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Studies on metabolic regulation of *Schizosaccharomyces pombe* biomass production for glucan yield improvement

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Abstract

Schizo-saccharomyces pombe (*S.pombe)* has been playing a pivotal role in biotech industries as a source of glucans; a biological response modifiers (BRM) and also been studied as a model strain for recombinant proteins in molecular biology. Owing to the presence of > 60% glucans in their cell wall structure, the present research was designed to understand the effect of nutrient regulation on growth of *S.pombe (Italic form* to economize the glucan production. The study was performed using two different media; yeast extract with supplements (YES) and yeast extract-peptone-dextrose (YPD) and the YPD medium was noticed to be the best and cost-effective for maximum growth of *S.pombe* Thereafter, a sequential optimization studies, starting with one factor at a time (OFAT) methodology followed by two step statistical approach Placket-burmann design (PBD) and Response surface methodology (RSM) improved the biomass yield from 14-34.5 g/L (1.5-fold) at shake flask level. Mass transfer of media components, temperature, dextrose and yeast extract have played significant role in metabolism mediated growth of the *S.pombe*. The major finding of the present study is non-significance of peptone in enhancing the *S.pombe* biomass. Validation of the above at bioreactor level with dextrose at 4%, rpm at 200, temperature at 28°C and yeast extract at 2% increased the biomass yield from 34.5 - 52 g/L.

Keywords

Schizosaccharomyces pombe, One factor at a time, Placket-burmann design, Response surface methodology, Dextrose, Yeast extract.

Introduction

The genus *Schizosaccharomyces pombe (S.pombe)*- ancient fission yeast, is a unique species of yeast that has been using in brewing industries since very long time (1). It is an eukaryote with the shortest genome and divides by medial fission to produce off springs of equal size. These characteristics attract the researchers to use it as a model organism to study the eukaryotic genetics and molecular processes (cell cycle) (2) and to produce the

recombinant proteins (Human lipocortin I, Human papillomavirus-type-16 and industrial enzymes) (3). Now a days, *S.pombe* has been gaining importance in health sector as a source of glucans that are used mainly as nutraceuticles and biological response modifiers (BRM) (4-5).

The *S.pombe* is a typical yeast proliferates normally in a haploid state. The cells are grown by extension of tip to attain a critical cell length (7-15 µm) which is followed by mitosis (6). However, under nitrogen limiting conditions, the growth arrests in G2 phase and diploid zygote formation occurs due to the conjugation of opposite cells that can proceed directly to meiosis and divides into four haploid spores (7). Therefore, controlling the cell physiology is the essential factor as it influences the growth state as well as the yield of the *S.pombe* biomass. In general, *S. pombe* cell physiology depends on the choice of nutritional components and culture conditions. It can grow on various nutrient media including complex and diverse sporulation-media which can support sexual differentiation. The rich medium, Yeast extract with supplements (YES) is the preferable media for vegetative growth (8).

The one-factor-at-a-time (OFAT) approach is the traditional method used to optimize the process parameters. However, the drawbacks associated with the OFAT approach compared to factorial designs, resulted the use of statistical methods for improved production yields (9-10). Placket-Burmann design (PBD) helps in understanding the significance of each variable and selects the significant growth parameters in initial scale up studies whereas the central composite design (CCD) of response surface methodology (RSM) (11) assist to understand the combinatorial role of media components at different concentrations to improve the productivity. Considering the above, the present study aimed to recognize the optimal conditions to improve the *S.pombe* biomass using multi-factorial based designs in sequential manner where OFAT approach followed by combination of PBD and RSM analysis to improve the yield of biomass as well as glucan production followed by validation at Bioreactor level. This is the first report on maximization of *S.pombe* biomass as a source of glucan.

Materials and methods

The media components such as peptone, yeast extract, malt extract, dextrose, agar used in the present study were obtained from Himedia, India. All chemicals and reagents used were of analytical grade.

Culture collection and morphological characteristics

The glycerol stocks of yeast strain *S. pombe* NCIM 3360 were procured from NCL, Pune. The culture was streaked on MGYP (Malt extract, Glucose, Yeast extract and Peptone) agar slants and incubated at 30°C. Further, the strain was sub-cultured in the same agar medium slants frequently for maintenance and preserved at 4°C.

Media and growth conditions

The growth of *S.pombe* was studied in different yeast media such as YES medium (W/V) containing yeast extract 0.5%, glucose 3%, nitrogen bases as described in ATCC 2064and YPD medium (W/V) containing 1%, yeast extract, 2%, peptone, 2%, glucose at 30°C,and shaking at 200 rpm for a period of 60 hrs. However, further studies were progressed in YPD medium as it supported the growth of *S. pombe* similar to YES medium.

Growth curve

The growth curve of *S.pombe* was constructed by measuring optical density at different time intervals using double beam UV-Visible spectrophotometer SL 210-Elico at 595 nm.

Morphological characterization

The morphology of *S.pombe* was observed by microscopic method using

Scanning electron microscope (SEM).

Optimization of growth conditions for S.pombe biomass production

Influence of and nutritional factors by OFAT approach

The influence of various fermentation physical parameters such as age of inoculum (12, 24, 36, 48, 60 and 72 h), incubation temperature (24, 26, 28, 30, 32, 34 and 36°C), initial pH of the medium (3,4,5,6, 7 and 8), agitation speed (120, 150, 180, 200, 220 and 250 rpm) concentration of inoculum (0.5, 1, 2, 3, 4 and 5%) as well as different carbon (1%) sources (sucrose, dextrose, maltose, fructose, galactose) and various nitrogen (1%) sources (peptone, meat extract, yeast extract, malt extract and beef extract) were investigated by supplementing one factor at a given experiment. Based on the optimized condition, further experiments were performed using best carbon and nitrogen source in the concentration range of 1 to 4% at optimized physiological conditions. Triplicate experiments were performed and the data represented as mean ± standard deviation.

Identification of significant factors by PBD

In the designing phase, A total of nine variables based on one-at-a-time factorial study were selected and used. Considering each factor concentration at OFAT, the higher level (+1) and lower level (-1) for were selected and used for this study.

Response surface methodology

Only those factors which showed significance in the PB design i.e., dextrose, RPM, temperature and yeast extract were considered for RSM studies. The CCD design was developed with four factors and six replicates at the centre point. The optimum concentration obtained with the OFAT approach was considered as 0 code and two levels below and two levels above were predicted based on the parameter importance on growth of the organism; and coded as -2,-1, 0, +1, and +2. Based on the software input, a total of 31 experiments were conducted and the response values (Y) were recorded in terms of biomass yield. The presented yield value is the average of the triplicates. By this approach each variable level for maximum response was measured. Thereafter, an experiment was performed to verify the validity of the model using the combination of different optimized variables in order to yield the maximum response. Furthermore, the optimized conditions were evaluated at the level of Bioreactor Biostat B Plus 2010 (Sartorious).

The experimental results of RSM on biomass yield was verified by coducting ANOVA and incorporated into the response surface regression, using the second order polynomial equation:

$$Y = \beta 0 + \Sigma\, \beta iXi + \Sigma\, \beta iiXi2 + \Sigma\, \beta ijXiXj$$

Here *Y* represents response (biomass, g/L), *Xi* and *Xj* represent independent variables, *β*0 is the intercept; *β*i, and *β*j are linear coefficients; *β*ii and *β*jj are squared coefficients; *β*ij is interaction coefficients and the independent variables were coded as *X1, X3, X7* and *X9*. Thus, the second order polynomial equation can be presented as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_3 X_3 + \beta_7 X_7 + \beta_9 X_9 + \beta_{11} X_1 X_1 + \beta_{13} X_1 X_3 + \beta_{17} X_1 X_7 + \beta_{19} X_1 X_9 + \beta_{33} X_3 X_3 + \beta_{37} X_3 X_7 + \beta_{39} X_3 X_9 + \beta_{77} X_7 X_7 + \beta_{79} X_7 X_9 + \beta_{99} X_9 X_{9\,3.}$$

Results and discussion

Growth curve

S.pombe is mostly evaluated as a strain for alcohol production at industrial sector and most of the studies are aimed to improve the alcohol yield rather than improving the biomass yield. Hence the present study is aimed to improve the *S.pombe* biomass and the experiments were conducted to optimize the biomass growth under aerobic conditions. It is evident from the literature that the *S.pombe* growth was studied

in YES medium preferably and a few studies are associated with YPD medium. However, it was proved that YES medium is highly effective in *S.pombe* fermentation studies (8). By considering the above, in the present study, the growth of *S.pombe* was evaluated in YES as well as YPD media at common physiological and nutritional conditions. The data revealed that both the media supported the growth of *S.pombe* in comparable manner; the biomass yield was observed to be 13.5 g/L and 14 g/L with YES and YPD media respectively. The cost effective analysis of the both media components revealed that YES ($127/ 500g) medium is all most a three-fold higher in cost compared to YPD ($ 45/500g) medium indicating YPD is the best economic media for *S.pombe* biomass production at industrial sector. Since glucan is the major component (>60%) of *S.pombe* cell wall, the optimization of YPD media components has been the next promising step to reduce the glucan production cost by improving the yield of *S.pombe* biomass. Furthermore, it is apparent to understand the effect of fermentation parameters (physiological, nutritional and biochemical) on the growth of *S.pombe.*

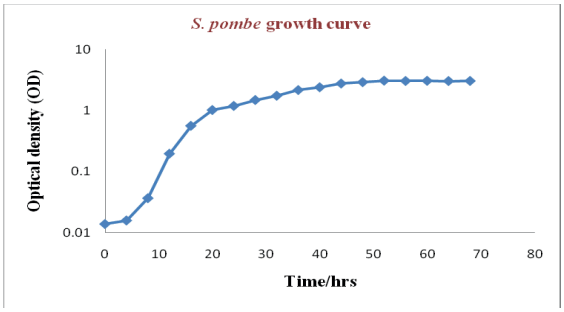

Fig. 1:*S. pombe* growth curve in YPD medium.

The growth curve of *S.pombe* in YPD medium is represented in (Fig 1). It is evident from the data that the growth of *S.pombe* has a lag phase of 0 to 6 hrs followed by 6 to 40 hrs log phase, thereafter the culture entered slowly into stationary phase. Critical observation of the growth pattern at log phase further denoted that it is bi-phasic in nature; initial fast growth phase (6-12hrs) followed by the second slower growth phase (12 to 40 hrs). This is further evidenced from the growth curve doubling time which was observed as 3.02 hrs at initial fast growth phase, while the same was noticed to be 16.07 hrs at second slower growth phase suggesting nutritional imbalance during the log phase of growth. Further, the specific growth rate (R) was observed to be 0.331/hr and 0.062/hr for faster and slower growth phases respectively. The above data is an indicative of influence of nutrient concentration on growth of *S.pombe* (12).This data is in accordance with the observations noticed by Hayles and Nurse (13) where the authors explain that, initial fast growth phase represent the growth of *S. pombe* through vegetative reproduction (fission) and the second slower growth phase may be associated with meiosis. To evaluate the same, the SEM analysis was performed to understand the morphological nature of the *S. pombe* cells during log phase at initial fast and slower second log phase. (Fig. 2) clearly is an indicative for difference in reproductive pattern (fission type at initial fast log phase (Fig. 2a) and meiosis type at slower second log phase (Fig 2b) regulated by nutritional status of *S.pombe* growth environment.

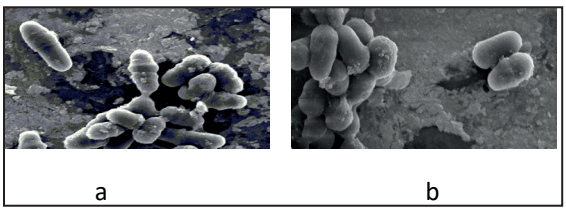

Fig. 2: SEM image of *S. pombe:* a) Fission b) Meiosis.

Effect of bioprocess parameters on S.pombe biomass

Any microbial growth is influenced by the fermentation factors and growth of the organism significantly varies based on nutritional status of the medium (14-15) as well as each microbial strain is unique in their

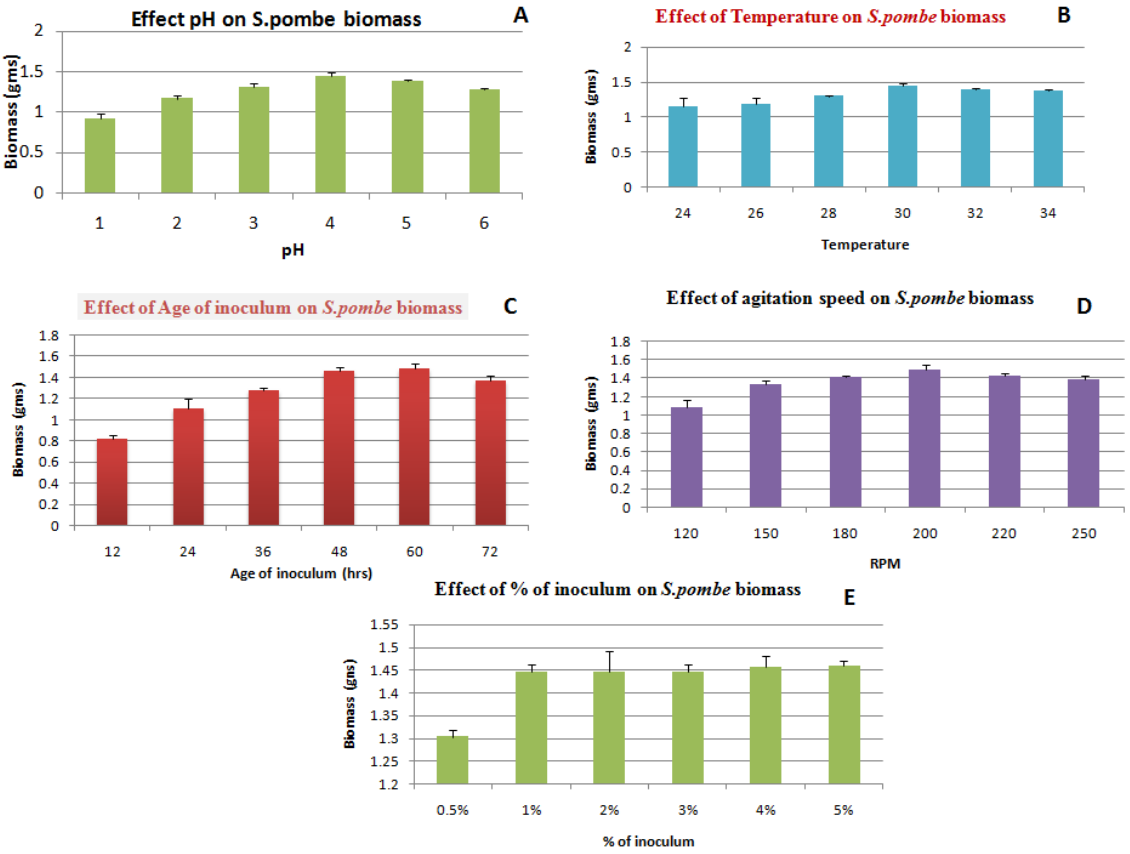

Fig. 3: Effect of physical parameters A) pH B) Temperature C) Age of inoculum D) Agitation speed E) inoculum concentration on *S. pombe* biomass.

growth requirements. Considering the above, the growth of *S.pombe* in terms of biomass yield was evaluated by varying physiological, biological and nutritional parameters one at a time at different experimentation conditions.

Effect of temperature on S. pombe biomass

Incubation temperature play vital role in growth and metabolite production in every microbe. Mostly fission yeast cells can grow between the temperature ranges of 18 to 37°C, however, the growth at 37°C or below 20 °C is not ideal for growth (8). Nevertheless, this feature may not be uniform to all strains. Therefore, the effect of incubation temperature on *S.pombe* growth was studied between the temperature ranges of 24 to 34°C. From the experimental results, the optimum temperature for the maximum biomass production (14.4±0.03) was noticed to be 30°C

Effect of inoculum concentration on S.pombe growth

The inoculum concentration is another principal factor which controls the growth state of *S. pombe*. In any microbial growth its effect on growth and metabolite production is significant as inoculum concentration is low, if the culture exhibit longer incubation time and if it is high, there is shorter incubation time these results in faster increase in biomass which ultimately leads to nutrient limitation that effects the product

formation. However, in the case of *S.pombe* the nutrient limitation arrests the growth in G_2 phase and inhibits the mitosis (16).Hence, the effect of inoculum concentration on the growth of *S.pombe* biomass was studied by inoculating the 24 hrs culture at different concentrations (0.5 to 5%). After 60 hours of incubation at 30°C the wet biomass was collected and weighed. The results suggested that growth of biomass was increased from 0.5 to 1.0% inoculum; thereafter the biomass concentration did not show any improvement or remained constant may be due to nutrient limitation.

Effect of initial pH

The medium pH is one of the significant factors that influence the microbial growth mostly by modulating the membrane transport of different nutrient and physiological growth factors. It has been reported earlier that the optimum pH for the growth of yeast is reported between 4.0 to 5.0. Here, the effect of pH on *S.pombe* at 30°C was investigated at the pH range of 3.0 to 8.0 with an increment of 1 pH unit for 60 hours. The results from the study suggested that biomass yield was increased from pH 3 to 6, there after the yield was constant over a broad pH range (6.0 to 8.0). However, a variable trend was reported by Meena et al. (2014), where the growth rate of *S.pombe* in the absence of malate was constant over a broad pH range of 3 to 8 indicating the nutritional parameters especially carbon source do control the growth of *S.pombe* (17).

Effect of agitation speed

The agitation speed of the incubation plays a major role in the mass transfer of gaseous elements and other fermentation components throughout the culture growth. This impacts the uniform supply of oxygen to the cell culture and leads to appropriate growth rate. The growth of *S.pombe* at different rate of agitation 120 to 250 rpm was studied and results were given in the (Fig.3)**.** From the (Fig. 3) it was evident that 200 rpm is the optimum for maximum biomass production which is found to be 14.9± 0.051 g/L.

Effect of different carbon sources

The glucose is the simplest and easy metabolizable carbon source for most of the microbial cells and routinely using carbon source in growth of *S.pombe.* However, it can also use glycerol, sucrose, raffinose, maltose as an energy source (18). Su et al in 1996 explored that absence of glucose arrest the cell division indicating strain dependent variation of growth parameters (12). Considering the above, the effect of different carbon sources like sucrose, fructose, dextrose, maltose and galactose at different concentrations (1 to 4%) were studied. However, the maximum biomass was obtained at 3% concentration of dextrose (17±0.03 g/L) (Fig 4). This result mimics the reports of dextrose effect on ATP production by *S.pombe* (19).

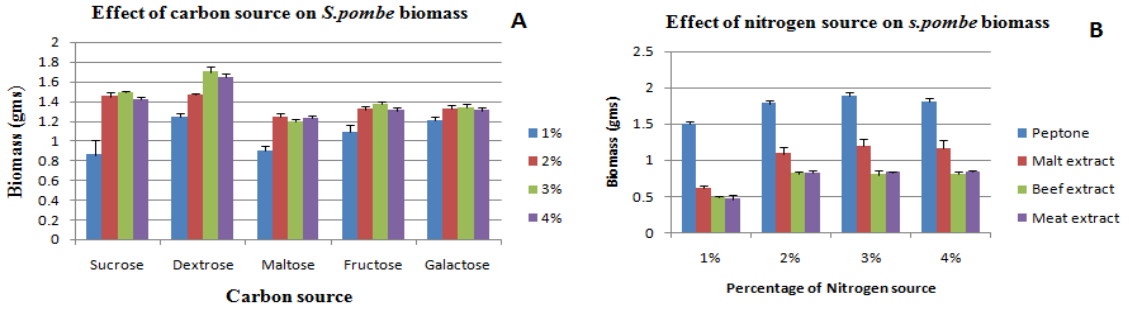

Fig. 4: Effect of nutritional factors A) Carbon source B) Nitrogen source on *S. pombe* biomass.

Effect of nitrogen source and yeast extract

The nitrogen plays a significant role in the cellular processes such as growth and metabolite production of microbes. Each distinct nitrogen source induces significant fluxes through the TOR (expand) and Sty1 growth control pathways to alter the architecture and flux of most cellular processes. Similarly, *S.pombe* growth is also influenced by different nitrogen sources. It is reported that all promote the cell proliferation, but the rate is distinct for different nitrogen sources (20). However, the absence of nitrogen source will arrest the growth in G_1 phase and stops the proliferation (21). Therefore, the present study assessed the influence of various nitrogen sources like peptone, malt extract, beef extract, meat extract on the *S.pombe* growth at different concentrations (1 to 4%). Among the different nitrogen sources peptone showed maximum biomass production (17.5±0.5 g/L) at 3% concentration and thereafter the yield was decreased (Fig 4).

The yeast extract can also used as a complex source to enrich the culture medium with some trace elements and growth factors at low cost to promote the growth of yeast (22). Hence, the effect of yeast extract on *S.pombe* was studied at different concentrations ranging from1 to 4%. From the results, it was concluded that yeast extract at the concentration of 1% has given higher yield (13.00 g/L) (Fig 5). The result was in accordance with the report of Perez et al.

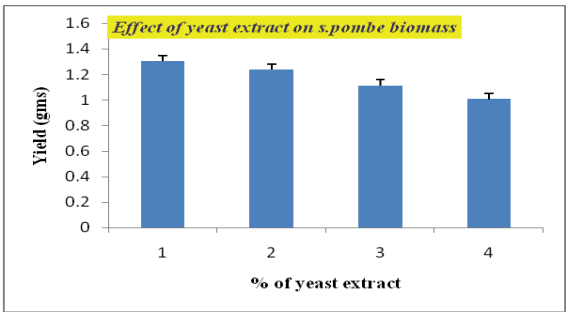

Fig. 5: Effect of yeast extract on *S.pombe* biomass.

(1992) where the effect of yeast extract on growth was influenced by the concentration of glucose. As the concentration of glucose increases, the yeast extract could increase the biomass at low concentration (1%) after that it could not support. However, the absence of yeast extract could not support the growth. Considering the above, it has concluded that yeast extract is one of the essential requirement for the growth of *S.pombe* upto limited concentration only (23).

Selection of significant variables by PBD

Based on the above studies, a total nine variables, i.e., physical parameters such as % of inoculum, age of inoculum, temperature, pH, incubation time and agitation speed as well as nutritional parameters such as dextrose, peptone and yeast extract were found to be effective in *S.pombe* growth. Considering the above and to understand which are the significant factors that influence the *S. pombe* biomass production, all

Table 1: Experimental variables at different levels used for the production of *S. pombe* biomass using Plackett–burmann design.

Variables	Units	Symbol codes	Experimental values	
			Lower	Higher
Temperature	°C	X1	28	32
pH	---	X2	5	7
RPM	---	X3	150	2
Age of inoculum	Hrs	X4	36	60
% of inoculum	Ml	X5	0.5	2
Time of growth	Hrs	X6	36	60
Dextrose	% (w/v)	X7	2	4
Peptone	% (w/v)	X8	2	4
Yeast extract	% (w/v)	X9	0.5	2

Table 2: Plackett-burmann design for growth studies of *S. pombe* and yield of the biomass for corresponding trail.

Run order	X1	X2	X3	X4	X5	X6	X7	X8	X9	Biomass (gm/100ml) (Y)	
										Experimental	Predicted
1	32	5	200	36	0.5	36	4	4	2	2.80	2.88
2	32	7	150	60	0.5	36	2	4	2	2.13	2.04
3	28	7	200	36	2	36	2	2	2	1.93	2.13
4	32	5	200	60	0.5	60	2	2	0.5	2.09	2.29
5	32	7	150	60	2	36	4	2	0.5	2.64	2.74
6	32	7	200	36	2	60	2	4	0.5	2.42	2.22
7	**28**	**7**	**200**	**60**	**0.5**	**60**	**4**	**2**	**2**	**3.10**	**2.91**
8	28	5	200	60	2	36	4	4	0.5	2.75	2.66
9	28	5	150	60	2	60	2	4	2	1.86	1.95
10	32	5	150	36	2	60	4	2	2	2.95	2.86
11	28	7	150	36	0.5	60	4	4	0.5	2.13	2.32
12	28	5	150	36	0.5	36	2	2	0.5	1.83	1.63

the above effective factors and their initial test ranges were studied and these factors were further subjected to statistical optimization by using PB design to identify the significant variables (Fig.6). All nine variables, each at two levels (lower and higher) were selected for the experimentation. The statistical designing tool

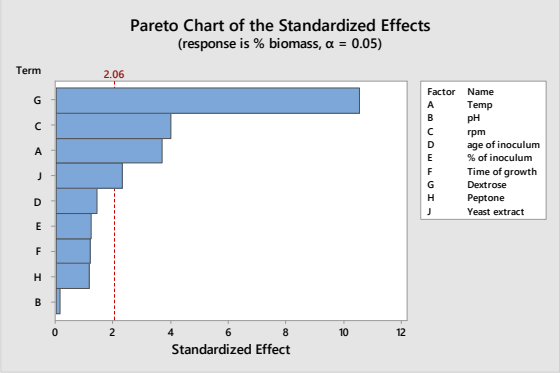

Fig. 6: Pareto chart for estimation of standardized effect of variables.

(P value) of each variable was determined using Student's t-test.

The data obtained by PBD experimentation indicated two-fold increased wet biomass (31g/L) than traditional OFAT (17 g/L) (Table 1; Run order 7) which also further confirmed by validation experiment. The efficiency of the model was evaluated, Mintab 18 version used to design the experiment. (Table 1 and 2) represent the design of the experiment (variable name, symbol code, and level of variables. The principal effect of each variable on the biomass yield was calculated by difference between averages of higher and lower level measurement. The significance level

Table 3: Estimated effects and coefficients for *S. pombe* biomass (g/l).

Term	Effect	Coef	T-Value	P-Value
Constant		2.3846	73.44	0.000
Temperature	0.2401	0.1200	3.70	0.001
pH	0.0104	0.0052	0.16	0.874
RPM	0.2596	0.1298	4.00	0.000
Age of inoculums	0.0928	0.0464	1.43	0.165
% of inoculums	0.0803	0.0401	1.24	0.227
Time of growth	0.0778	0.0389	1.20	0.241
Dextrose	0.6859	0.3430	10.56	0.000
Peptone	-0.0768	-0.0384	-1.18	0.247
Yeast extract	0.1497	0.0749	2.31	0.029

and the statistically significant variables were further reassured via student's t-test by ANOVA (Table 3). From the (Table 3), it has been concluded that the factors representing $P < 0.05$ were considered as significant factors for the production of biomass and further subjected for optimization studies. Dextrose, agitation

speed with a probability value of 0.000 and temperature, with a probability value of 0.001 were considered as more significant followed by yeast extract (0.029).

The Pareto chart demonstrated influence of each factor represented by alpha value equals to 0.05 i.e., 95% of confidence level and 8 degrees of freedom and t-value (equal to 2.06). The degree of each effect represents the length of the column. In the present study dextrose, revolution per minute, temperature and yeast extract, revealed a significant effect on biomass yield and subjected for further optimization while age of inoculum, incubation time, pH, peptone and % of inoculum did not show any significance.

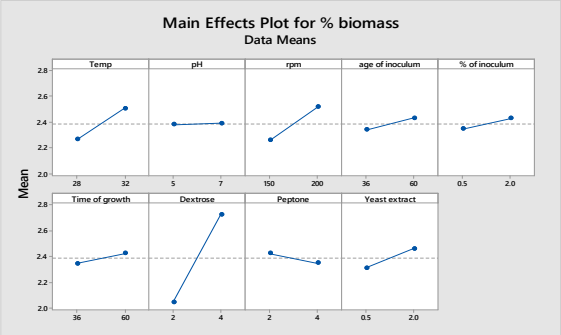

Fig. 7: Main effect plot for *S. pombe* biomass production.

Further the results of the main effect plot (Fig 7) indicated that dextrose at the highest level has a major effect on biomass yield whereas temperature, rpm and yeast extract were significant at lower level while pH, % of inoculum, age of inoculum, incubation time and peptone did not show any significant role on the biomass yield.

Optimization of significant variables using RSM

Considering significant factors observed by PBD, further optimization studies i.e., interaction between significant variables and their optimum levels for maximum production was studied by the statistical design RSM using CCD design. A quadratic model consisting of 31 trials were applied for experimental studies. The significant variables selected for this purpose include: dextrose, RPM, temperature and yeast extract were represented in (Table 4).

The design and results (mean predicted values) of RSM experiments were shown in (Table 5). The ANOVA data (Table 6) indicated that the most of the model terms, were significant (represent $P < 0.05$), except a few insignificant terms like, $X_7 * X_3$, $X_3 * X_3$, $X_7 * X_9$, $X_7 * X_1$ *and* $X_3 * X_9$. Further, it explores a significant linear effect of dextrose, yeast extract and temperature ($P < 0.001$) than the rpm. These results inferred a direct relationship between the concentration of the carbon, yeast extract with respect to a temperature in biomass production. Furthermore, the higher F- and P-values (<0.0001) and lack of fit (0.000) indicated the suitability and good fit of the model for the present study (Table 7). The calculated regression equation (express the response, biomass production (Y) by *S.pombe)* coefficient values were found to be following second-order polynomial equation.

$$Yield = 2.88952 + 0.36472\ X_7 - 0.16639\ X_1 + 0.06361\ X_3 + 0.11861\ X_9 - 0.10092\ X_7X_7 - 0.10092\ X_1\ X_1 - 0.02842X_3\ X_3 - 0.11176\ X_9\ X_9 + 0.07375X_7$$

Table 4: Experimental codes, range of levels of independent variables for RSM experiment.

Variables	Symbol codes	Range of levels				
		-2	-1	0	+1	+2
Temperature	X1	26	28	30	32	34
RPM	X3	125	150	175	200	225
Dextrose	X7	1	2	3	4	5
Yeast extract	X9	0.5	1	1.5	2	2.5

Table 5: CCD matrix with experimental values of biomass production from *S. pombe*

StdOrder	X7	X3	X1	X9	Biomass (Y) (g/100ml)	
					Experimental	Predicted
1	2	150	28	1	2.24	2.31
2	4	150	28	1	3	2.83
3	2	150	32	1	1.87	1.83
4	4	150	32	1	2.6	2.65
5	2	200	28	1	2.23	2.04
6	4	200	28	1	2.73	2.73
7	2	200	32	1	2.05	2.01
8	4	200	32	1	2.84	3.0
9	2	150	28	2	3.09	2.71
10	4	150	28	2	2.95	3.18
11	2	150	32	2	1.61	1.78
12	4	150	32	2	2.58	2.55
13	2	200	28	2	2.48	2.68
14	**4**	**200**	**28**	**2**	**3.45**	**3.26**
15	2	200	32	2	2.18	2.13
16	4	200	32	2	2.96	3.07
17	1	175	30	1.5	1.62	1.75
18	5	175	30	1.5	3.32	3.25
19	3	175	26	1.5	2.6	2.82
20	3	175	34	1.5	2.34	2.15
21	3	125	30	1.5	2.62	2.65
22	3	225	30	1.5	2.89	2.91
23	3	175	30	0.5	2.15	2.21
24	3	175	30	2.5	2.71	2.76
25	3	175	30	1.5	2.91	2.89
26	3	175	30	1.5	2.9	2.89
27	3	175	30	1.5	2.85	2.89
28	3	175	30	1.5	2.96	2.89
29	3	175	30	1.5	2.84	2.89
30	3	175	30	1.5	2.86	2.89
31	3	175	30	1.5	2.85	2.89

X_1 + 0.04375 $X_7 X_3$ – 0.01208$X_7 X_9$ +0.11042 X_1 X_3 -0.11292 $X_1 X_9$ + 0.04292 $X_3 X_9$

S = 0.163560 PRESS = 3.17079

R-Sq = 88.98% R-Sq(pred) = 83.25% R-Sq(adj) = 87.00%

The Fisher's test helps to evaluate the statistical significance of the model- equation and term. The coefficient of determination (R^2) and

Table 6: Analysis of variance (ANOVA) for quadratic model.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	16.8473	16.8473	1.20338	44.98	0.000
Linear	4	12.8752	12.8752	3.21881	120.32	0.000
Square	4	2.3265	2.3265	0.58162	21.74	0.000
Interaction	6	1.6456	1.6456	0.27426	10.25	0.000
Residual Error	78	2.0866	2.0866	0.02675		
Lack-of-Fit	10	1.5467	1.5467	0.15467	19.48	0.000
Pure Error	68	0.5400	0.5400	0.00794		
Total	92	18.9339				

the adjusted R^2 values were determined to understand of the model (For a good statistical model, the R^2 value should be in the range of 0 to 1.0), The closer the R^2 (near to 1.0), the stronger the model and the better in the prediction of the response (10).

The quality of fit (second-order polynomial model equation) which was expressed in three-dimensional surface plots. These plots aid to study the interactive relationship between the responses and each variable experimental level utilized in this experiment in terms of biomass production.

The regression equation also displayed the R^2 [multiple correlation coefficient] value as 0.8898 which is an estimate of the fraction of the overall variation in the data whereas the 'adjusted R^2' is 87%. This suggested the capability of model which can explain the 88.98 % of the variation in response as well as fitness

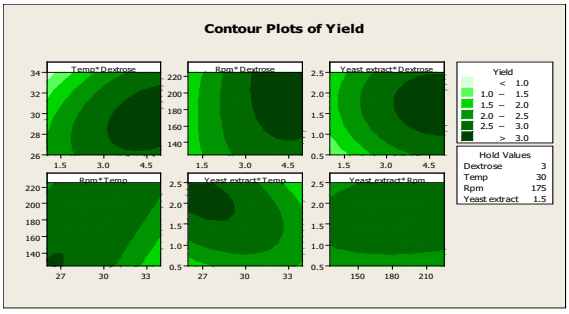

Fig. 8: Contour plots for *S.pombe* biomass optimization.

Table 7: Model coefficient estimated by multiple linear regressions.

Term	Coef	SE Coef	T	P
Constant	2.88952	0.03569	80.958	0.000
X7	0.36472	0.01928	18.921	0.000
X1	0.16639	0.01928	-8.632	0.000
X3	0.06361	0.01928	3.300	0.001
X9	0.11861	0.01928	6.153	0.000
X7*X7	-0.10092	0.01766	-5.715	0.000
X1*X1	-0.10092	0.01766	-5.715	0.000
X3*X3	-0.02842	0.01766	-1.610	0.112
X9*X9	-0.11176	0.01766	-6.329	0.000
X7*X1	0.07375	0.02361	3.124	0.003
X7*X3	0.04375	0.02361	1.853	0.068
X7*X9	-0.01208	0.02361	-0.512	0.610
X1*X3	0.11042	0.02361	4.677	0.000
X1*X9	-0.11292	0.02361	-4.783	0.000
X3*X9	0.04292	0.02361	1.818	0.073

Two-dimensional contour plots showed in (Fig. 8), denote the interaction effect of each independent variable against the biomass yield. For example, contour of glucose (x-axis) and temperature (y-axis) helps in evaluating the contributory role of these two factors at different concentrations on biomass production (>3 g/100 ml) (Fig 8). It also elucidates the direct interaction between dextrose and temperature in maximum biomass production which is further evidenced by their p-value of 0.003 (Table 6) which is significant. Simultaneously, the (Fig. 8) also depicts the significant interaction of temperature with rpm and yeast extract (Table 6). The surface in the other plots indicates that there is no interaction between other factors. Finally, the physical and nutritional parameter variation effect on *S.pombe* biomass production was depicted in (Table 7). It describes the step-by-step improvement of biomass production, where the initial yeast extract-peptone-glucose medium composition by using OFAT method has given the yield of 17 g/L followed by the statistical optimization (PBD & RSM) which yielded 34 g/L. At this step a 2.5-fold rise in biomass production explains the importance of statistical methods to achieve the higher yields of biomass compared to conventional OFAT method.

Validation of model

From the studies it was evident that the experimental biomass production of 34.5 g/L was produced where as predicted value was 32.6 g/L. This indicates a strong agreement between experimental values and predicted values. The optimum levels of tested variables were determined as dextrose (4%), rpm (200), temperature (28° C) and yeast extract (2%). Furthermore, the model was validated by repeating the experiment three times at optimum conditions. This results a biomass yield of 35 g/L, thus proving the validity of the model.

Verification of statistical model by 5L Bioreactor

The optimized levels of significant variables obtained by RSM were examined using 5L Bioreactor with working volume of 3L. The maximum biomass concentration of 52g/L was produced with in the 10hrs, whereas, the shake flask method produced 34g/L within 60hrs of incubation. This might be due to the oxygenation (0.75vvm) which could resulted in rapid growth rate and high biomass concentrations of *S.pombe* in bioreactor. The same type of effect was observed on *S.pombe* in an ethanol production. From the study, it was evident that aerated cultures were shown higher growth rates in comparison to non-aerated cultures (17).

As several researchers have studied on the growth kinetics of *S.pombe* in relation to ethanol production (17), the studies to improve the biomass were not performed earlier. The present study focused on the optimization studies of *S.pombe* to improve the biomass yield which in turn increases the yield of glucans (Table 8). From these studies, the production media under optimized conditions was designed to have only yeast extract (2%), dextrose (4%) which replaces the commercially used media for the growth of yeast (YPD). Therefore, the production cost of the media for biomass was reduced and that simultaneously reduces the cost of glucan production. Eventhough, the glucans were extracted from different species of higher fungi (*Lentinus edodes, Ganoderma lucidium*) (24-25), the simplest structure and rapid growth of *S.pombe* under laboratory conditions will reduce the production cost of glucans compared to higher fungi which require variable environmental conditions for their growth.

Conclusion

To improve the yield of glucan economically, biomass yield should be improved at low production cost. This can be achieved only by using optimization studies of growth as it is directly proportional to yield of glucan. Gene manipulation could not be suitable for economization of glucan production as it is a

Table 8: An overview of step-wise progress in *S. pombe* biomass concentration under batch culture conditions.

Method of optimization	Physical parameters	Nutritional parameters % (w/v)	Biomass (g/L)
OFAT	30^0C, 150 rpm, 1% inoculum, 48hrs of age of inoculum, pH -6, 60hrs of incubation time	dextrose-3%, Peptone-2%, Yeast extract-1%,	17
Plackett-Burman design	28^0C, 200 rpm, 0.5 % inoculum, 60 hrs of age of inoculum, pH-7, 60hrs of incubation time	dextrose-4%, Peptone-2%, Yeast extract-2%,	31
Response surface methodology	28^0C, 200 rpm, 0% inoculum, 48 hrs of age of inoculum, pH-7, 60hrs of incubation time	dextrose-4%, Yeast extract- 2%,	34.5
Bioreactor	26^0C, 175 rpm, 0.5% inoculum, 48 hrs of age of inoculum, pH-7,0.75 vvm	dextrose-4%, Yeast extract- 2%,	52

structural component of cell wall. Therefore, the present combination of statistical optimization and scale up studies could plays a significant role in the maximum production (52g/L) of biomass using *S.pombe* which was three fold raised compared to OFAT studies (17g/L).

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Conflicts of interest

The authors declared that there are no conflicts of interest.

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