International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN : 0974-4290
Vol.6, No.7, pp 3478-3486, Sept-Oct 2014

Optimum production of γ-decalactone by *Sporidiobolus salmonicolor* using Response Surface Methodology

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Abstract: Lactones come under aroma compounds, extensively used in flavor industry. The present paper comprises of an old and regenerative method for the production of γ–decalactone by using *Sporidiobolus salmonicolor,* which is influenced by incubation time, inoculum level, pH, salt solution concentration, carbon sources, and nitrogen sources. The γ–decalactone production was obtained at optimum incubation time and pH of 72 hrs and 6.5 repspectively for a maximum production of γ–decalactone. γ–decalactone and biomass were estimated by using NMR spectra and spectrophotometer method respectively. Residual concentration estimated by using DNS method. STATISTICA 6.0 is used to optimize the medium components.
Keywords: Optimization, γ–decalactone, *Sporidiobolus salmonicolor*, RSM

Introduction

Lactones are aroma compounds extensively used in the flavoring industry [1]. They have been isolated from a wide variety of foods and have been observed to be associated with aromas described as fruity, coconut like, buttery, sweet or nutlike [2]. Production of Lactones from oil derivatives and yeast has been extensively done at an industrial level. Generally, the patents and papers on this subject describe the bioconversion of a hydroxyl fatty acid and the position of the hydroxyl group determines which particular Lactones ([gamma] or [delta]) will be produced. Sporidiobolus spp. is capable of producing [gamma]-decalactone, an important flavor compound responsible for the flavor. [gamma]-decalactone is of interest owing to its outstanding detection threshold in water (0.088 ppm). Its concentration is very low, mostly less than 1 ppm in the finished products [3]. γ–decalactone is an essential ingredient for the human life to cover in many aspects including food processing and natural fragrances. However, the large amounts of production were due industrially. Microbial fermentation is regarded as a potential means to produce natural flavor substances and has attracted a great deal of research interest [4, 5]. Till Now various flavoring compounds such as acids, alcohols, esters, lactones, pyrazines and terpenes have been reported for the production by many organisms including fungi, yeasts and bacteria [6-9]. Most industrial processes use the bioconversion of ricinoleic acid by yeasts to produce γ-decalactone [10,11]. It has been since 1930 that γ–decalactone ($C_{10}H_{18}O_2$), which is an aroma compound present naturally in many fruits and fermented products, is produced by members of the genus Sporiodiobolus and this fact was confirmed by the identification of 4-decanolide and cis-6-dodecanolide in 1972 and 1973 on

the peach like odour of sporobolomyces odorus [12]. In this study we reported the influence of incubation time, pH, salt sources, carbon sources and nitrogen sources on the production of γ-decalactone by *S.salmonicolor* and also optimization of process variables for the production of γ-decalactone by *S.salmonicolor* by using RSM. In this study we reported the growth behavior of and γ-decalactone production by Sporidiobolus salmonicolor. The experiments were carried out in shake flask and the results were were used in fermentor studies.

Materials and Methods

Microorganism

Sporidiobolus salmonicolor MTCC 485 obtained from the Microbial Type Collection Centre, Chandigarh, India, was used throughout the study. It was rejuvenated by culturing with YM agar media slants and stored at refrigeration temperature $4^{O}C$ and Sub culturing was done once in 15 days.

Inoculum preparation

Medium components were sterilized in an autoclave at 121^{o}C for 15 min. A full loop of 24 h slant culture (3% v/v) was transferred aseptically to a 250 mL Erlenmeyer flask containing seed medium (100 mL) with the following composition (g/L): Glucose: 15; Peptone: 0.5; yeast extract: 1.0; malt extract: 1.0; KH_2PO_4: 2.0; $CaCl_2.2H_2O$: 0.1; $FeSO_4.7H_2O$: 0.01; $MgSO_4.7H_2O$: 3.0. The flasks were incubated in an orbital shaker at 180 rpm and 30^{o}C for 48 h and pH was adjusted to 7.0 with addition of 2 N NaOH.

Fermentation Experiments

The experiments were conducted in shake flask. The strain Sporidiobolus salmonicolor was used for the fermentation of γ-decalactone. The culture was inoculated in to the fermentation medium and kept for incubation under aerobic condition. Readings were taken at periodic time intervals and the samples were tested for γ-decalactone production and bio mass growth.

Analytical techniques

Biomass estimation

Optical densities of collected samples are measured using spectrophotometer at 600 nm with blanks of appropriate growth medium. Curves relating OD to dry weight are constructed by harvesting culture at room temperature, washing with distilled water, and resuspending the cells in distilled water to about 10mg of dry weight per ml.

γ-decalactone and Glucose estimation

γ-decalactone determination was estimated by NMR spectra method. Two ml of the culture was removed and centrifuged for 10 min. The supernatant were separated and acidified to pH 6.5. Then it is extracted with 2ml diethyl-ether in 5ml glass vials by shaking for 2 min. Then samples were analyzed in a liquid state using NMR spectra and were recorded at room temperature. Residual glucose concentrations were estimated by DNS method.

Results And Discussion

Effect of Incubation time on γ-decalactone production

The incubation time is an important factor for the production of extracellular γ-decalactone production by the microorganisms. In this study with *Sporidiobolus salmonicolor* MTCC 485, γ-decalactone concentration increased from 24 h onwards and reached maximum production (34.82 mg/l) after 72 h of incubation (Fig. 1). At longer incubation periods, the γ-decalactone concentration decreased which might be due to the depletion of nutrients, accumulation of toxic end products, and the change in pH of the medium, or loss of moisture.

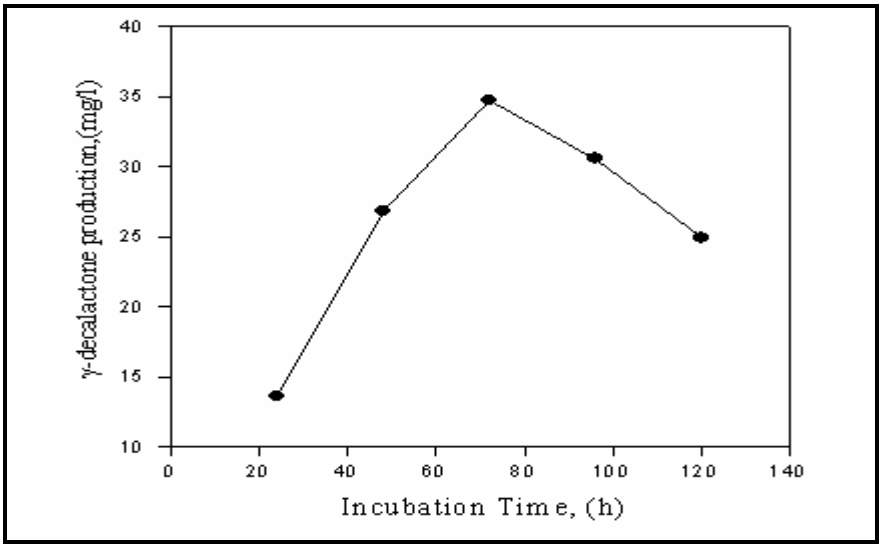

Fig. 1 Effect of Incubation time on γ-decalactone production

Effect of inoculum level on γ-decalactone production

Different levels of the inoculum were tried to study their effect on γ-decalactone concentration (Fig. 2) so as to find an optimum inoculum level in the fermentation process. A lower inoculum may give insufficient biomass causing reduced product formation, whereas a higher inoculum may produce too much biomass leading to the poor product formation. In our study, the maximum γ-decalactone concentration (34.56 mg/l) was obtained with 2 ml (20 % v/v) inoculum level.

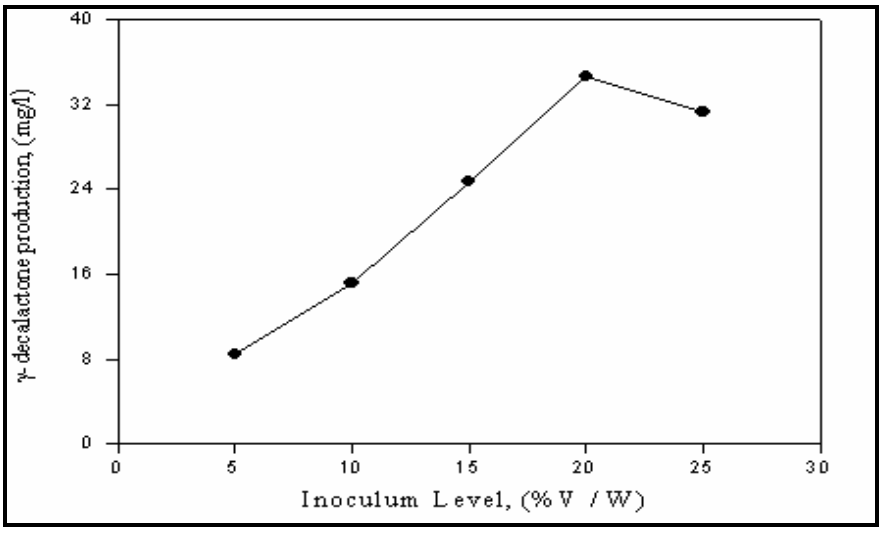

Fig. 2 Effect of inoculum concentration on γ-decalactone production

Effect of pH on γ-decalactone production

Different pH were tried to study their effect on γ-decalactone concentration (Fig. 3) so as to find an optimum pH in the fermentation process. In our study, the maximum γ-decalactone concentration (54.93 mg/l) was obtained with pH 6.5. A lower production of cell mass and γ-decalactone were obtained in acidic and alkaline pH values compared to neutral pH may be due to a lower metabolic activity of the organisms.

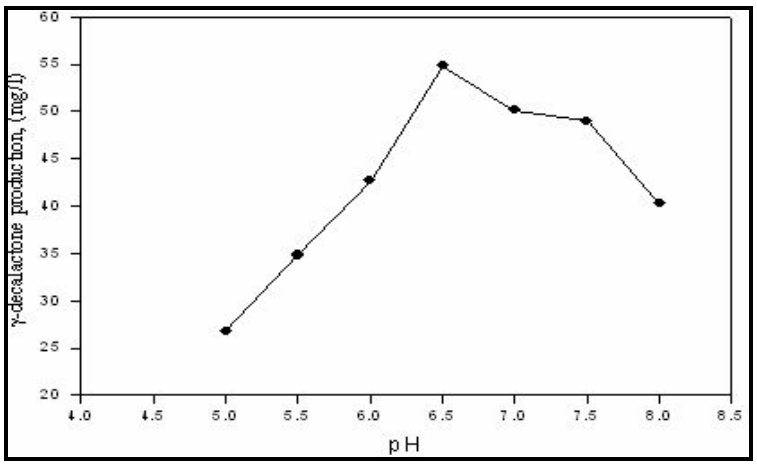

Fig. 3 Effect of pH on γ-decalactone production

Effect of salt solution concentration on γ-decalactone production

The influence of salt solution concentration on γ-decalactone concentration by Sporidiobolus salmonicolor MTCC 485 was presented in Fig 4. Salt solution concentration of 15 %v/v had shown maximum γ-decalactone production.

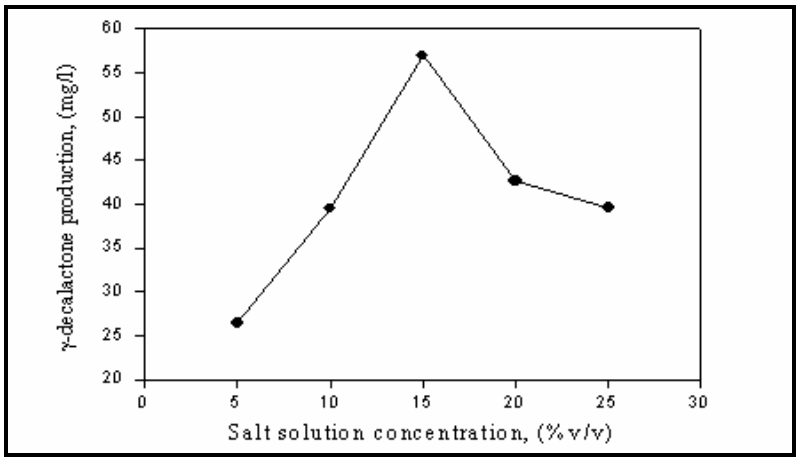

Fig.4 Effect of salt solution concentration on γ-decalactone production

Effect of carbon sources on γ-decalactone production

To study the effect of various carbon sources on γ-decalactone production, different carbon sources at 6 % (w/v) level were tried. The results are presented in Fig. 5. Carbon to the medium increased γ-decalactone production (Glucose - 59.50 mg/l) with some carbon sources like glucose while other carbon sources showed decreased γ-decalactone production probably due to catabolite repression.

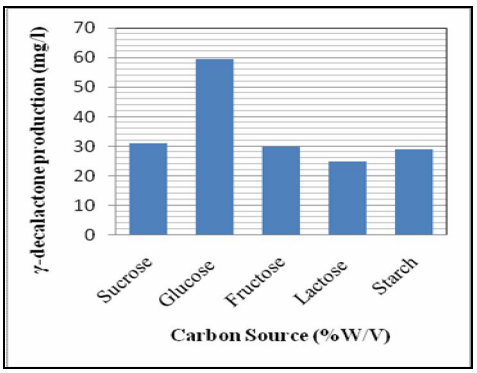

Fig.5. Effect of carbon sources on γ-decalactone production

Optimization of selected medium constituents using CCD

RSM is a sequential procedure with an initial objective of leading the experimenter rapidly and efficiently to the general vicinity of the optimum. Since the location of the optimum is unknown prior to running RSM experiments, a design that provides equal precision of estimation in all directions is employed. The base points for the design were selected from a single parameter study. The three variables which influence highly the fermentative production of γ-decalactone were glucose, peptone, and salt solution concentration.

Table.1 Independent variables in the experimental plan

Variables	Coded levels				
	–1.682	–1	0	+1	+1.682
Glucose (%w/v), X_1	**2.636**	**4**	**6**	**8**	**9.364**
Peptone (%w/v), X_2	**0.659**	**1.0**	**1.5**	**2.0**	**2.341**
Salt solution concentration (%v/v), X_3	**6.59**	**10**	**15**	**20**	**23.41**

A CCD was employed to analyze the interactive effect of these parameters and to arrive at an optimum. A 2^3-factorial central composite experimental design, with six axial points, and six replications at the center points leading to a total number of 20 experiments (Table 1 and 2) was employed for the optimization of the parameters.

Table. 2 CCD matrix having real values along with the experimental and predicted values of γ-decalactone production

Run No.	X_1	X_2	X_3	γ-decalactone production (mg/l)	
				Experimental	Predicted
1	-1	-1	-1	25.72	31.99934
2	-1	-1	+1	40.89	40.76667
3	-1	+1	-1	52.68	50.82820
4	-1	+1	+1	61.23	63.41054
5	+1	-1	-1	55.19	56.81179
6	+1	-1	+1	33.52	39.17413
7	+1	+1	-1	32.31	36.23566
8	+1	+1	+1	24.89	22.41300
9	-1.682	0	0	46.07	44.04783
10	1.682	0	0	33.79	30.43618
11	0	-1.682	0	52.17	46.01752
12	0	1.682	0	46.98	47.75649
13	0	0	-1.682	54.58	50.48277
14	0	0	1.682	47.51	46.23124
15	0	0	0	68.12	68.28644
16	0	0	0	68.13	68.28644
17	0	0	0	68.12	68.28644
18	0	0	0	68.10	68.28644
19	0	0	0	68.18	68.28644
20	0	0	0	68.15	68.28644

The calculated regression equation for the optimization of medium constituents showed that γ-decalactone production (Y) is a function of the concentration of glucose (X_1), peptone (X_2), and salt solution concentration (X_3). By using multiple regression analysis (STATISTICA 6.0) the coefficients of equation (1) was estimated, and gave the following equation.

$$Y = -287.056 + 55.575X_1 + 145.187X_2 + 11.589X_3 - 2.743X_1^2 - 30.256X_2^2 - 0.282X_3^2 - 9.851X_1X_2 + 0.382X_2X_3 - 0.66X_1X_3 \quad \ldots\ldots\ldots\ldots (1)$$

The predicted γ-decalactone concentration resulted from equation (1) are in close agreement with the experimental values as evident from last column of Table. 2, and hence the above equation was deemed to be

adequate in representing the submerged fermentation of γ-decalactone production under the specified range of experiments.

The significance of each coefficient in equation (1) was determined by student's t-test and p-values which were also listed in Table 3. The larger the magnitude of the t-value and smaller the p-value, the more significant is the corresponding coefficient. This implies that the linear and quadratic effects of glucose, peptone, and salt solution concentration were highly significant as is evident from their respective p-values. This indicates that they can act as limiting nutrients and small variations in their concentration will alter either growth rate or product formation rate or both to a considerable extent. The interaction effect of peptone and salt solution concentration was found to be insignificant (p>0.05).

Table. 3 Model coefficients estimated by multiple linear regression (significance of regression coefficients)

Term , Coefficient	**Value**	***t*-value**	***p*-Value**
Constant, b_0	**–287.056**	**–9.70618**	**0.000002**
Glucose, b_1	**55.575**	**12.06281**	**0.000000**
Peptone, b_2	**145.187**	**7.87834**	**0.000013**
Salt solution concentration, b_3	**11.589**	**6.28860**	**0.000090**
Glucose ×glucose, b_1^2	**–2.743**	**–9.92750**	**0.000002**
Peptone ×peptone, b_2^2	**–30.256**	**–6.84319**	**0.000045**
Salt solution concentration ×salt solution concentration, b_3^2	**–0.282**	**–6.37311**	**0.000081**
Glucose ×peptone, b_{12}	**–9.851**	**–6.63916**	**0.000058**
Peptone ×salt solution concentration, b_{23}	**0.382**	**0.64277**	**0.534832***
Salt solution concentration × glucose, b_{31}	**–0.660**	**–4.44885**	**0.001237**

The results of the second order response surface model fitting in the form of Analysis of Variance (ANOVA) were given in Table 4. The ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test (F_{model} = 26.18214) and a very low probability value (P_{model} > F=0.000008).

Table. 4 ANOVA for the entire quadratic model

Source of variation	**Sum of squares (SS)**	**Degree of freedom (d.f.)**	**Mean squares (MS)**	***F*-value**	**Probe>*F***
Model	4150.437	9	461.1597	26.18214	0.000008
Error	176.135	10	17.6135		
Total	4326.572	19			
R= 0.9794; R^2= 0.95928; Adjusted R^2= 0.92265					
$P_{model} > F$ = 0.000008					

The goodness of the fit of the model was checked by the determination coefficient (R^2). The closer the R^2 value to 1, the stronger the model is and the better it predicts the response. In this case, the value of the determination coefficient (R^2 = 0.9592) indicates that 95.92% of the variability in the response could be explained by the model. Also a higher value of the correlation coefficient (R^2=0.9794) justifies an excellent correlation between the independent process variables.

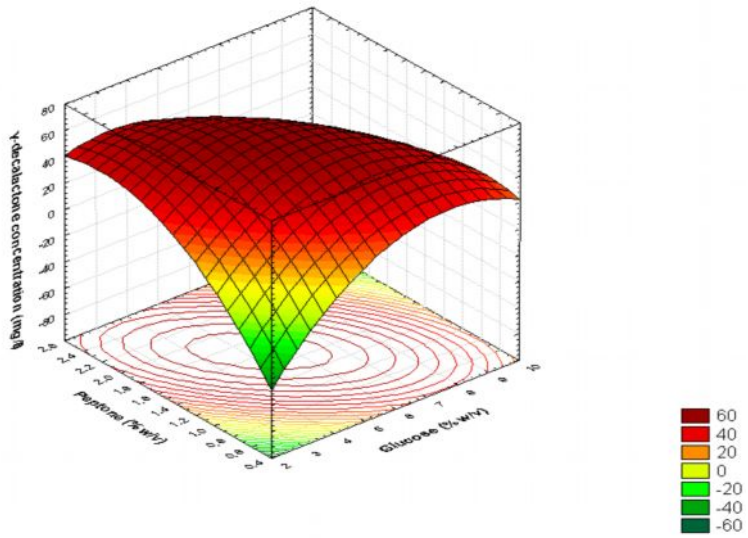

Fig. 6. Response surface and contour plot of glucose vs. peptone on γ-decalactone production (salt solution concentration was kept constant at 15 (%v/v)).

Figs. 6–8 represent the surface contour plots for the optimization of medium constituents of γ-decalactone production. The optimum values of the medium constituents for maximum γ-decalactone concentration can be attained at 5.3652 (% w/v) of glucose, 1.6228 (% w/v) of peptone, and 15.3781 (% v/v) of salt solution concentration. At these optimum medium constituents, maximum γ-decalactone concentration of 68.9442 mg/l was obtained.

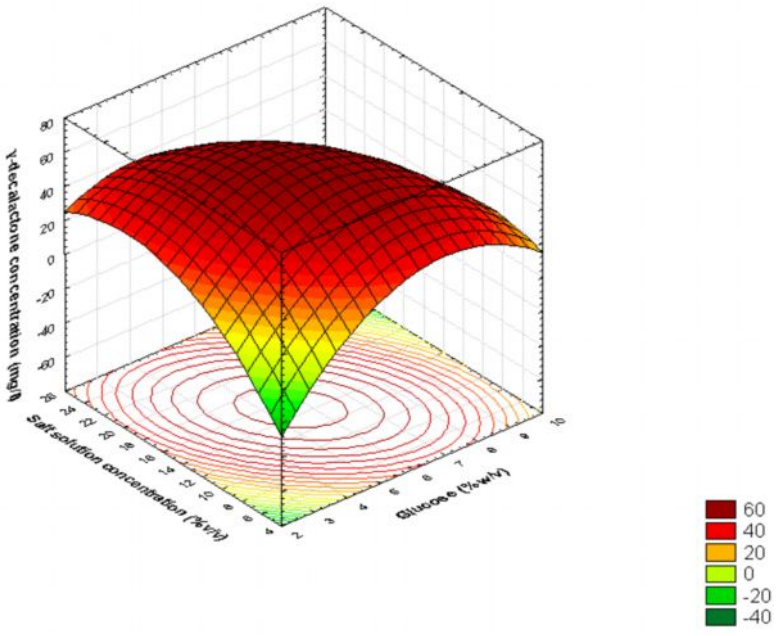

Fig. 7. Response surface and contour plot of glucose vs. salt solution concentration on γ-decalactone production (peptone was kept constant at 1.5 (%w/v)).

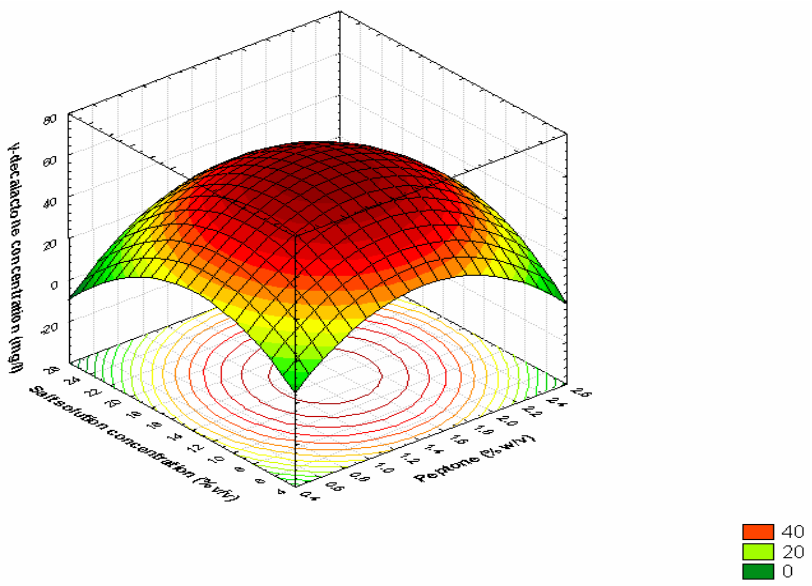

Fig. 8. Response surface and contour plot of peptone vs. salt solution concentration on γ-decalactone production (glucose was kept constant at 6 (%w/v)).

Conclusions

The present study enabled the RSM with CCD to find the importance of factors at different levels. The optimum values of medium constituents found are: incubation time of 72 hrs (34.82 mg/l), inoculums level of 20 % v/v (34.56 mg/l), pH of 6.5 (54.83 mg/l), Salt solution concentration of 15 %v/v (56.93 mg/l), carbon sources of 6%w/w (glucose – 59.50 mg/l) and the γ–decalactone production obtained with the above optimum values are 408.8844 µg/ml. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for γ–decalactone production. The results of this study have clearly indicated RSM is an effective method for maximum production of γ–decalactone using *Sporidiobolus salmonicolor* MTCC 485.

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