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CO-OPTIMIZATION OF *BACILLUS LICHENIFORMIS* 208 BIOMASS AND ALPHA AMYLASE SYNTHESIS USING RESPONSE SURFACE METHODOLOGY

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Abstract

Among the prokaryotic species, amylases from *B. lichenformis* (BLA) have gained considerable interest pertaining to their broad industrial applications. Although a huge number of studies report the amylase production from *B. licheniformis*, none addressed the simultaneous optimization of BLA enzyme with biomass using statistical modelling. The main objective of the present study was to co-optimize biomass production together with alpha amylase synthesis from *B. licheniformis* 208 strain through response surface methodology. In this connection, the effect of 6 independent variables including temperature, pH, incubation time, concentration of peptone, yeast extract, and starch was studied at 5 different levels. According to the results, an optimized set of parameters for alpha-amylase production were found as pH (7.5), temperature (50˚C), incubation time (72 hrs), 1.5% concentration of substrate (starch) and tested nitrogen sources (peptone and yeast extract). Amylase production was significantly influenced by the interaction of peptone and starch at the concentration of 15g/l. However, biomass production was maximally optimized at a parametric combination of pH 5, temperature 30˚C, 24 hrs of incubation time, and 1% of the tested carbon and nitrogen sources. Both the biomass and alpha-amylase synthesis models ($p<0.05$) were found highly significant at 95% confidence interval. Results revealed that RSM mediated optimization enhanced the alpha-amylase production by 26% when compared with one variable at a time optimization. Furthermore, the current data may also provide insights into the potential use of *B. licheniformis* as a probiotic where RSM-assisted biomass optimization may help in exploring growth-promoting interactions.

Key words: Response surface methodology, Biomass optimization, Central composite design, Alpha-amylase, *Bacillus licheniformis.*

Introduction

Alpha amylases cleave α-1, 4 glycosidic linkages of starch in an endo-acting fashion thereby, liberating the low molecular weight subunits such as mainly maltose and glucose (Ojha *et al.,* 2020). Although amylase synthesis is ubiquitous among microbes, production yield from most of these microbial species falls below the levels suitable for industrial applications. Nevertheless, *B. licheniformis* is one of those prolific amylase-producing species markedly employed in industries pertaining to their reduced fermentation cycles, GRAS status, and extracellular release of the enzyme. Provided with optimized operating conditions, these industrial biobanks can release as much as 20-25g/L of the proteins (Schallmey *et al.,* 2004).

Generally, optimization of such industrial enzymes on a global scale often employs one variable at a time (OVAT) approach (Asad *et al.,* 2014). However, several shortcomings (including reproducibility failures, over time consumption, and high cost) are usually observed on a lab scale. This may be because of the ignorant interaction effects of variables while following this method (Sánchez Blanco *et al.,* 2016). Alternatively, response surface methodology can successfully be employed for improving yields and reproducibility. RSM can help to unmask the interaction effect of the parameters and selecting the right combination of variables at appropriate levels thereby, facilitating a better response/ yield (Singh *et al.,* 2017).

As per annual estimations, amylases hold ~25% of the share in the enzyme trade market at a global level. The spectrum of alpha-amylases has not only been widened to industrial applications and clinico-medical practices but rather its universal presence in the living domains makes it an ideal molecule to investigate the history of evolutionary events (Souza & Magalhães, 2010, Ilyas *et al.,* 2020). Unfortunately, no industrial setup is currently being run in Pakistan for the bulk production of these industrially relevant enzymes, and a huge foreign exchange is incurred on the import of these enzymes. Thus, attempts were made to optimize the alpha-amylase production from *Bacillus licheniformis* 208 strain together with its biomass using statistical modeling. To the best of our knowledge, this is the first report of optimization of amylase production coupled with biomass yield from a *B. licheniformis* strain via response surface methodology.

Materials and Methods

Isolation, maintenance, and identification of the amylolytic bacterial strain: Thirty amylolytic strains were isolated from a local hot spring and stove ash samples using the spread plate method (Saleem *et al.,* 2012). Upon screening of their amylolytic potential, *Bacillus* 208 strain (later identified as *Bacillus licheniformis* 208 with GenBank accession no; KC332218) showed maximum enzyme index (3.14±0.25) during plate screening assay, thus selected for parametric optimization. Maintenance of the culture was done at 50°C using LB agar slants (Bacto-yeast extract 5g; Bacto-tryptone 10g; NaCl 10g; agar 2% and distilled H2O 1L).

The miscellaneous zymogenic potential of the *B. licheniformis* 208 strain: The selected amylolytic *B. licheniformis* 208 strain was further evaluated for its potential of producing different enzymes of industrial interest such as lipase, xylanase, ß-galactosidase, esterase, caseinase, carboxymethyl cellulase, pectinase, keratinase, and dextranase using the respective substrates as per Kiran *et al*., (2015).

Enzyme assay: With 100µl of 1% (w/v) starch solution (prepared in 20mM sodium phosphate buffer, pH 7.0), 100µl of the crude amylase preparation was mixed and incubated at 50˚C for 15 min. The reaction was stopped by adding 100µl of 90mM DNS reagent and the reaction mixture was kept on boiling for exactly 15 min and then cooled on ice down to room temperature. Absorbance was measured at 540nm using a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme which released 1.0 mg of maltose from starch in 15 min at pH 7.0 at 50°C (Bernfeld, 1955). International Units were calculated by applying the following formula (Ikram-ul-Haq *et al.,* 2003):

$$\text{IU/ml/min} = \frac{\text{Enzyme activity (U/ml)}}{\text{Mol. wt. of maltose x incubation time (min)}} \text{ X } 1000$$

Thin layer chromatography: Enzyme assay was carried out using 100µl of enzyme solution (cell-free supernatant) and 100µl of substrate solution containing 1% starch. The mixture was incubated for 15 min at 50˚C and thereafter heated to boil for 15 min. Then 5 µl of this hydrolysate was spotted on TLC plate with standard sugars (controls). After air drying, a one-dimensional ascend was done using the solvent system of butanol: ethanol: H2O (v/v). After a complete run, TLC plate was removed, air-dried, and further subjected to a second run in the same solvent system. Followed by two ascends, TLC plate was sprayed with freshly prepared methanol: H2SO4 mixture (v/v), air-dried, and kept at 100˚-110˚C for 10 min. The appeared sugar spot was identified using the standards (Teka, 2006).

Optimization of the biomass and production of alpha-amylase enzyme: For RSM based optimization, a CCD statistical design was generated by Minitab software (release 16), and six (06) parameters were chosen including temperature, substrate (starch) concentration, yeast extract concentration, peptone concentration, pH and incubation time. All the parameters were investigated at five different levels (Table 1).

These experiments were conducted in 100ml Erlenmeyer flasks containing 25ml of sterilized LB medium. These flasks were incubated at varying culture conditions at 150 rpm as per the randomized design. Inoculum size of 8% (v/v) of 24 hrs grown culture was used. Post-fermentation, the cell-free crude enzyme extract was obtained by cold centrifugation (4°C) at 11448x g for 20 min and assayed for amylase activity (Zar & Haq, 2012).

For biomass estimation, 1 ml of the cultured broth was taken into the pre-weighed microfuge tube and centrifuged at 11448x g for 20 min. The supernatant was removed post centrifugation and the weight (mg/ml) of microfuge tube was remeasured after drying the pellets at 95℃ until constant weight (Madrid & Felice, 2005, Khusro *et al.,* 2017).

Statistical analysis: All the experiments were conducted in triplicates and the results were calculated as mean ± S.D. Multiple linear regression was applied for the response prediction. The student's t-test was used to calculate coefficient parameters and the statistical significance of the model success (p-value) was evaluated by applying one-way ANOVA at 95% confidence interval.

Results

According to the results, the amylolytic *B. licheniformis* 208 strain can efficiently degrade a variety of the complex substrates tested including, xylan, casein, lactose, dextrin, tween 20, tween 80, and keratin. However, the selected strain was found unable to hydrolyze cellulose and pectin (Table 2).

When the responses obtained after running the complete experimental design were carefully analysed (Table 3), maximum amylase yield was observed in experimental run 19 (54.8U/ml/min) when the medium pH was pre-adjusted at 9 with 15g/l of all the tested carbon (starch), and nitrogen sources (peptone & yeast extract) and incubation was done at 50°C for 72 hrs. Nonetheless, the lowest amylase titer was measured after run 25 (2.598U/ml/min) when the initial pH of the fermentation broth was kept slightly acidic (6) with 20g/l of the starch and 10g/l of each nitrogen source and incubated for 96 hrs at 40°C.

Table 1. Levels of factors used in the experimental design.

Factor	Name	Levels				
		-2	-1	0	+1	+2
X1	pH	5	6	7	8	9
X2	Temperature (°C)	30	40	50	60	70
X3	Incubation time (hrs)	24	48	72	96	120
X4	Peptone (g/l)	5	10	15	20	25
X5	Yeast Extract (g/l)	5	10	15	20	25
X6	Substrate concentration (g/l)	5	10	15	20	25

Table 2. Enzyme production profile of the *Bacillus licheniformis* 208.

S. No.	Enzyme screened	Enzyme index
1.	Amylase	3.03 ± 0.152
2.	Lipase	2.75 ± 0.478
3.	Esterase	2.15 ± 0.240
4.	Pectinase	0.0
5.	CMCase	0.0
6.	Xylanase	1.50 ± 0.184
7.	ß-galactosidase	2.3 ± 0.265
8.	Caseinase	2.36 ± 0.572
9.	Keratinase	2.31 ± 0.150
10.	Dextranase	1.88 ± 0.385

Maltose was found as the main hydrolysis product when starch hydrolysate was run on TLC plate after keeping the reaction mixture under standard assay conditions (Fig. 1).

Key: G: Glucose, M: Maltose, ES: Enzyme substrate extract
Fig. 1. Thin layer chromatography.

When the correlation between *B. licheniformis* 208 proliferation and amylase production was evaluated, it was noticed that biomass concentration was at maximum (0.158) when the incubation conditions were set as per experimental run 06 with 7.7 amylase units. On the other hand, the lowest value for biomass (0.015) with no amylase units was witnessed when the fermentation was carried according to the incubation parameters set in experimental run 39. It indicates that amylase production was not initiated in the early lag phase and even in the exponential phase of growth, amylase yield was not much appreciable. Thus, the data suggest that being a secondary metabolite, maximum amylase production from *B. licheniformis* 208 depends on the incubation conditions at which the organism achieves early stationary phase.

Parity plots of both the RSM models display a good synergy between predicted and observed responses (Fig. 2; Table 3).

Analysis of RSM models revealed that the amylase production model was found highly significant with a p-value (0.0000005) being less at a 1% level of significance. The model terms of all regression coefficients with p-values <0.01 were assumed to be significant. The significant linear terms were temperature and yeast extract and significant squared terms included temperature, peptone, yeast extract, and starch, whereas, the interaction term of peptone and starch was found significant (Tables 4a & b).

On the other hand, when the biomass of *B. licheniformis* 208 was modelled as a function of process parameters, a significant effect of temperature, peptone, yeast extract, and starch was observed. Interestingly, the model also explains the significant interaction between time and temperature for achieving the maximum biomass yield. Overall, the RSM biomass model was also found statistically significant (p<0.01) as shown in Tables 5a & b.

As per the estimated coefficient of determination (R^2) in alpha-amylase rsm model, 87.7% variability in the responses can be explained. The adjusted R^2 value of 74.3% suggests a good agreement with R^2. Signal to noise ratio of 26.24 indicates the adequate precision of the model as a value greater than 4 is desirable. The coefficient of variation was calculated as 26.4% (Table 6).

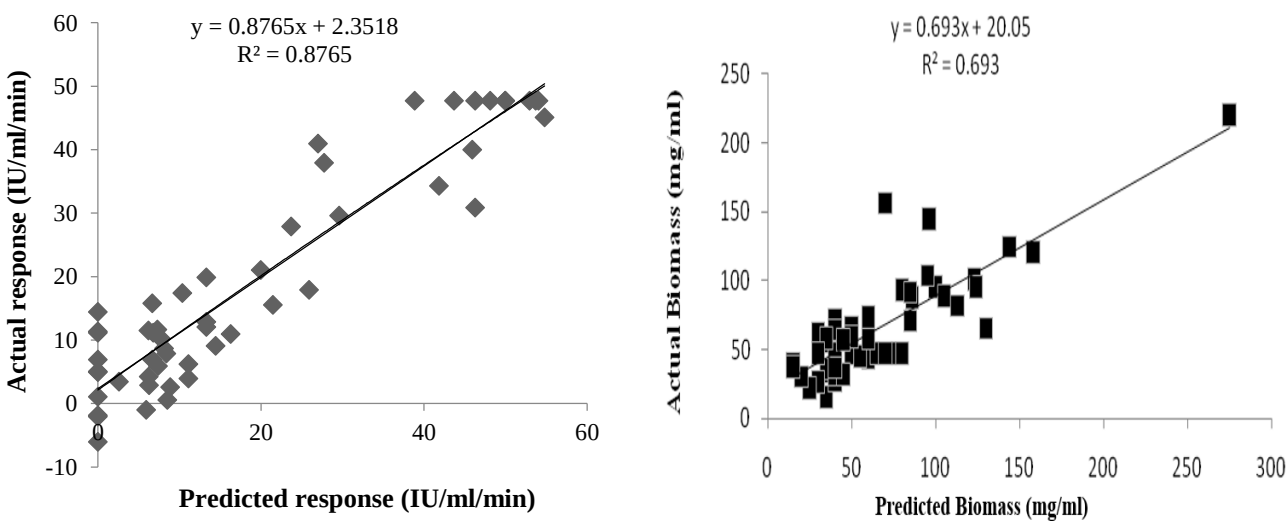

Fig. 2. Parity plots between Observed Vs Predicted responses of *B. licheniformis* biomass and alpha-amylase synthesis.

Table 3. Comparison of the predicted Vs observed responses.

Run order	pH	Temperature (°C)	Time (hrs)	Peptone (g/l)	Yeast extract (g/l)	Starch (g/l)	Amylase (units/ml/min)		Biomass (mg/ml)	
							Actual	Predicted	Actual	Predicted
1	7	50	72	15	5	15	23.7	27.9	0.030	0.062
2	6	60	96	10	20	20	6.3	2.9	0.040	0.071
3	8	40	96	10	10	10	20.0	21.0	0.050	0.057
4	7	50	72	15	15	15	54.1	47.7	0.030	0.047
5	6	40	96	20	20	20	14.4	9.1	0.113	0.082
6	8	40	48	20	10	10	7.8	10.2	0.158	0.121
7	8	60	96	10	20	10	0.0	6.9	0.035	0.016
8	7	50	72	15	15	15	50.0	47.7	0.075	0.047
9	6	60	96	20	10	20	0.0	14.4	0.085	0.071
10	8	60	48	20	10	20	13.3	19.9	0.040	0.050
11	7	50	24	15	15	15	27.8	37.9	0.035	0.056
12	5	50	72	15	15	15	27.0	41.0	0.040	0.065
13	8	60	48	10	10	10	7.3	11.6	0.015	0.040
14	8	40	48	20	20	20	10.4	17.4	0.123	0.101
15	8	60	96	20	10	10	7.4	5.9	0.035	0.034
16	8	40	48	10	10	20	13.3	12.1	0.144	0.125
17	7	50	72	15	15	15	53.7	47.7	0.050	0.047
18	6	60	48	20	20	20	6.3	4.2	0.040	0.028
19	9	50	72	15	15	15	54.8	45.1	0.040	0.038
20	7	50	72	25	15	15	11.1	3.9	0.060	0.059
21	6	40	96	10	10	20	0.0	1.0	0.124	0.096
22	8	40	96	20	20	10	0.0	5.0	0.060	0.044
23	7	50	120	15	15	15	45.9	40.0	0.035	0.036
24	6	60	48	10	20	10	6.8	11.1	0.030	0.027
25	6	40	96	20	10	10	2.6	3.4	0.086	0.088
26	6	40	48	10	20	20	8.1	8.6	0.100	0.095
27	6	60	96	20	20	10	6.7	6.9	0.060	0.074
28	6	60	48	20	10	10	8.9	2.6	0.025	0.022
29	6	60	96	10	10	10	25.9	17.9	0.050	0.066
30	7	50	72	15	15	15	46.3	47.7	0.065	0.047
31	6	40	48	10	10	10	21.5	15.5	0.275	0.221
32	8	60	48	10	20	20	0.0	-1.9	0.040	0.032
33	6	40	96	10	20	10	41.9	34.3	0.105	0.089
34	8	60	96	10	10	20	8.4	7.9	0.045	0.032
35	7	50	72	15	15	15	38.9	47.7	0.070	0.047
36	6	60	48	10	10	20	0.0	-6.1	0.020	0.031
37	7	30	72	15	15	15	0.0	11.1	0.070	0.157
38	7	50	72	15	25	15	29.6	29.6	0.055	0.045
39	8	60	48	20	20	10	0.0	-2.1	0.015	0.038
40	8	40	96	20	10	20	16.3	10.9	0.060	0.058
41	7	70	72	15	15	15	5.9	-1.0	0.130	0.066
42	6	40	48	20	10	20	8.5	0.6	0.080	0.094
43	7	50	72	15	15	15	43.7	47.7	0.040	0.047
44	7	50	72	15	15	15	53.0	47.7	0.030	0.047
45	8	40	96	10	20	20	6.2	11.5	0.040	0.037
46	6	40	48	20	20	10	13.3	12.8	0.085	0.092
47	7	50	72	15	15	25	11.1	6.2	0.050	0.060
48	7	50	72	5	15	15	0.0	11.3	0.035	0.059
49	7	50	72	15	15	5	6.7	15.8	0.045	0.058
50	7	50	72	15	15	15	50.0	47.7	0.030	0.047
51	7	50	72	15	15	15	48.1	47.7	0.080	0.047
52	8	40	48	10	20	10	46.3	30.9	0.095	0.104
53	8	60	96	20	20	20	0.0	4.9	0.096	0.145

Table 4(a). ANOVA of the response surface model for amylase enzyme production.

Sources of variation	Sum of squares	df	Mean square	F value	P-value	P<0.01
Model	15663.3	27	580.1222222	6.5725802	<0.0000005	Significant
A-pH	42.2	1	42.2	0.4781111	0.279686	
B-Temperature	369.2	1	369.2	4.1829058	<0.0000081	Significant
C-Time	10.6	1	10.6	0.1200943	0.199276	
D-Peptone	136.8	1	136.8	1.5498958	0.06244	
E-Yeast extract	7.5	1	7.5	0.0849724	<0.002484	Significant
F-Starch	229.5	1	229.5	2.6001541	0.321537	
AB	36.8	1	36.8	0.416931	0.524144	
AC	129.6	1	129.6	1.4683223	0.236874	
AD	0.4	1	0.4	0.0045319	0.948838	
AE	141.8	1	141.8	1.606544	0.216597	
AF	124.3	1	124.3	1.4082752	0.246451	
BC	49.9	1	49.9	0.5653494	0.459098	
BD	161.2	1	161.2	1.8263392	0.188703	
BE	287.3	1	287.3	3.2550077	0.08325	
BF	69.9	1	69.9	0.7919424	0.381977	
CD	21.8	1	21.8	0.2469863	0.623605	
CE	7.7	1	7.7	0.0872383	0.769537	
CF	0	1	0	0	0.987338	
DE	33.9	1	33.9	0.384075	0.541162	
DF	701.4	1	701.4	7.9466147	<0.009286	Significant
EF	14.8	1	14.8	0.1676788	0.685963	
AA	335.3	1	335.3	3.7988308	0.475666	
BB	4852.5	1	4852.5	54.977114	<0.0000001	Significant
CC	378.4	1	378.4	4.2871386	0.188843	
DD	3814.1	1	3814.1	43.212408	<0.0000002	Significant
EE	886.9	1	886.9	10.048264	<0.007355	Significant
FF	2819.6	1	2819.6	31.945074	<0.000007	Significant
Residual	2206.6	25	88.264			
Lack of Fit	2003.8	17	117.8705882	4.6520212	<0.016441	Insignificant
Pure error	202.7	8	25.3375			
Corrected total	17869.9	52				

Table 4(b). Estimated regression coefficients for IU/ml/min.

Term	Coefficients	StDev	T	P
Constant	-530.1	140.736	-3.767	0.001*
pH	29.0	26.234	1.105	0.280
Temperature	10.5	2.238	4.702	0.000*
Time	1.1	0.808	1.319	0.199
Peptone	7.6	3.878	1.950	0.062
yeast extract	13.0	3.878	3.363	0.002*
Starch	3.9	3.878	1.011	0.322
pH*pH	-1.2	1.625	-0.724	0.476
temperature*temperature	-0.1	0.016	-6.564	0.000*
time*time	-0.0	0.003	-1.351	0.189
peptone*peptone	-0.4	0.065	-6.165	0.000*
yeast extract*yeast extract	-0.2	0.065	-2.917	0.007*
Starch*Starch	-0.4	0.065	-5.652	0.000*
pH*temperature	-0.1	0.166	-0.646	0.524
pH*time	-0.1	0.069	-1.212	0.237
pH*peptone	0.0	0.332	0.065	0.949
pH*yeast extract	-0.4	0.332	-1.268	0.217
pH*Starch	0.4	0.332	1.187	0.246
temperature*time	0.0	0.007	0.752	0.459
temperature*peptone	0.0	0.033	1.351	0.189
temperature*yeast extract	-0.1	0.033	-1.804	0.083
temperature*Starch	0.0	0.033	0.890	0.382
time*peptone	-0.0	0.014	-0.497	0.624
time*yeast extract	-0.0	0.014	-0.296	0.770
time*Starch	-0.0	0.014	-0.016	0.987
peptone*yeast extract	-0.0	0.066	-0.620	0.541
peptone*Starch	0.2	0.066	2.819	0.009*
yeast extract*Starch	-0.0	0.066	-0.409	0.686

*Statistically significant (α=0.01)

Table 5(a) ANOVA of the response surface model for biomass (mg/ml).

Sources of variation	Sum of squares	df	Mean square	F-value	P-value	P<0.01*
Model	72823	27	2697.1481	2.0921749	<0.0033709**	Significant
A-pH	1778	1	1778	1.3791927	0.354783	
B-Temperature	20580	1	20580	15.963883	<0.000222*	Significant
C-Time	1013	1	1013	0.785783	0.103225	
D-Peptone	0	1	0	0	<0.014522**	Significant
E-Yeast Extract	678	1	678	0.5259239	<0.047329**	Significant
F-Starch	11	1	11	0.0085327	<0.028493**	Significant
AB	1361	1	1361	1.0557262	0.314015	
AC	1476	1	1476	1.1449316	0.294902	
AD	2677	1	2677	2.076546	0.161968	
AE	522	1	522	0.4049148	0.530172	
AF	1781	1	1781	1.3815197	0.250977	
BC	12932	1	12932	10.031338	<0.004026*	Significant
BD	2615	1	2615	2.0284526	0.166709	
BE	2751	1	2751	2.1339477	0.15653	
BF	2300	1	2300	1.7841075	0.193672	
CD	2101	1	2101	1.6297434	0.213459	
CE	1841	1	1841	1.4280617	0.243332	
CF	1701	1	1701	1.3194638	0.261577	
DE	2124	1	2124	1.6475845	0.211073	
DF	1449	1	1449	1.1239877	0.299272	
EF	1284	1	1284	0.9959974	0.327851	
AA	165	1	165	0.1279903	0.87622	
BB	8996	1	8996	6.9781873	<0.016434**	Significant
CC	0	1	0	0	0.965358	
DD	314	1	314	0.2435695	0.650045	
EE	100	1	100	0.0775699	0.798531	
FF	272	1	272	0.2109901	0.650045	
Residual	32229	25	1289.16			
Lack of fit	28824	17	1695.5294	3.9824531	<0.026349*	Insignificant
Pure error	3406	8	425.75			
Corrected total	105052	52				

Table 5(b). Estimated regression coefficients for *B. licheniformis* 208 Biomass.

Term	Coef	SECoef	T	P
Constant	2364.03	537.864	4.39522	0.000*
pH	-94.53	100.261	-0.94284	0.355
Temperature	-36.88	8.553	-4.31193	0.000*
Time	-5.22	3.087	-1.69122	0.103
Peptone	-39.04	14.819	-2.63459	0.014**
Yeast extract	-30.91	14.819	-2.08611	0.047**
Starch	-34.45	14.819	-2.32486	0.028*
AB	0.65	0.635	1.02752	0.314
AC	-0.28	0.264	-1.06986	0.295
AD	1.83	1.269	1.44109	0.162
AE	0.81	1.269	0.63660	0.530
AF	1.49	1.269	1.17522	0.251
BC	0.08	0.026	3.16725	0.004*
BD	0.18	0.127	1.42435	0.167
BE	0.19	0.127	1.46078	0.157
BF	0.17	0.127	1.33573	0.194
CD	0.07	0.053	1.27665	0.213
CE	0.06	0.053	1.19492	0.243
CF	0.06	0.053	1.14864	0.262
DE	0.33	0.254	1.28354	0.211
DF	0.27	0.254	1.06002	0.299
EF	0.25	0.254	0.99798	0.328
A^2	0.98	6.212	0.15737	0.876
B^2	0.16	0.062	2.57218	0.016**
C^2	-0.00	0.011	-0.04387	0.965
D^2	0.11	0.248	0.45922	0.650
E^2	0.06	0.248	0.25798	0.799
F^2	0.11	0.248	0.45922	0.650

** Significant at p<0.05; S = 35.9052; R-Sq = 69.32%; R-Sq(adj) = 36.19%

Regression equation for amylase production is given below:

Y (IU/ml/min) = - 530 + 29.0 A + 10.5 B + 1.07 C + 7.56 D + 13.0 E + 3.92 F - 0.107 AB - 0.0839 AC + 0.022 AD - 0.421 AE + 0.394 AF + 0.00520 BC + 0.0449 BD - 0.0599 BE + 0.0296 BF – 0.0069 CD - 0.0041 CE - 0.0002 CF - 0.0412 DE + 0.187 DF - 0.0272 EF - 1.18 A^2 - 0.107 B^2- 0.00381 C^2 - 0.401 D^2 - 0.190 E^2 - 0.367 F^2

where A= pH, B= Temperature, C= Incubation time, D= Peptone concentration, E= Yeast extract concentration and F= Starch concentration

whereas, in case of the biomass model, 69.3% of the response deviation can be predicted by the RSM model. However, this model indicates the presence of more independent terms as the value of adjusted R^2 (36.19%) is not in conformity with the coefficient of determination R^2. A high signal-to-noise ratio together with a lower coefficient of variance value also advocates for adequate precision of the biomass model (Table 6).

Table 6. RSM Model parameters.

Model parameters	Amylase model	Biomass model
R^2	0.877	0.693
Adjusted R^2	0.743	0.361
Signal to noise ratio (S/N)	26.24	24329.4
Coefficient of variance	26.4%	0.03%

Following regression equation was obtained when the biomass responses were fitted to second-degree polynomial;

Response (Biomass mg/ml) = 2364.03 – 94.5307 A - 36.8792 B - 5.22138 C - 39.0426 D - 30.9146 E - 34.4526 F + 0.652187 AB – 0.282943 AC + 1.82938 AD + 0.808125 AE + 1.49188 AF + 0.083763 BC + 0.180813 BD + 0.185437 BE + 0.169563 BF + 0.067526 CD + 0.0632031 CE + 0.0607552 CF +0.325875 DE + 0.269125 DF + 0.253375 EF + 0.977509 A^2 + 0.159775 B^2 - 0.000473075 C^2 + 0.1141 D^2 + 0.0641004 E^2 + 0.1141 F^2

where A= pH, B= Temperature, C= Incubation time, D= Peptone concentration, E= Yeast extract concentration and F= Starch concentration

Interactive effects of the variables (2 at a time) are graphically presented by employing 3D surface plots while holding all other variables at their middle (0) level range. A significant interaction for improving the amylase yield was witnessed between starch and peptone at the concentration of 15g/l (Fig. 3) whereas, the interaction term of time and temperature was found significant for maximizing the biomass production as shown in Fig. 4.

As per the observed RSM results, the retrieved set of optimum parameters for amylase production included pH (7.5), temperature (50°C), incubation time (72hrs), starch (15 g/l), peptone (15 g/l), and yeast extract (15 g/l). Predicted amylase units according to this optimized set of variables were 27.34 IU/ml min. Upon validation, a yield of 62 amylase units was observed. This further justifies the satisfactory precision and adequacy of the model (Table 7). Nevertheless, optimized parameters for *B. licheniformis* 208 biomass were found as pH 5, temperature 30°C, incubation time 24 hrs, and 0.5% of all the tested carbon and nitrogen sources.

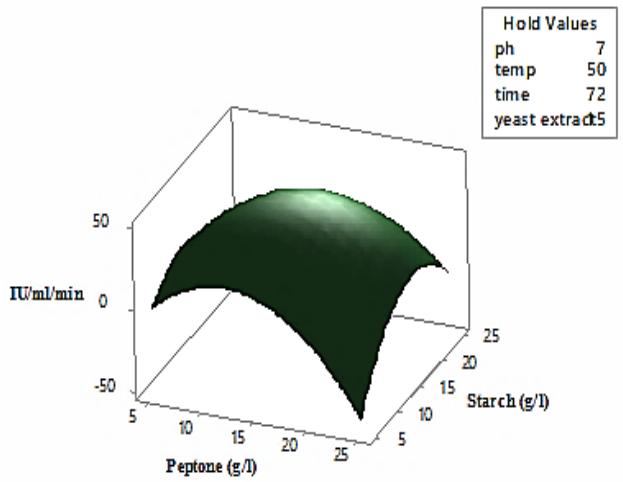

Fig. 3. Response surface plot between alpha amylase production (IU/ml/min), peptone and starch.

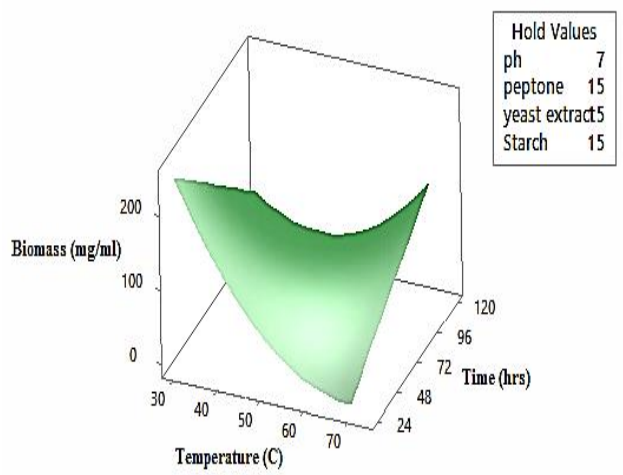

Fig. 4. Response surface plot between B.licheniformis biomass (mg/ml), time and temperature.

Table 7. Comparison of optimized parameters through RSM Vs OVAT analysis.

Factor	OVAT	RSM	
		Amylase production	Biomass
Temperature (°C)	50	50	30
pH	7	7.5	05
Incubation time (hrs)	24	72	72
Starch conc. (g/l)	10	15	05
Peptone extract conc. (g/l)	15	15	05
Yeast extract conc. (g/l)	20	15	05

Discussion

Diverse hydrolytic enzyme production by a single microbe can be exploited in waste management and biodeterioration where undefined and complex substrates are the target. When the zymological profile of *Bacillus licheniformis* 208 was analysed, it was

noticed that other than being amylolytic, it can potentially degrade various substrates including tween 20, tween 80, xylan, keratin, casein, dextrin, and lactose. Microbes and products possessing such activities have been the remedy and have already been commercialized through medical and pharmaceutical sectors, where deficiency arising in patients due to genetic disorders for not producing hydrolytic enzymes are tackled (Goud *et al.,* 2009, Ashraf *et al.,* 2018).

RSM is a collective integration of statistical and mathematical applications to advance understanding of the modelling and analysis of the influence exerted by multiple variables thus, encouraging a better response approach. So far, many reports have been published thereby, reporting the optimization of various enzymes following the response surface methodology (Ilyas *et al.,* 2020, Ojha *et al.,* 2020). Biomass optimization via statistical optimization facilitates in exploring the multivariable interactions for growth promotion particularly when probiotics are the target as these cells need to be procured in large numbers. The Probiotic potential of the *B. licheniformis* strains has already been evidenced contributing to boosting gut mucosal immunity in animals (Li *et al.,* 2007, Zhang *et al.,* 2011, Gao *et al.,* 2018). Even though these reports demonstrate the GRAS status of these industrial biofactories, the data about the statistical growth optimization is missing. The present findings might (further) help in establishing the baseline data in this regard.

Applicability of an RSM model for response prediction can be determined by the coefficient of determination (R^2). Generally, high values for R^2 are considered as the model success. However, it may be because of an increased bias induced by a small sample size or more number of independent parameters. This bias can be corrected by calculating adjusted R^2 which is a comparatively more precise measure of goodness of fit. In the current study, the statistical model for optimization of alpha-amylase from *B. licheniformis* 208 was found highly significant ($p<0.0000005$) with R^2 (87.7%) and R^2_{adj} (74.3%). Conformity in these values suggests the significance of the model. Zambare, (2011) reported the model significance (0.009) with an R^2 value of 0.818 and an adjusted R^2 value of 0.655. In case of biomass production, model significance was also found at the confidence level of 99% ($p=0.0033709$) with the value of R^2 as 0.69 which means the model can explain nearly 70% of the variability in responses. It is reported that an R^2 value up to 0.61 is enough to explain the model adequacy and significance. However, a distant value of adjusted R^2 (0.369) indicates towards the presence of more independent factors not included in the study. Interestingly, despite the advantages offered by approaching RSM modelling, Gonçalves *et al.,* (2012) experienced some shortcomings such as high titers of the enzymes pectin lyase and polygalacturonase were observed with significantly lower model values of raw and the adjusted coefficient of determination (0.4437, 0.2984) respectively.

The coefficient of variance (CV) determines the measure of dispersion in the data, thus, relatively a lower value demonstrates better precision. Presently, the coefficient of variance for amylase and biomass production model was calculated as 26.4% and 0.03% respectively (Zar & Haq, 2012). Adequate precision of the model provides a measure of signal to noise ratio (S/N) which can be used to judge the model adequacy to navigate through the design space or to predict the response. A value >4 is desirable. Adequate precision or signal to noise ratio (S/N) of the amylase production model was found to be 26.24 while for the biomass model (S/N) ratio was calculated as 24329.4. Soni *et al.,* (2012) found an adequate precision value of 46.98 of an RSM model for amylase production from *B. subtilis*. Adequacy of both the models was also evidenced by plotting the predicted versus actual responses where they tend to lie close on a diagonal line.

Another parameter check for adequacy of the model is to test for "Lack of fit" which was found insignificant for both amylase (0.016) and biomass model (0.026) relevant to pure error at 1% level of significance. Lack of fit (0.015) that was close to the current findings was reported by Tamilarasan *et al*., (2012) during RSM optimization of amylase by *A. oryzae* MTCC 1847 with R2=0.912.

Among all the tested variables, temperature and yeast extract exerted a significant effect on the amylase production synthesis. This indicates that the amylase production will be enhanced at increased varying operating parameters. It is reasoned that temperature markedly affects the release of extracellular enzymes possibly by modifying the physical properties of the cell membrane (Dinarvand *et al.,* 2013).

These results imply the significant role of temperature and preference for yeast extract over peptone for amylase production from *B. licheniformis* 208. The most significant interaction was found between peptone and starch ($p=0.009$). It is reported that both peptone and yeast extract exert considerable influence on the cell walls of gram-positive bacteria mostly by altering the surface charges, carbon to nitrogen ratio, and surface hydrophobicity (Schär-Zammaretti *et al.,* 2005). Similar results were reported by Sumrin *et al*., (2011) for yeast extract and starch by *B. subtilis.*

Contrary to the amylase production model, coefficients of all the factors were found as negative when the student's t-test was applied to the biomass model. It implies that cell proliferation will be maximum preferably at low levels of process parameters. The temperature along with peptone and yeast extract was observed as significant at $p<\alpha=0.05$. This may be because of the fact that batch fermentation often results in the accumulation of acetates in the medium and the presence of yeast extract in the medium facilitates the acetate assimilation and growth once the carbon sources are exhausted. In addition, peptone stabilizes the activities of vital enzymes (Nancib *et al.,* 1991). Our results are also endorsed by the findings of Shafi *et al*., (2018) during RSM mediated optimization of dry cell mass of *Bacillus* sp.

Graphical representation of the responses in the form of surface plots and contour plots provides a quick and convenient way to visualize the pattern towards the maximum response (Tanyildizi *et al.,* 2005). Infinite combinations between the two variables can be illustrated by a contour plot keeping the other variables at a constant level. A circular-shaped contour explains no interaction

whereas, an ellipse in the contour plot demonstrates an ideal interaction between two independent variables (Muralidhar *et al.,* 2001). The maximum or minimum profile of the ellipse in the contour plot can be indicated by 3D surface plots. As shown by these graphic aids, amylase production was optimum at 50°C and is the same as previously reported in OVAT analysis (Rasooli *et al.*, 2008; Asad *et al.,* 2014). However, in contrast to the results of the OVAT approach, statistical optimization resulted in maximum amylase titer when the fermentation broth incorporated with 15g/l concentration of starch and nitrogen sources (yeast extract and peptone) was incubated at 50°C, pH 7.5 for 72hrs. The difference (that lies) may be because of the unnoticeable interaction effect of the studied parameters during OVAT optimization. Our results are in agreement with those of Divakaran *et al*., (2011), who reported the maximum amylase production by *B. licheniformis* after 72 hrs of inoculation. Usually, the percentage of starch in combination with the nitrogen sources in the media formulations varies from 1-2% (Qader *et al.,* 2006; Riaz *et al.,* 2009). These findings are also in accordance with the reports of Waghode and Garode, (2013) for peptone concentration (15g/l) and Rao & Satyanarayana, (2007) for starch concentration (15g/l) being maximum for amylase production by *B. licheniformis*. Contrary to these findings, optimum amylase production at a highly alkaline pH of 9 and 11.35 was observed by Alkando *et al.*, (2011) and Zambare, (2011) respectively. When compared with conventional optimization, 26% improved enzyme yield was obtained after following the RSM model.

Interestingly, according to the biomass model prediction, optimum temperature and pH for growth were 30°C and 5 respectively. These results contradict the optimized temperature-pH conditions found after the OVAT approach (50°C and pH 7). Although *B. licheniformis* is known to have a mesophilic growth pattern (Thaniyavarn *et al.,* 2003, Shinde *et al.,* 2014), a thermophilic pattern is no surprise when isolated from thermal niches such as hot-spring (Tamariz-Angeles *et al.,* 2014). These results show a lack of agreement with the findings of Alkando *et al*., (2011) who reported optimum growth of *B. licheniformis* at 70°C. Our results are in compliance with the findings of Vijayalakshmi *et al.,* (2013) who found 5(g/L) peptone as the best for the growth of *B. licheniformis*. The predictions of the biomass model are also consistent with the report of El Hadj-Ali *et al*., (2007) who observed increased growth of *B. licheniformis* NH1 between 2-5 g/L concentration of yeast extract. Božić *et al*., (2011) reported 5(g/L) of starch concentration favouring the maximum growth. It is reasoned that higher concentrations of starch may restrict bacterial growth by interfering with the oxygen solubility by increasing the medium viscosity.

In general, enzyme production is presumed to be growth-associated.

On the contrary, predicted optimized parameters for biomass synthesis differed from those of amylase production. These results are supported by the findings of Chmelová & Ondrejovi, (2013) who found the highest laccase titer from white rot fungi *Ceriporiopsis subvermispora* at the lowest biomass yield.

When a comparison between the optimized parameters after OVAT and RSM was established (Asad *et al.,* 2014), minor differences were noticed for amylase production except for incubation time. However, a sharp distinction was observed in case of biomass optimization, it may be attributed to the inclusion of more independent terms in the model or ignorance of cause-effect relationship in OVAT analysis, thus some other parameters affecting biomass need to be evaluated. However, when the optimum parameters for biomass and α-amylase from *Kluyveromyces marxianus* after OVAT and RSM approach were compared by Stergiou *et al*., (2014), minor differences in the results were observed.

Conclusions

In the present study, RSM was found effective in elevating the reproducible amylase yields from *B. licheniformis* 208 strain and improving the growth-promoting interactions. The temperature along with starch, yeast extract, and peptone concentration was noticed as playing the most significant role during the amylase production. Furthermore, a significant interaction for upscaling the amylase yield was displayed between starch and peptone concentration. Nevertheless, the Time-temperature relationship was witnessed as the most influential interaction for the proliferation of these industrial biofactories. These findings may help in exploring the potential of *B. licheniformis* strains to be employed as probiotics where the most desired target is to achieve the maximum biomass under optimized conditions.

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