Optimization of Lipid Production from Paddy Straw Using Dilute Acid Pretreatment and Fermentation by Rhodosporidium Toruloides

Dr.S. Sumathi

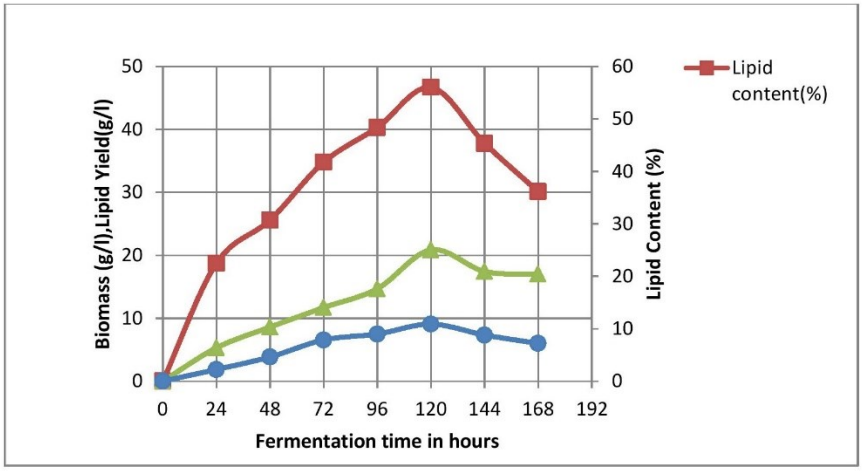
Assistant Professor, Department of Civil Engineering, Government college of Engineering, Thanjavur-613402, Tamil Nadu, INDIA

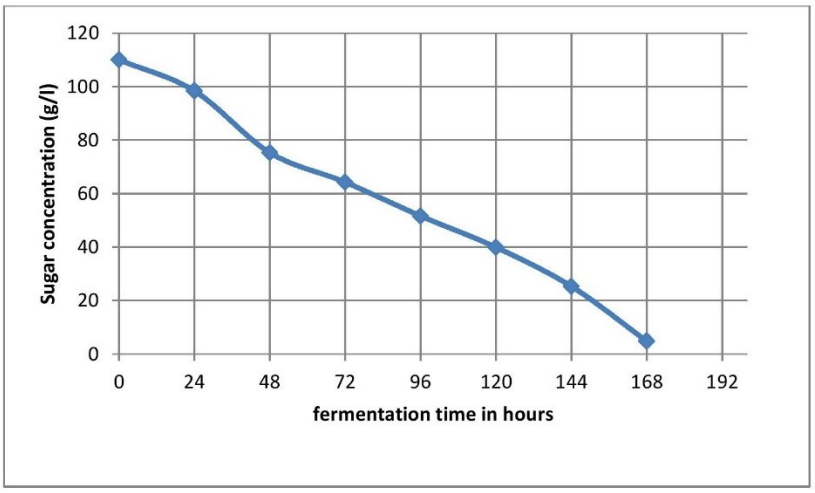
sumathigcetnj@gmail.com

Dr.C Meiaraj

Professor, Department of Civil Engineering, Government college of Engineering, Bodinayakanur, Theni-625582, Tamil Nadu, INDIA. mejarajc@gmail.com

GRAPHICAL ABSTRACT





Abstract

Among the lignocellulose wastes Paddy Straw is the most abundant one which have the ability of being used as a feedstock for production of biofuel. In this work, paddy straw is subjected to dilute acid pretreatment, detoxification and fermentation processes for the production of lipids. Rhodosporidium Toruloides, an oleaginous yeast strain is used for fermentation and produce biomass and lipids. Straw of paddy is pre-processed with 2% (v/v) the sulfuric acid at 121° C for an hour that is 60 minutes and then detoxified. Fermentation process has to be optimized in order to achieve higher lipid yields and higher cellulose content. In this study, optimization is carried out by using response surface process and Box Behnken Design (BBD) for learning impact of concentration of inoculum, pH and temperature on fermentation. During hydrolysis and fermentation processes, sugar concentration is analysed using dinitro salicylic acid reagent and standard curve of glucose. Observations show that highest yield of sugar from the dilute acid pre-treatment is 3.3 g/L. Optimum conditions for lipid yield from fermentation process is 10.88 g/L at a pH of 6, at a temperature of 25°C and at 10% inoculum loading.

Keywords: pre-treatment, detoxification, fermentation, hydrolysis, paddy straw

1. Introduction

Lignocellulosic biomass contains cellulose, hemicelluloses and lignin. It is widely known as a viable source of sugars and useful for biofuel production and value-added biomaterials [1-2]. As a reliable source of production of biofuel, rice straw has earned considerable scientific attention. Rice Straw (RS) is considered the most suitable candidate for the production of biofuels as it is renewable and abundantly available throughout the world [2-4]. Production of rice results in 650 to 975 million tons of rice straw annually. While most of the rice straw is used to feed the cattle and the remaining is allowed to go waste [5]. In order to make a better use of the valuable rice straw, many researchers use it to produce biofuels like ethanol, methane and biodiesel [6]. When compared to other carbon sources that use oleaginous microorganisms for lipid accumulation, rice straw shows great promise due to its easy availability and low-cost [7-8]. Oleaginous organisms like fungi, bacteria, microalgae and yeast have been used as fermenting agents in biodiesel production. Research shows that accumulation and composition of lipids and fatty acids is directly influenced by the micro organic strain, substrate and culture environment. Rhodosporidium toruloides, anoleaginous yeast is a red basidiomycete derived from the wood

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pulp of coniferous trees. It synthesizes fatty acids and plays a vital part in designing lipid production pathways. It is used widely in the production of lipids where agro waste is used as a substrate.

This study proposes the use of paddy straw as an alternative to carbon energy sources. The concentration of sugar is measured to optimize acid pretreatment process and various detoxification processes are used to remove inhibitors from paddy straw hydrolysate. Rhodosporidium toruloides is used to produce biomass, lipid content and lipid yield through fermentation. Response surface methodology with BBD is used to optimize fermentation parameters and production of lipid.

2. Materials and Methods

2.1.Paddy Straw Hydrolysate (PSH)

Paddy straw is collected from local farmland, air-dried, milled and screened. Particles with sizes lesser than 0.75 mm are selected and stored at a temperatire of 4° C until they are used. Pretreated paddy straw is mixed with 2% (v/v) H_2SO_4 with a rate of 10% (w/v) of the solid loading. The amalgamation is treated under an autoclave at 121°C and 15 psi for 60 minutes. After thr process of hydrolysis, liquid fraction (PSH) is filtered and its pH is then adjusted as 5.5 using 10M NaOH. The filtrate is analysed for sugar concentrations using DNS method.

2.2.Detoxification of Paddy Straw Hydrolysate (DPSH)

Detoxification of paddy straw involves over-liming and adsorption processes. During detoxification, liquid hydrolysate is heated up to 70°C for 40 minutes and $Ca(OH)_2$ is added to raise its pH to 10.5 and resulting mix is heated to 50°C for half an hour. During the adsorption process, Activated Charcoal (AC) is mixed with the hydrolysate in the ratio of 3% (w/v) and resulting combination is incubated at an temperature of 30°C and shaken at 150 rpm for an hour in an incubator shaker. The final AC detoxified liquid is obtained by adjusting the pH to 5.5 and carrying out the filtering process.

2.3.Microorganism, Media Preparation, Pre-cultivation and Cultivation

Rhodosporidium toruloides (MTCC 9565) is acquired from MTCC, CSIR Institute of Microbial Technology, Chandigarh, India. The obtained sample is revived in distilled water. The

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cells are maintained in a medium of yeast agar at 4°C. Pre-culture is done on precultivation containing (glucose) C6H12O6 (10.0 g/L), peptone (5.0 g/L), extract of yeast (5.0 g/L) and malt extract (3.0 g/L) at 25°C and shaken at 150 rpm for 24 hours. Then, the pre-culture is injected into the medium of culture in ratio of 1:10 (v/v).

Batch fermentation experiments are done in 500 ml of flasks that has 250 ml of nitrogen limiting liquid medium to study the impact of inhibitors on the growth of yeast and also the accumulation of lipid. Further hydrolysate, the medium of culture also contains 0.1 g/L $(NH_4)_2SO_4$, 0.75 g/L extract of yeast, 0.4 g/L KH_2PO_4 and 1.5 g/L $MgSO_4.7H_2O$. The value of pH of medium of liquid is then adjusted to 5.5, 6.0 and 6.5 to suit needs of various trials. Then, 5ml, 10ml and 15ml of inoculum are put to medium and culture is maintained at 25 °C and shaken in an orbital shaker at 150 rpm for about 10 days.

2.4.Optimization of Lipid Production using Box Behnken Design (BBD)

A model is developed using 2-level 3-factor design of Box Behnken for assessing effects of concentration of inoculum (A), pH value (B) and time of fermentation (C) on lipid production using R toruloides and DPSH. This study involves 17 trials of the experimental plan and the variables that are independent are studied at various levels, called low (-1) and the high (+1), and their values are tabulated in the Table 1. Experimental readings are obtained in triplicate and the response variable (Y) is obtained by averaging the lipid concentration produced after fermentation. Table 2 shows the design of experiment employed in this research. Variable (Y) is integrated into a model of second order to compare variables of response with the variables that are independent. The software called Design Expert (Version 13.0) is used to determine and analyse the second order polynomial coefficients. The analysis of variance is used to formulate the statistical model. This analysis includes Fisher's F test for determining overall significance of model, its probability p (F), coefficient of correlation R and coefficient of determination R^2, that estimates suitability of a regression model. 3D contour plots are prepared for each variable and for the design of quadratic models. Design Expert software is employed to generate response surface curves.

Table 1: Coding and the levels of factors of experiment

Factor	Name	Units	Coded	Coded

			Low	High
			-1	+1
A	Inoculum concentration	%	5.00	15.00
B	pH		5.50	6.50
C	fermentation time	days	7.00	9.00

Run	Coded Level	Lipid yield (g/l)

Biomass, content of lipid and profile of fatty acid of lipid are taken as given in [9]. The hydrolysate samples are analysed using HPLC and using the method described in [10] and total concentration of reducing sugar is measured using the method DNS [11].

3. Results and Discussion

3.1.Effects of H_2 SO_4 Concentrations on Paddy Straw Pretreatment

Hydrolysis is performed on 1%, 2%, 3% and 4% (v/v) H_2SO_4 at a loading rate of 10% (w/v) at 121°C for 30, 45, 60 and 120 minutes to optimize conditions and obtain maximum fermentable sugars (particularly, glucose) from paddy straw. Formation of glucose from paddy straw is found to be at its highest with 2% H_2SO_4 hydrolysed for 60 minutes. Acid hydrolysis breaks down the complex polysaccharides in the rice straw to simple fermentable sugars. Dilute acid breaks down the cellulose and hemicelluloses into fermentable sugars, which can be used by microorganisms. When hemicelluloses degrade, glucose, acetic acid, mannose, xylose and galactose are produced [12] and cellulose is hydrolysed to glucose. When hydrolysis is carried out at higher temperatures, higher acid concentrations and with prolonged pretreatment periods, xylose is degraded to furfural. 5-hydroxymethyl furfural is formed by the degradation of hexose and such by-products inhibit fermentation [13]. Therefore, in the subsequent experiments 2% H_2 SO_4 is used to prepare the PSH. During the detoxification process, glucose concentration increases and the concentration of inhibitors like HMF and furfural are reduced by 99%.

TABLE 2: Arrangement and response of design of Box–Behnken

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	A	B	C	Actual value	Predicted value
1	0	0	0	10.88	10.88
2	0	1	-1	7.80	7.65
3	1	0	1	8.20	8.29
4	0	-1	-1	7.23	7.43
5	0	0	0	10.88	10.88
6	0	0	0	10.88	10.88
7	1	-1	0	8.60	8.36
8	0	-1	1	7.03	7.18
9	0	0	0	10.88	10.88
10	-1	1	0	8.60	8.84
11	-1	-1	0	7.68	7.57
12	1	0	-1	7.50	7.54
13	-1	0	-1	8.30	8.21
14	-1	0	1	7.60	7.56
15	0	0	0	10.88	10.88
16	1	1	0	8.00	8.11
17	0	1	1	8.20	8.00

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Figure 1a shows time course of growth of cell and accumulation of lipid of R Toruloides in DPSH. Figure 1b shows sugar concentration during fermentation process. In previous work [10], during fermentation of rice straw hydrolysate a lag occurs on the first day and makes growth and the accumulation of lipid are hardly detectable. This can be attributed to the existence of inhibitors in the fermentation medium. However, the biomass begins to grow and increase during 2nd day, denoting adaptation of the cells to new environment of fermentation. From the second

day, glucose, galactose, xylose and arabinose are used as carbon sources simultaneously. These four sugars are completely used by R toruloides cells in different orders and rates. From day three, the content of lipid of yeast cells increases sharply and reaches a higher level of 46.7% on day six. The maximum concentration of lipid of 10.88 g/L is obtained on sixth day. After this period, lipid content does not increase but the biomass grows continuously. Studies conducted in [9-10] report a similar condition and use of the lipid that is accumulated for the growth of cell can lead to this condition. Compared to [9], the observations are similar to those obtained using already treated hydrolysate of paddy straw (11.5 g/L).

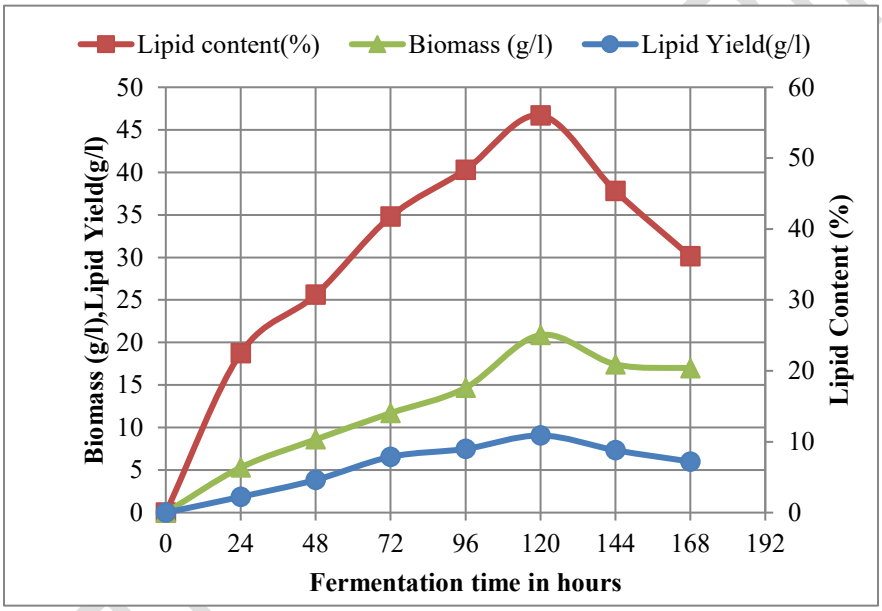

Figure 1a: Production of oil of microbial on Paddy straw that is pre-treated using sulphuric acid hydrolysate by Rhodosporidium Toruloides (**a**) course of time of growth of cell and accumulation ( )lipid content ( ) Biomass ( ) yield of lipid and total sugars

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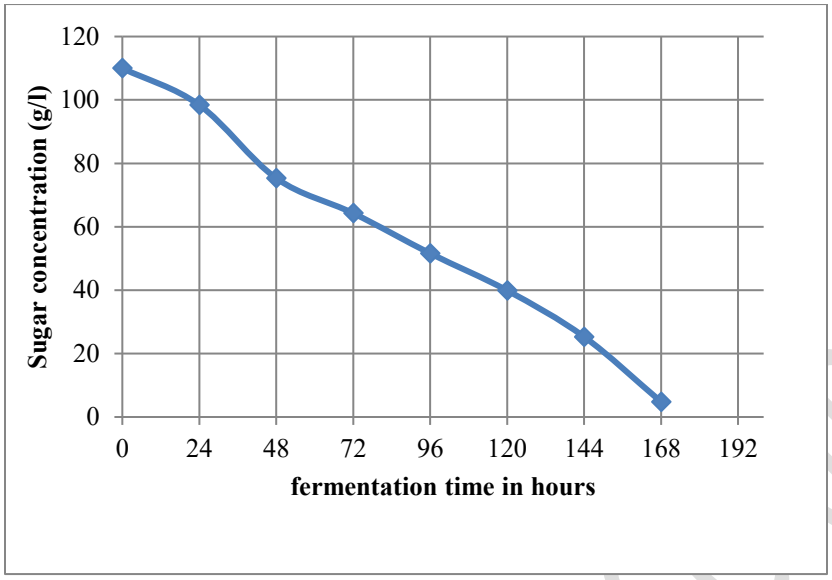

Figure 1**b**: time course of sugar utilization

Conditions of Fermentation: concentration of inoculum10%, pH 6.0, temperature 25 °C, 150 rpm.

3.2.Optimization of Lipid Yield

Design of Box Behnken and RSM are used for studying optimum conditions for lipid yield and higher glucose concentrations. A 2-level 3-factor BBD is used for evaluating effects of three factors discussed in previous sections on lipid production. Table 2 shows the BBD experimental data and proves that lipid concentration is much influenced by conditions maintained during the process of fermentation. Regression equation is obtained after variance analysis, provides the response level as a function of three variables that are independent using analysis of multiple regression. A quadratic approach for concentration of lipid of R toruloides is shown below (regarding coded factors):

Lipid yield = 10.88 + 0.015 * A + 0.2575 * B + 0.025 * C -0.38 * AB + 0.35 * AC + 0.15 * BC –1.1625 * A^2 - 1.4975 * B^2 -1.8175 * C^2

In this, Y represents predicted concentration of lipid (g/L) and A, B and C represent the inoculum concentrations as (%), pH and time of fermentation (in days) respectively. A combination of triglycerides, free fatty acids, and other lipid classes like phospholipids and sterols are typically present in the microbial lipids that Rhodosporidium toruloides produces. For accurate detection of lipid profile, the HPLC or the GC-MS models are employed for this process.

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Table 3: Response: lipid yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Method	34.00	9	3.78	90.92	< 0.0001	significant
A-Inoculum concentration	0.0018	1	0.0018	0.0433	0.8410	
B-pH	0.5305	1	0.5305	12.77	0.0091	
C-Fermentation duration	0.0050	1	0.0050	0.1203	0.7389	
AB	0.5776	1	0.5776	13.90	0.0074	
AC	0.4900	1	0.4900	11.79	0.0109	
BC	0.0900	1	0.0900	2.17	0.1846	
A^2	5.69	1	5.69	136.95	< 0.0001	
B^2	9.44	1	9.44	227.25	< 0.0001	
C^2	13.91	1	13.91	334.75	< 0.0001	
Residual	0.2909	7	0.0416			
Lack of Fit	0.2909	3	0.0970			
Pure Error	0.0000	4	0.0000			
Core Total	34.29	16				

coding of factor is **Coded**
Sum of squares is **Type III - Partial**

Model F-value of 90.92 denotes that method is significant. There is only a 0.01% chance that a value of F of this large can happen because of noise.

Table 3 shows findings of model quadratic polynomial in analysis as variance carried out using Design Expert software. The table demonstrates that the proposed model is significant in terms of very high F-value (90.92) and lesser P-value ($P<0.0001$). The reliability of the proposed

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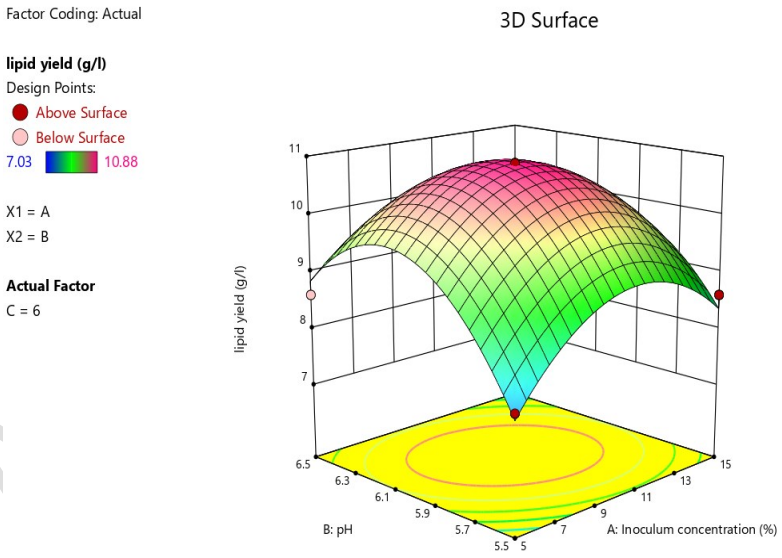
model for lipid production confirmed by a R^2 value of 0.9915 indicating that it is on good terms between values of experiment and values which are predicted. Adj-R^2 value of 0.9806 suggests that the variation in total of 98.06% during the concentration of lipid is attributed to variables which are independent. The missing of an optimal value is not important. P=0.0505 suggests that equation is sufficient enough to predict the concentrations of lipid under all situations. Among method terms B, AB, AC, A^2, B^2 and C^2 are significant with 99% of level of probability and other terms are not significant as they have lower probability levels. A #-D surface of response plot representing the equation of regression mentioned is used to confirm relationship among response and factor levels of each of the variable. Optimal levels of variables are determined visually using the plots [16]. Figure 2 indicates the response surface curves. An observable interaction between each pair of variables is clearly shown by the surface plots. Interactions between the pH and the concentration of inoculum, the concentration of inoculum and fermentation duration is important (P-value<0.05). Design Expert software analysis shows that optimal values of three important variables for lipid fermentation using R toruloides are 10% inoculum concentration, 6.0 initial pH and six days of fermentation.



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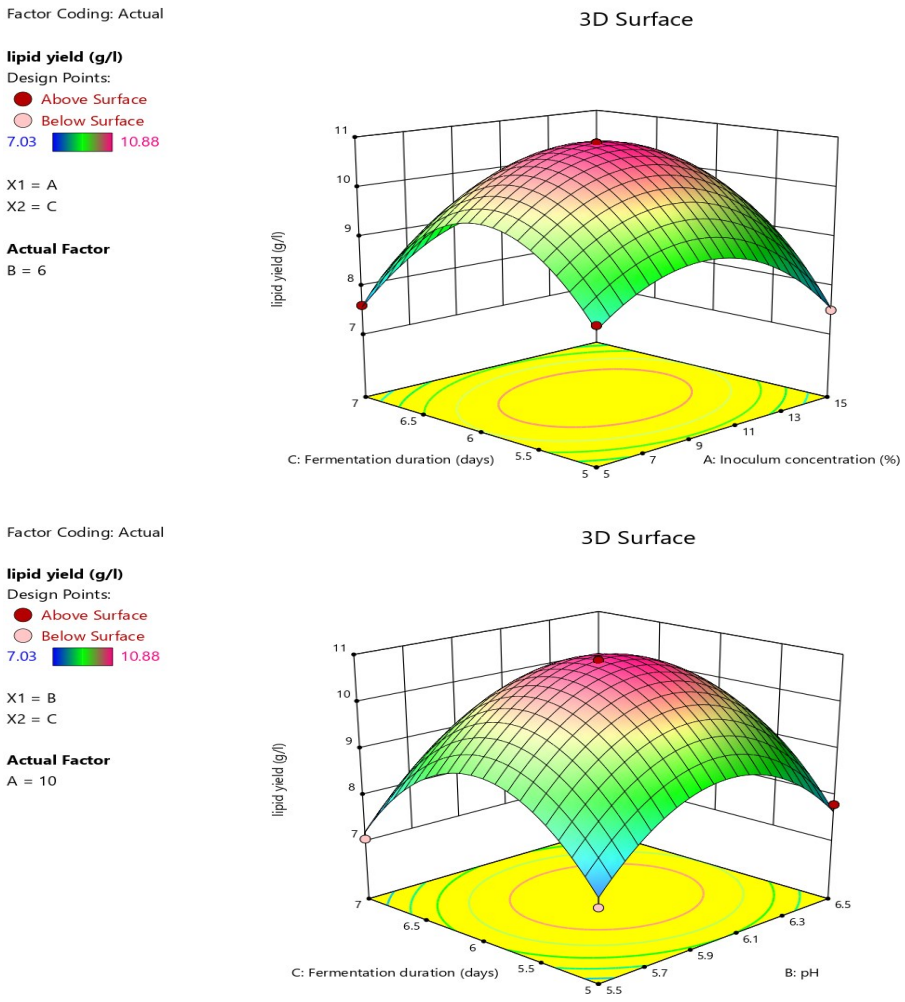

Figure 2: Plots of surface of Response showing the interaction of various variables. The interaction among (a) concentration of inoculum and fermentation duration (b) pH and inoculum concentration (c) fermentation duration and pH

3.3.Production of lipid under Optimal Conditions

The parameters of fermentation are given as follows: 10% inoculum concentration, pH value of 6.0 and 6 days of fermentation period. Under such conditions, the predicted concentration of lipid of 10.88 g/L can be achieved. Table 6 shows results of the present work and those carried out with various residues of agro-industries as feedstock. Although lipid productivity of R toruloides using paddy straw hydrolysate will be as maximum as that of Y, lipolysis of industrial fats using R toruloides can produce even higher lipid concentrations. This makes R toruloides a

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very reliable candidate for production of lipid using inexpensive and abundantly available lignocellulosic materials.

TABLE 4: Production of lipid on different residues of agro-industries by several microbes

Strain	**source of carbon**	**Glucose Concentration (g/L)**	**Lipid concentration (g/L)**	**Reference**
Y. lipolytica	Industrial fats	10	3.8	Papanikolaou, S. et al. (2001)
M. isabellina	Glycerol	26.8	3.3	Fakas, S. et al. (2009)
L. starkeyi	Sewage sludge	40	6.4	Angerbauer, C. et al. (2008)
T. fermentans	Molasses	150	12.8	Zhu, L.Y, et al. (2008)
T. fermentans	Rice straw hydrolysate	116.9	11.5	Huang, C. et al., (2009)
T. fermentans	Sugarcane bagasse hydrolysate	123.5	15.8	Huang C,et al.,(2012)
R.toruloides	Paddy straw hydrolysate	110	10.88	This study

4. Conclusion

Owing to its effectiveness, low-cost and abundance, PSH is highly suitable for microbial lipid production. PSH prepared with 2% H_2SO_4 produces optimal glucose concentration, maximum growth of biomass and lipid accumulation. Detoxified paddy straw hydrolysate is employed for growth of cell and also for accumulation of lipid using R toruloides. Optimization of parameters of fermentation using RSM results in 10.88 g/l lipid yield, with a pH of 6.0 and with a fermentation period of 6 days and produce a maximum lipid yield of 46.7%. R toruloides proves to be a very promising yeast strain for the production of microbial lipids using PSH.

Declaration

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Conflict of Interest

The authors declare no conflict of interest.

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