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RESEARCH ARTICLE

OPTIMIZATION OF MEDIUM COMPONENTS FOR PHYTASE PRODUCTION ON ORANGE PEEL FLOUR BY *KLEBSIELLA* sp. DB3 USING RESPONSE SURFACE METHODOLOGY

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Abstract

Statistical experimental designs were applied for the optimization of phytase production by a bacteria *Klebsiella* sp. DB3 in orange peel flour. Four factors i.e., orange peel flour, ammonium dihydrogen phosphate, temperature and pH were optimized using central composite design (CCD). The optimum levels of variables that supported maximum enzyme activity were orange peel bran 2.0%, sucrose 2.0% and ammonium dihydrogen phosphate ($NH_4H_2PO_4$) 0.1% and pH 5.5. The validity of the model in optimized conditions was verified. An overall 5.25-fold enhancement in phytase activity (0.60 to 3.15 U) was attained due to the optimization.

Keywords: Phytase, *Klebsiella* sp., central composite design, response surface methodology

Introduction

Phytase hydrolyzes phytic acid to myo-inositol and phosphoric acid. Phytases are found in many plants, certain animal tissues and microorganisms. Phytases are of particular interest due to its hydrolyzing capacity for the reduction of phytate in feedstuffs. When phytase is added to feed, it increases the bioavailability of phosphate and decreases the phosphorus pollution caused by animal farming (Wodzinski and Ullah, 1996). Phytase also acts as antinutrients by complexing with proteins and inhibiting enzymes such as amylase, trypsin, acid-phosphatases and tyrosinase (Boling *et al.*, 2000). Supplementations with inorganic phosphorus, along with phytate phosphorus excretion, impose a global ecological problem (eutrophication).

In the last decade, phytate-degrading enzymes of bacteria such as phytase also have received increasing attention as they can be easily incorporated into feed diets and are rich in nutrients. Of the 120 bacteria strains isolated, 8 strains showed comparatively higher phytase activity (Mittal *et al.*, 2011). We found that *Klebsiella* sp. DB3, one of the bacteria, could produce more phytase than any other bacterial strains tested.

The **C**onventional method to analysis the effect of some factors (independent variables) upon responses is to investigate one factor at a time, while keeping the others constant. This approach

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for optimization of phytase parameters are extremely tedious, time-consuming, expensive and do not take into consideration the interactions among the factors (Vohra and Satyanarayana, 2002; Bogar *et al*. 2003; Stowe and Mayer, 1999). A statistical approach provides an efficient alternative, which is economical and allows the study of interactions among the factors, and at the same time predicts the optimum values of the variables. Response surface methodology (RSM) is efficient in handling large number of design parameters (Stanbury *et al*., 1997). RSM is the most widely used statistical technique for bioprocess optimization. Response surface experiments identify the response of a system as a function of explanatory variables. RSM is most often used to determine the optimum response for the specific range of variable conditions. The interaction among the possible influencing parameters can be evaluated with limited number of experiments. RSM in biotechnological processes is gaining immense importance for optimization of enzyme production (Vohra and Satyanarayana, 2002; Rao and Satyanarayana, 2003; Chadha *et al*., 2004). Several researchers have applied these conditions in biotechnology for optimization of culture conditions, determination of optimal values of process parameters and feeding rates (Kalil *et al*., 2000; Sunitha *et al*., 2000; Vohra and Satyanarayana, 2002). These techniques also have been successfully applied to optimization of medium components and cultivation conditions for phytase production by bacteria and other microorganisms (Vohra and Satyanarayana, 2002).

Orange peel is an organic fruit waste which is a completely waste and not used anywhere so we showed more interest in upgrading this waste for some value added products along with solving the disposal problems of waste. Phytate content in orange peel is 0.062-0.082 % dry weight. Although phytate content in orange peel is less but still our isolate utilizes this phytate very efficiently and giving higher production of phytase. Chemical composition as well as some trace elements, ascorbic acid, carotenoids dietary fiber, total polyphenols and their antiradical efficiency, using the 2,2-diphenyl-1-picrylhidracyl (DPPH) were assessed in the dried peels of orange (*Citrus sinensis*) (Rincon *et al*., 2005). Orange peel is also a good source of dietary fiber and phenolic compounds, whose use could be useful in the formulation of functional foods, taking advantage of the presence of dietary fiber and antioxidant compounds in only one ingredient. It is reported a yearly world production of 106 million tons of citrus fruits. The orange fruits represented the 63% of the world citrus production**.** In this investigation, an attempt was made to optimize the cultural conditions for maximizing the production of phytase from the bacteria. *Klebsiella* sp. DB3 in an inexpensive orange peel flour medium using statistical approaches. Further, a feasibility of the phytase production was attempted in shake-flasks level. In this study we reported the use of statistical methods for improving a bacterial phytase in orange peel bran medium.

Materials and methods

Bacterial strain

The bacterial strain used in the present study was isolated from poultry field soil and identified as *Klebsiella sp*. DB-3 FJ711774.1 by Xcelris Labs Ltd, Ahmedabad, India and has been given National Center for Biotechnology Information (NCBI) accession. It was cultivated on a Nutrient Agar (NA) medium (Hi media, India) and maintained at 4^{o}C by transfer on to a fresh medium after every 4-6 weeks. The morphological, physiological and biochemical characteristics of the isolated bacterial strain are already published (Mittal *et al*., 2011).

Cultivation medium and culture conditions

One loop of cells of the bacterial strain was transferred to 50 ml of nutrient broth medium prepared with distilled water in 250 ml flask and aerobically cultivated for 24 h. The cell culture (1.0 ml) was centrifuged at 10000 g and 4^{0}C, for 10 min and washed three times with sterile saline water. The washed cells were transferred to 50 ml of the production medium which contained 2.0% orange peel bran, 2.0% sucrose, 0.1% ammonium dihydrogen phosphate, pH 5.5 and grown by shaking at 120 rpm and 50^{0}C, for three days. Crude enzyme were harvested by centrifugation at 10,000 X g for 10 min at 4^{0}C and the clear supernatant

was used for measuring phytase activity. Phytase activity was determined by measuring the amount of liberated inorganic phosphate.

Phytase assay

Phytase activity was assayed in a reaction mixture containing 0.5% w/v sodium phytate (Sigma) prepared in 0.2 M sodium acetate buffer (pH 5.5) and suitably diluted enzyme (Gulati *et al.*, 2007). The reaction was stopped by adding an equal volume of 15% trichloroacetic acid after 30 min of incubation, at 50^0C. The liberated phosphate ions were quantified by mixing 100 µl of assay mixture with 900 µl of 1.0 M H_2SO_4- 10% ascorbic acid-2.5% ammonium molybdate (3:1:0.1) (Gulati *et al.*, 2007). After 20 min of incubation, at 50^0C, absorbance was measured at 820 nm. Control for the enzyme assay was run simultaneously that contained all the reagents but the reaction was terminated prior to the addition of heat inactivated enzyme. One enzyme unit was defined as the amount of enzyme liberating 1µmol of inorganic phosphate in 1min under the assay conditions.

Optimization of phytase biosynthesis

The optimization of medium components and cultural conditions for phytase production by the bacterial strain of *Klebsiella sp*. DB-3 FJ711774.1 was carried out in the following steps.

Optimization of components

Response surface methodology (RSM) was employed to optimize the four most significant factors, orange peel bran concentration (A), ammonium dihydrogen phosphate concentration (B), temperature (C) and pH (D), for enhancing phytase production. The three independent variables were studied at five different levels (-α, -1, 0, +1, +α). All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated are listed in Table 1 and a set of 30 experiments were carried out (Table 2). The statistical software package 'Design Expert 8.0.4' was used to analyze the experimental data.

Table 1. *Ranges of the three independent variables variation used in RSM*

Code	Independent variables	Levels				
		-α	-1	0	+1	+α
A	Orange peel bran (% w/v)	-1	1	2.5	5	7
B	Ammonium dihydrogen phosphate (% w/v)	-0.1	0.1	0.25	0.5	7
C	Temperature (^{0}C)	15	30	45	60	75
D	pH	2.75	4	5.25	6.5	7.75

Upon completion of experiments, the average maximum phytase biosynthesis yield were taken as the responses (Y). A multiple regression analysis of the data was carried out for obtaining an emphirical model that relates the response measured to the independent variables. A second order polynomial equation for a four factor system is:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{23} BC + \beta_{34} CD + \beta_{13} AC + \beta_{14} AD + \beta_{24} BD$$

Where Y is the predicted response, β_0 intercept, β_1, β_2 , β_3, β_4 linear coefficients, β_{11}, β_{22}, β_{33}, β_{44} squared coefficients, β_{12}, β_{23}, β_{34}, β_{13}, β_{14}, β_{24} interaction coefficients and A, B, C, A^2, B^2, C^2, D^2, AB, BC, CD, AC, AD, BD are levels of the independent variables. The response surface curves were obtained the 'Design Expert 8.0.4' software for determining the optimum levels of the variables for maximum production of phytase.

Validation of the experimental model

The statistical model was validated with respect to phytase production under the conditions predicted by the model in shake-flasks level containing 2.0% orange peel bran, 0.1% ammonium dihydrogen phosphate concentration, pH 5.5 and grown by shaking at 120 rpm and 50^0C, for three days. Samples were drawn at the desired intervals and phytase activity was determined as described above.

Table 2. *Experimental plan for optimization of phytase production using RSM*

Runs	A	B	C	D	Extracellular Phytase Activity (U/ml)	
					Observed	Predicted
1	1.00	0.50	60.00	4.50	1.22	1.01
2	-1.00	0.30	45.00	5.25	2.75	2.68
3	1.00	0.50	30.00	6.00	0.94	1.01
4	3.00	0.70	45.00	5.25	2.89	2.61
5	1.00	0.10	60.00	6.00	1.05	1.23
6	3.00	0.30	45.00	5.25	2.01	2.23
7	3.00	0.30	45.00	5.25	2.34	2.65
8	1.00	0.10	30.00	6.00	1.02	0.99
9	3.00	0.30	45.00	5.25	2.22	2.39
10	1.00	0.10	30.00	4.50	1.27	1.20
11	3.00	0.30	45.00	5.25	2.40	2.56
12	5.00	0.10	60.00	4.50	1.35	1.21
13	1.00	0.50	60.00	6.00	1.01	1.11
14	5.00	0.10	60.00	6.00	1.25	1.12
15	5.00	0.10	30.00	6.00	1.37	1.43
16	3.00	0.30	45.00	5.25	2.38	2.42
17	5.00	0.10	30.00	4.50	0.97	1.07
18	1.00	0.10	60.00	4.50	1.19	1.39
19	5.00	0.50	30.00	4.50	0.59	0.71
20	3.00	0.30	45.00	3.75	0.47	0.32
21	5.00	0.50	60.00	4.50	0.88	0.56
22	3.00	0.30	75.00	5.25	0.08	0.01
23	5.00	0.50	30.00	6.00	0.79	0.57
24	5.00	0.50	60.00	6.00	0.34	0.26
25	3.00	0.30	45.00	6.75	0.09	0.03
26	3.00	0.30	45.00	5.25	3.01	3.00
27	7.00	0.30	45.00	5.25	0.89	0.74
28	3.00	0.30	15.00	5.2	0	0.13
29	1.00	0.50	30.00	4.50	1.06	0.93
30	3.00	-0.10	45.00	5.25	2.69	2.46

Results and discussion

Optimization of the selected variables

Response surface methology (RSM) using CCD was applied to determine the optimum levels of the four selected variables that affected phytase production, and the mean predicted and observed responses are presented in Table 2.

The regression equations obtained after the analysis of variance (ANOVA) provided the levels of phytase produced as a function of the values of orange peel bran concentration, ammonium dihydrogen phosphate concentration, temperature and pH. The production of phytase could be predicted by the model:

$Y= -14.25510 + 0.028411A + 0.43102B + 0.15724C + 4.72294D – 0.011152A^2 + 1.19229B^2 – 1.48653E\text{-}003C^2 - 0.43845D^2 – 0.17396AB – 2.08917E\text{-}004AC + 0.010424AD – 2.67869E\text{-}003BC – 0.14684BD – 3.855469E\text{-}003CD$

Where Y is enzyme response (U/ml), A is orange peel bran concentration (%), B is ammonium dihydrogen phosphate concentration (%), C is temperature (^{0}C) and D is pH.

The coefficient of determination (R^2) was calculated to be 0.93 for phytase production. This implies that 93% of experimental data of the phytase production was compatible with the data predicted by the model (Table 3). The R^2- value is always between 0 and 1, and a value >0.75 indicates aptness of the model. For a good statistical model, R^2-value should be close to 1.0 and all the four factors should be positive and close to each other, as is the case here. However, Pred R^2 of 0.65 is not as close to the Adj R^2 of 0.87, probably indicating a large block effect (Table 3). The 'adequate precision' measures signal to noise ratio and a value >4 is desirable. The 'adequate precision' value of 15.399 for phytase production indicates that the model can be used to navigate the design space.

Table 3. *Test of significance for regression coefficient*

Source	Sum of Squares	Df	Mean Square	F- Value	P-value	
Model	5.06	14	0.36	14.90	< 0.0001	
A	*0.20*	*1*	*0.20*	*8.15*	*0.0121*	
B	*0.068*	*1*	*0.068*	*2.82*	*0.1138*	
C	*0.017*	*1*	*0.017*	*0.71*	*0.4121*	
D	*0.061*	*1*	*0.061*	*2.50*	*0.1346*	
AB	*0.077*	*1*	*0.077*	*3.19*	*0.0941*	
AC	*6.285E-004*	*1*	*6.285E-004*	*0.026*	*0.8743*	
AD	*3.912E-003*	*1*	*3.912E-003*	*0.16*	*0.6936*	
BC	*1.033E-003*	*1*	*1.033E-003*	*0.043*	*0.8392*	
BD	*7.762E-003*	*1*	*7.762E-003*	*0.32*	*0.5799*	
CD	*0.030*	*1*	*0.030*	*1.24*	*0.2829*	
A^2	*0.055*	*1*	*0.055*	*2.25*	*0.1543*	
B^2	*0.062*	*1*	*0.062*	*2.57*	*0.1296*	
C^2	*3.07*	*1*	*3.07*	*126.53*	*< 0.0001*	
D^2	*1.67*	*1*	*1.67*	*68.79*	*< 0.0001*	
Residual	0.36	15	0.024			
Lack of Fit	0.31	10	0.031	2.77	0.1358	not significant
Pure Error	0.056	5	0.011			
Cor Total	5.42	29				

Values of "Prob > F" less than 0.0500 indicate model terms are significant while values greater than 0.1000 indicate the model terms are not significant. Therefore, among the model terms in this study, orange peel bran (A), temperature (C2), pH (D2)are significant model terms. Table 4 also indicate that the interaction between A and A, B and B, C and C, D and D, A and B, A and C, A and D, B and C, B and D, C and D, had very significant influence on phytase yield by the bacterial strain used in this study. The Model F-value of 14.90 implies the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise.

The "Lack of Fit F-value" of 2.77 implies the Lack of Fit is not significant relative to the pure error. There is a 13.58% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good and this makes the model to fit (Table 4).

The response curves were plotted to understand the interaction of the variables and to determine the optimum level of each variable for maximum response (Figs. 1, 2 and 3). The model predicted maximum phytase production (3.01 U/ml) in the medium containing 2% orange peel bran, 0.1% $NH_4H_2PO_4$, temperature 50°C, pH 5.5.

Table 4. *Analysis of variance (ANOVA) for regression*

Std. Dev.	0.16	R-Squared	0.9329
Mean	1.08	Adj R-Squared	0.8703
C.V. %	14.40	Pred R-Squared	0.6578
Press	1.86	Adeq Precision	15.339

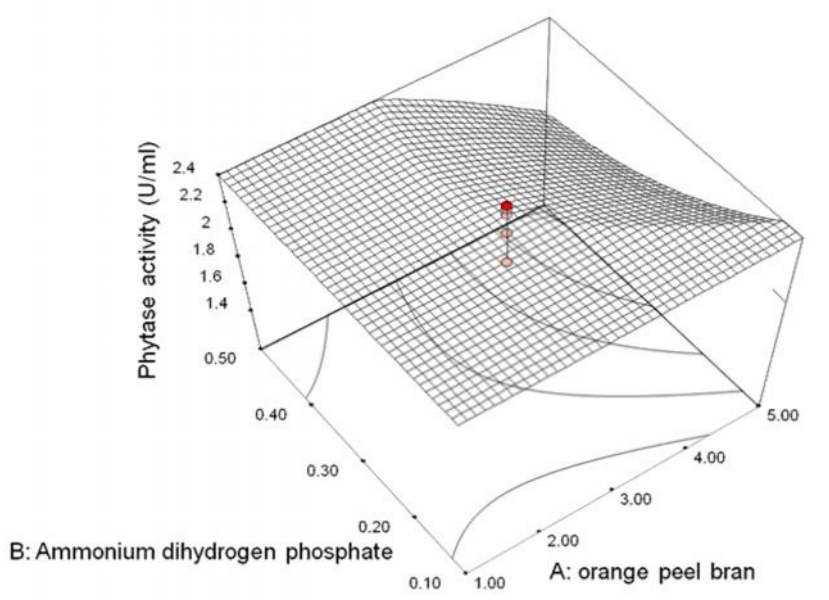

Figure 1. *Response surface plot of extracellular phytase production as a function of orange peel bran and ammonium dihydrogen phosphate concentration (temperature was set at 50^{0}C and pH was set at 5.5)*

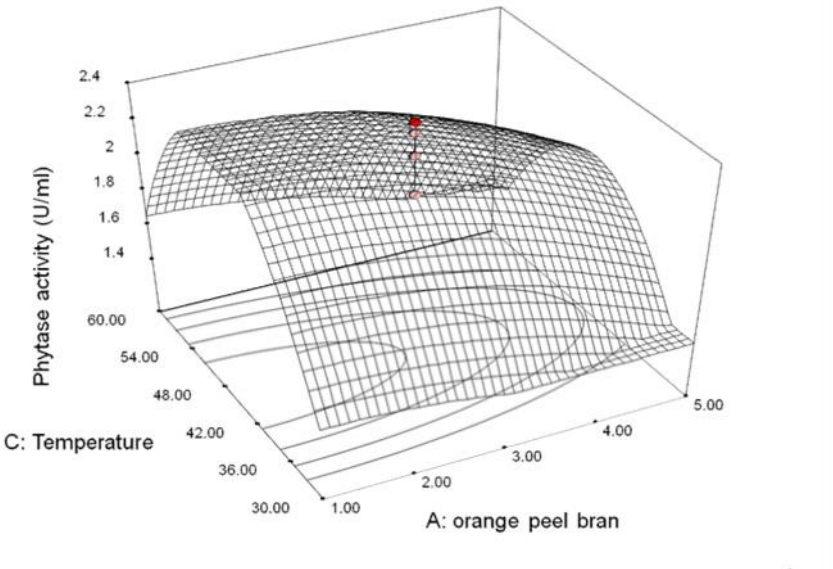

Figure 2. *Response surface plot of extracellular phytase production as a function of orange peel bran concentration and temperature (ammonium dihydrogen phosphate concentration was set at 0.1% and pH 5.5)*

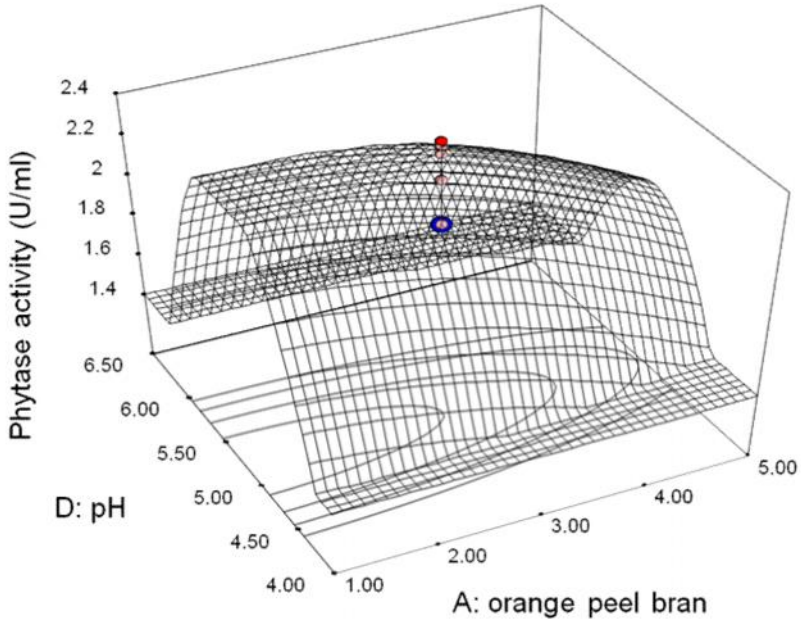

Figure 3. *Response surface plot of extracellular phytase production as a function of orange peel bran concentration and pH (ammonium dihydrogen phosphate concentration was set at 0.1% and temperature 50^{0}C)*

Validation of the experimental model

All the data have shown that the optimal medium for phytase production contained orange peel bran 2.0%, sucrose 2.0% (Mittal *et al.,* 2011) and ammonium dihydrogen phosphate 0.1% while the optimal cultivation conditions for phytase production were pH 5.5, a temperature of 50^{0}C and a shaking speed of 120 rpm. We also find that 1.0% (v/v) of 24 h old culture ($OD_{600\ nm}$ = 7.5) was the best inoculation size to produce the maximum phytase activity (data not shown). Therefore, time course of phytase production during the fermentation was checked under the conditions mentioned above. Our results indicate that 3.15 U/ml of phytase activity could be reached within 72 h of the fermentation (data not shown), which was almost equal to the actual predicted value (3.01 U/ml). These results demonstrate that the bacterial strain could produce high yield of extracellular phytase in the simple medium and this may have wide uses in phytase production.

Although it was reported that *K. ohmeri* BG3 strain could secrete over 575.5 U/ml in oat medium but it is not a waste product as orange peel flour (Li *et al.*, 2008). *A. adeninivorans* CBS 7377 and *A. adeninivorans* CBS 8335 strains also secrete over 1427 U/ml and 1921 U/ml of phytase respectively in the phosphate –depleted yeast extract medium (Sano *et al.,* 1999) but the medium and production process for phytase were not feasible in industries. *Klebsiella oxytoca* MO-3, intracellular phytase producer, reported to have phytase activity of approximately 40 munits/mgprotein (Jareonkitmongkol *et al.*, 1997). Till now all reports of *Klebsiella* spp. are of intracellular enzyme (Greiner *et al*., 1997; Wang *et al.,* 2004). We are reported first time the extracellular production of phytase from *Klebsiella* sp. Extracellular enzyme production has much advantage than intracellular enzyme production. Extracellular enzyme is already outside the cell so there is no need to broke the cell and to separate the enzyme from the mixture of all the cellular contents as is the case with intracellular enzyme. Moreover, extracellular enzyme is more robust so less likely to be broken down by heat of chemicals.

The optimum values of the critical components determined by central composite design of response surface methodology by *C. utilis* in submerged cultivation system for maximum phytase production were potassium dihydrogen phosphate 5g/L, ammonium sulfate 5g/L, calcium chloride 0.13 g/L, magnesium sulfate 0.5 g/L and

yeast extract 1.17g/L (Li *et al*., 2009). Highest enzyme production (20,925.6 U/g DMR) (Device Master Record) was attained by central composite design of RSM by mould *Sporotrichum thermophile* in SSF (Singh and Satyanaryana, 2008). After the optimization of the medium, we found that the bacterial strain could produce high yield of extracellular phytase (3.15 U/ml) in the simple medium which contained orange peel bran 2.0%, ammonium dihydrogen phosphate 0.1%, pH 5.5 and temperature of 50^0C.

Phytate content of orange peel bran was found to be 0.062-0.082 % dry weight of substrate (Gulati *et al*., 2007). Orange peel is an organic fruit waste which is a completely waste and not used anywhere so we showed more interest in upgrading this waste for some value added products along with solving the disposal problems of waste. Orange peel is also a good source of dietary fiber and phenolic compounds, whose use could be useful in the formulation of functional foods, taking advantage of the presence of dietary fiber and antioxidant compounds in only one ingredient. It is reported a yearly world production of 106 million tons of citrus fruits. So, we analyze that the production in orange peel medium will be highly cost-effective. Sucrose was also used as main carbon source for phytase production by *Aspergillus niger* and *Cryptococcus laurentii* during fermentation (Thyagarajan and Namasivayam, 2010; Gunashree and Venkateswaran, 2008; Pavlova *et al.* 2008). Hassouni *et al.* (2006) found that *Myceliophthora thermophila* showed the highest phytase activity when grown on a synthetic culture medium in which diammonium hydrogen phosphate $(NH_4)_2HPO_4$ was the sole carbon source. In our study, *Klebsiella sp*. DB-3 FJ711774.1 secreted high level of phytase in the medium with pH 5.5 and temperature of 50^0C. This feature is useful in animal feed industry for feed pelleting process.

Conclusions

RSM method have been proved to be effective in optimizing phytase production by the terrestrial bacterial strain of *Klebsiella* sp. DB-3 FJ711774.1 isolated from poultry field soil in submerged fermentation, which resulted in an overall 3.5-fold enhancement in phytase production. This is the first report of *Klebsiella* sp. which produce extracellular enzyme. Optimized conditions by RSM method enhanced enzyme production many fold. Till now all reports of *Klebsiella* spp. are of intracellular enzyme (Greiner et al., 1997; Wang et al., 2004). So, by biotechnological condition optimization was possible to increase of extracellular enzyme yield at *Klebsiella* sp. DB-3 FJ711774. Sodium phytate could be substituted with orange peel bran that resulted in sustained secretion of phytase, thus making the fermentation process cost –effective and more economical. Such a terrestrial bacterial strain may have highly potential application in animal feed industry for improving the nutritional status of feed and in combating environmental pollution.

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