	1.09 ± 0.00	5.45 ± 0.00	15.00 ± 5.00
9	2.33 ± 2.02	5.77 ± 0.06	24.33 ± 0.67
10	0.88 ± 0.00	5.25 ± 0.00	25.83 ± 2.50
11	1.03 ± 0.00	5.79 ± 0.01	36.67 ± 3.33
12	0.87 ± 0.00	5.24 ± 0.00	21.50 ± 2.17
13	0.91 ± 0.00	5.35 ± 0.00	17.83 ± 0.50
14	14.71 ± 2.83	6.65 ± 0.03	15.00 ± 1.67

* Mean ± standard error

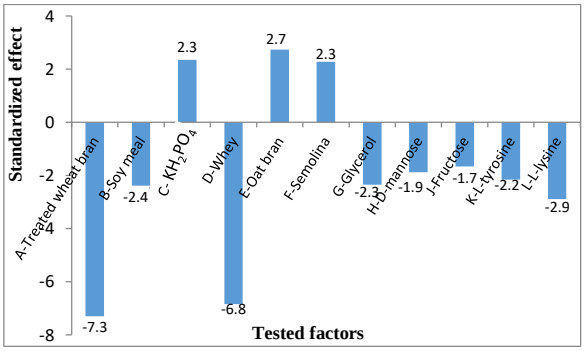

Fig. 3: Effect of different tested factors on rapamycin production through PBD

Table 3: ANOVA analysis for rapamycin production in PBD.

Source	Sum of Squares	df	Mean Square	F Value	p- value Prob > F
Model	445.79	11	40.53	2.026E+005	0.0017
A-Treated wheat bran	159.78	1	159.78	7.989E+005	0.0007
B-Soy meal	17.07	1	17.07	85361.84	0.0022
C-KH_2PO_4	16.49	1	16.49	82473.73	0.0022
D-Whey	140.25	1	140.25	7.012E+005	0.0008
E-Oat bran	22.34	1	22.34	1.117E+005	0.0019
F-Semolina	15.52	1	15.52	77623.08	0.0023
G-Glycerol	16.55	1	16.55	82757.92	0.0022
H-D-mannose	10.52	1	10.52	52617.11	0.0028
J-Fructose	8.26	1	8.26	41313.12	0.0031
K-L-tyrosine	14.00	1	14.00	70001.71	0.0024
L-L-lysine	24.99	1	24.99	1.250E+005	0.0018
Curvature	34.33	1	34.33	1.716E+005	0.0015

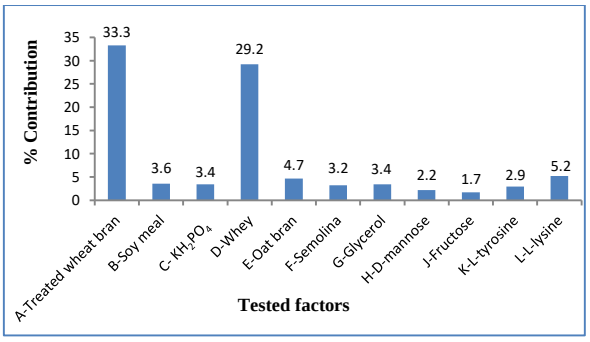

Fig. 4: Percent contribution of different tested factors on rapamycin production through PBD

3.4. Box-Behnken design (BBD) to model the production process.

Focusing on the three factors affecting positively on the production and with applying all other negative factors at the lowest limits, a BBD of 15 runs containing three center points was conducted (**Table 4**).

Table 4: Design and outputs of BBD.

Run	Factor A Oat bran (g/l)	Factor B Semolina (g/l)	Factor C KH_2PO_4 (g/l)	Rapamycin conc. (mg/l)*	Final pH*	PCV%*
1	30	20	7	15.43 ± 0.83	6.68 ± 0.00	33.33 ± 1.17
2	30	20	7	14.33 ±1.14	6.62 ± 0.06	32.33 ± 0.83
3	40	10	7	14.21 ±0.85	6.73 ± 0.02	33.33 ± 1.00
4	30	20	7	15.25 ± 0.77	6.73 ± 0.04	30.33 ± 0.67
5	40	20	9	17.53 ± 0.69	6.44 ± 0.00	36.67 ± 1.33
6	30	10	9	14.70 ± 0.82	6.46 ± 0.01	33.33 ± 1.20
7	20	10	7	16.16 ± 0.97	6.55 ± 0.05	30.00 ± 0.50
8	30	10	5	11.31 ± 1.08	6.8 ± 0.06	31.67 ± 0.33
9	30	30	5	10.65 ± 1.02	6.72 ± 0.00	38.33 ± 1.50
10	40	30	7	11.29 ± 1.45	6.55 ± 0.04	38.33 ± 1.80
11	40	20	5	9.51 ± 0.71	6.72 ± 0.00	40.00 ± 1.78
12	20	20	5	11.92 ± 1.05	6.92 ± 0.01	30.00 ± 0.60
13	20	30	7	13.84 ± 0.91	6.55 ± 0.01	34.33 ± 1.50
14	20	20	9	18.29 ± 1.10	6.43 ± 0.00	30.67 ± 0.80
15	30	30	9	19.60 ± 1.87	6.61 ± 0.00	34.33 ± 1.67

* Mean ± standard error

According to ANOVA analysis of BBD (**Table 5)**, a highly significant linear model was obtained. The Model p-value of 0.0003 and F-value of 15.00 revealed that the model is significant at confidence level of 99.97% and noise could largely interfere by only 0.03%. The "Lack of Fit F-value" of 7.28 implies the “Lack of Fit” is not significant which affirms the good fitness of the model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The current ratio of 11.353 indicates an adequate signal. This model can be used to navigate the design space and the final equation describing the model was as follow:

Rapamycin conc. = +5.69596 - 0.095695 * Oat bran - 0.012527 * Semolina + 1.67041 * KH_2PO_4

The three-dimensional-surface-plot for the relation of rapamycin production with combination of two of studied factors was shown in **Fig. 5**.

Table 5: ANOVA analysis for linear model of BBD

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	96.74	3	32.25	15.00	0.0003	significant
A-Oat bran	7.33	1	7.33	3.41	0.0919	
B-Semolina	0.13	1	0.13	0.058	0.8135	
C-KH_2PO_4	89.29	1	89.29	41.54	< 0.0001	
Residual	23.64	11	2.15			
Lack of Fit	22.94	9	2.55	7.28	0.1265	not significant
Pure Error	0.70	2	0.35			
Model specification						
R-Squared 0.8036	Adjusted R-Squared 0.7500		Predicted R-Squared 0.5986		Adeq Precision 11.353	

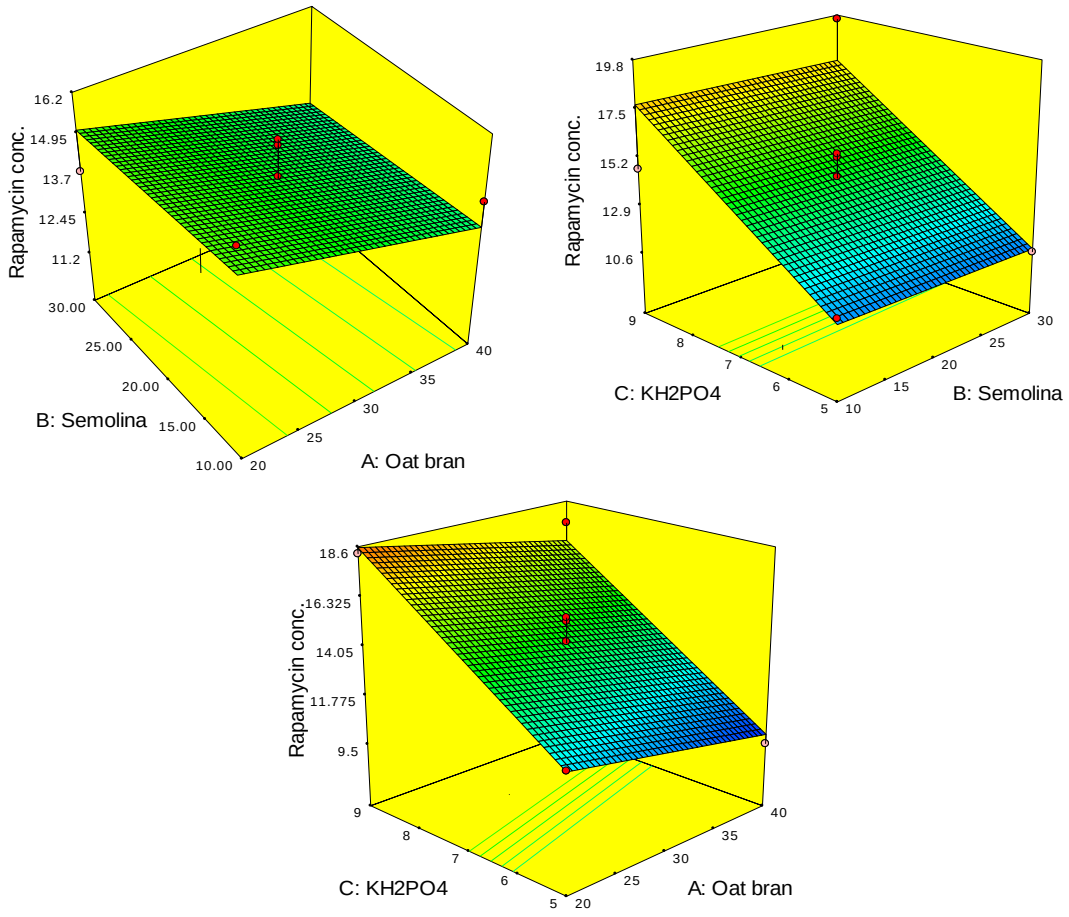

Fig. 5: The three-dimensional surface plot for rapamycin production in relative to the combination of two of the studied factors.

3.5. Prediction and validation of the optimum production medium.

After two successive significant designs, the optimum composition of production medium was predicted basing on BBD and response-surface-methodology (RSM), as shown in **Table 6**, the yield obtained practically (19.12 mg/l) was inside upper limit of 95% confidence interval (20.83 mg/l) which reflected the perfect predictability and reliability of BBD and RSM. The ability to achieve an impressive increase in the yield to 19.12 mg/l from a base of 11.2 mg/l in BM has verified more than 70% improve in productivity and realized the importance and the successful role of factorial design in medium optimization. Singh *et al*. [29] reviewed extensive studies that employed full or partial factorial design in optimizing production of several metabolites (included antibiotics, enzymes, polysacchardies and others) and recorded many fold increase in productivity ranged from 1.1 to 8.

Table 6: Optimum medium proposed basing on BBD-RSM

Medium components	**Amount**
A:Treated wheat bran (g/l)	10
B:Soy meal (g/l)	10
C:KH_2PO_4 (g/l)	9
D:Whey (ml/l)	10
E:Oat bran (g/l)	20
F:Semolina (g/l)	10
G:Glycerol (g/l)	0
H:D-mannose (g/l)	0
J:Fructose (g/l)	0
K:L-tyrosine (g/l)	0
L:L-lysine (g/l)	0
Predictions	
Rapamycin yield (mg/l)	18.6904
SE Mean	0.97
95% CI low	16.55
95% CI high	20.83
SE Pred	1.76
Practical verification	
Rapamycin yield (mg/l)	19.12 ± 1.2

Conclusion

Based on affordable natural substrates, a medium for the production of rapamycin has been successfully developed. Optimization of the medium was accomplished using a one-at-a-time strategy for testing variables and statistical designs of Plackett-Burman and Box-Behnken, which efficiently potentiated a more than 70% increase in drug yield. The newly developed medium highlighted, for the first time, the role of natural substrates such as oat bran and semolina in the production of rapamycin in submerged fermentations. It is thought that the obtained medium will lead to a significant decrease in the production cost of the drug and will aid in affording the drug in an economic price.

Conflict of interest

The authors declare that there is no conflict of interest.

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