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Isobutanol production by *Candida glabrata* – A potential organism for future fuel demands

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Abstract

Due to global concern on the sustainability of energy from fossil fuels, isobutanol as a biofuel has urged attention in recent years due to its high octane number, higher blending capacity, low vapour pressure and higher energy content. The present study reports a novel wild strain, *Candida glabrata*, which produces isobutanol under submerged fermentation condition. Different process parameters have been optimized by both conventional as well as statistical methods. The production of isobutanol is enhanced in the presence of valine and this attribute the degradation pathway of valine is highly depend on isobutanol production. Under optimized condition, the yield of isobutanol is increased from 0.19 g/L to 0.96 g/L. This strain has not yet reported for the production of isobutanol.

Key words: Isobutanol; Lignocellulose; Biomass; *Candida glabrata*; Fermentation

1. Introduction

Deteriorating global energy reserves and the non-reliability on fossil fuels has brought in a serious search for renewable energy source. Biofuel is the most promising candidate that can be considered as an efficient energy source in future. Butanol is an important commodity chemicals with a variety of applications [1]. Isobutanol is one amongst the biofuel that can be considered as the most suitable biofuel because of its properties such as high energy content, high octane number, diminished flammability and hygroscopicity and effective compatibility with gasoline [2, 3]. Butanol production through fermentation could be a possible strategy by which lignocellulosic biomass can be efficiently utilized [4]. Over these years there are numerous studies have been reported for the production of isobutanol from various microbes, but the major challenges that hinders its commercialization could be its less productivity and the toxicity associated with the microbes [5]. Wild type yeast strains are capable of producing isobutanol via its valine degradation pathway. Keto acids (keto-isovalerate) with the α-carbon is the most common intermediate in the degradation or production of amino acids in Ehrlich pathway which is the major metabolic pathway for the production of higher alcohols in many microbes including yeast. The production of isobutanol by *Candida glabrata* through the valine degradation pathway is first of its kind and the genetics related to the pathway is poorly understood

As compared to the existing microbial pathway, yeast has reported a very well established native pathway for the production of isobutanol, but in very trace amounts in its native form [6]. Considering *Candida glabrata,* the higher tolerance towards isobutanol and its active native pathway for isobutanol synthesize is an added advantage for utilizing it as an excellent host. For the best production of microbial metabolite, a well identified production media is required with well established utilization profiling which effectively identifies the key media

components and hence it could improve the overall yield. Being a wild type strain, *Candida glabrata* has higher alcohol tolerance.

In *Candida* the valine degradation pathway is not clearly understood but the mechanism of action can be the uptake of valine as nitrogen source as it is indicated that the yeast generally utilize amino acids for their growth [7]. It is well established that the yeast strains are capable of degrading branched chain amino acids and aromatic amino acids through the Ehrlich pathway [8]. The initial stages of the valine degradation mainly occur in the mitochondrial complex and the proceeding Ehrlich pathway that initiate the conversion of the 2-ketoisovalerate to isobutanol occurs in the cytosol [9]. Pyruvates produced through glycolysis are transferred into the mitochondria through the mitochondrial pyruvate complex [10]. The available pyruvate in the mitochondria is the initial source through which the valine degradation pathway proceeds and the first conversion from pyruvate to acetolactate is catalyzed by the acetolactate synthase ILV2. The second step is the NADPH dependent step catalyzed by the enzyme ketoacidredectoisomerase ILV5 and it converts acetolactate to 2, 3-hydroxyisovalerate. The final step of the mitochondrial complex is the conversion of the 2, 3-hydroxyisovalerate to form 2-ketoisovalerate and process is a vital process because the end product of this reaction is the precursor for the valine biosynthesis and also for the isobutanol production via Ehrlich pathway in the cytosol, the reaction for the formation of ketoisovalerate is carried out by dihydroxyacid dehydrogenase ILV3.6 [11]. BAT1 and 2 are a very important genes that involved in the valine biosynthesis pathway that act an intermediate along 2-ketoisovalerate and BAT1 is associated with the mitochondrial complex and BAT2 is the cytosolic counterpart known as mitochondrial and cytosolic branched chain amino acid transaminases with reverse reaction activity [12].

2-ketoisovalerate can be considered among the most important intermediate which is crucial in the formation of isobutanol through a two stage process involving decarboxylation followed by a reduction reaction of the intermediate compound. The cytosolic phase of the isobutanol production is catalyzed by the activity of ketoacid decarboxylase and alcohol dehydrogenases. The oxo acid availability to the Ehrlich pathway is attributed through the activity of pyruvate decarboxylase and the decarboxylase activity in the genome is mainly due to the expression of the following genes PDC1, PDC5 and PDC6, 89 [13,14] but there are also two similar genes in the yeast such as ARO10 and THI3 which are also involved in the decarboxylation reaction in the Ehrlich pathway.10 [15]. Alcohol dehydrogenase that catalyze the conversion of isobuteraldehyde to the end product isobutanol is encoded in yeast genome with different genes with similar sequence similarity and the commonly encoded genes are ADH1, ADH2, ADH3, ADH4, ADH5 and SFA1.

In the present study isobutanol production by *Candida glabrata* was evaluated. Media components are the key entity in the fermentation process and thus understanding the key components that go into the production process is the most significant part for improved production from the wild type strain. Hence the concentrations of various media components were optimized through conventional as well as statistical means. Central Composite Design (CCD) is a statistical tool that helps to identify and optimize the important parameters in a process. The designs provided a critical insight into the necessary media components that are being involved in the pathway kinetics that lead to the production of compound of interest [16–18]. The process parameters optimization through CCD and its validation were carried out.

2. Materials and methods

2.1. Equipment specification and analysis method

Quantitative and qualitative analysis of isobutanol was performed using High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC). HPLC was performed using Phenomenex Rezex ROA organic acid column (300 x 7.8 mm). The mobile phase was 0.01N sulphuric acid at a flow rate if 0.6 mL/min. Oven temperature was maintained at 55°C. The sample injection volume was 20µL and samples were detected using Refractive Index (RI) detector.

For GC analysis, Shimadzu GC-2014 fitted with Zebron ZB- wax plus™ column (30 meter x 0.25 mm x 0.25 µm) and equipped with Flame ionization detector (FID) was used. Injector and detector temperature was set to 150°C and 250°C respectively. Oven temperature was initially set at 35°C and this temperature was maintained for 2 min and then it is increased to 100°C at an increment of 5°C/min and final temperature was 160°C with an increment of 60°C /min. Nitrogen was used as carrier gas.

UV spectrophotometer (Shimadzu UV-1601) was used for growth curve analysis of the organism. Growth over time of cell population was measured at 600 nm.

2.2. Isolation and screening of microbes

Soil samples were collected from Sikkim Himalayan regions and deep well soil of Kerala. Microbial samples were also isolated from Marcha (ethnic fermented beverages of Sikkim Himalayan Region) and standard yeast cells were also evaluated in the study. The samples were stored aseptically at 4°C. One gram of soil samples were enriched with YPD medium along with 0.1% isobutanol and incubated for about 48 hrs at 30°C at 200 rpm. Serial dilutions were done to obtain individual colonies which were further purified. Individual strains obtained were transferred to a production media containing YPD in combination with

different amino acids like valine and glycine [19, 20]. All the strains were checked for isobutanol production using HPLC.

2.2.1. Identification and characterization of isobutanol producing microorganisms

Morphological as well as microscopic characteristics were analysed using lacto-phenol cotton blue staining technique. Molecular identification was done using Qiagen Fungal DNA isolation kit. ITS sequence was amplified using universal set of primers. The reaction condition for the above reaction include master mix 10 µL, forward and reverse primer 1µL, 0.5 µL of the DNA template and 7.5 µL of nuclease free water. The reaction condition includes initial denaturation at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 1 min annealing at 54°C for 1 min elongation at 72°C for 1 min with a final elongation at 72°C for 10 min. The amplified products were purified using QIAGEN PCR purification kit and the sample was sequenced using Sanger's method. The sequenced product was aligned using clustal omega and the sequence was identified using the basic local alignment search tool.

2.2.2. Phylogenetic analysis of the isolate

To understand the related species phylogeny was analysed using UPGMA method. Fourteen different related species of yeast were taken for analysis. Literature report suggests that *Candida glabrata* is closely related to *Candida albicans* and also *Saccharomyces cerevisiae* [21]

2.3. Growth curve analysis of the isolate

To understand the growth characteristic of the isolate growth curve was checked. Initially microbial colony was inoculated into fresh Potato Dextrose Broth (PDB) media with an initial OD of 0.05. Incubation was carried out in a rotating shaker at 30°C for about 84 hrs.

OD was checked at a regular interval of 12 hrs at 600 nm using the UV spectrophotometer [22].

2.4. Optimization of fermentation media and conditions using single parameters

Different media like YPD (yeast extract peptone dextrose) with 0.5% valine and PDB with 0.5% valine were compared for the production of isobutanol. To understand the optimum culture conditions, single parameter optimization was performed initially. Parameters such as valine concentrations, pH, inoculum age, nitrogen source, and carbon concentrations were selected.

Valine concentration: Valine concentration of 0.5%, 1.0%, 1.5%, 2.0% were added to production media and the concentration change in isobutanol were analysed using gas chromatography after 96 hrs of inoculation.

pH optimization: 2% of seed medium was inoculated to the production media containing PDB and valine at different pH range of 4.0 to 9.0. Inoculated medium was incubated for 96 hrs and samples were collected and isobutanol concentration was quantified by HPLC.

Inoculum age optimization: Seed medium of different ages (12 hrs, 16 hrs, 20 hrs and 24 hrs) were inoculated and checked for production of isobutanol at different hours.

Effect of various nitrogen sources: Seed medium was inoculated to the production media containing different nitrogen sources like ammonium sulphate, ammonium chloride, yeast extract and peptone. The culture was incubated at rotating shaker at 200 RPM for 96 hrs.

Carbon concentration optimization: For the optimization of the concentration of glucose, different glucose concentrations ranging from 2% to 8% were selected and the production was quantified using gas chromatography.

2.5. Statistical analysis using design of experiment

Design of experiment was performed using Response Surface Methodology (RSM). Central composite design was selected to study the combinational effect of components like glucose, peptone and valine in potato dextrose broth. Total of 20 experiments were conducted based on design matrix shown in Table 1.

3. Results and discussion

3.1. Isolation and screening of microbes

Total of fifteen isolates were collected from different sources, out of which only three isolates were positive for isobutanol (Table 2). Isolate from Marcha sample showed highest isobutanol production as compared to other two isolate and hence it was selected further in the subsequent studies.

3.1.1. Identification and characterization of isobutanol producing microorganism.

Morphological and microscopic characteristics were visualised using Thermo Scientific™ Invitrogen™ evos™ fl Auto 2 Imaging System (Supplementary Fig 1). Molecular identification using BLAST showed 99% similarity towards *Candida glabrata*. The sequence received was submitted in GenBank and the GenBank accession number is MW218511.1

3.1.2. Phylogenetic analysis of the isolate

The evolutionary history was inferred using the UPGMA method. The optimal tree is shown in Supplementary Fig. 2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 14

nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 946 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

3.2. Growth curve analysis of the *Candida glabrata*

Growth curve analysis revealed that the lag phase is too short such that it enters the exponential phase within few hours and has its exponential phase till 36 hrs after which it enters the stationary phase as shown in Fig. 1. *C. glabrata* was reported the fastest growing species among *Candida*[23].

3.3. Optimization of fermentation media and condition using single parameters

Isobutanol production using PDB supplemented with valine was found to be 0.4 g/L while in media containing YPD; it showed a production of 0.2 g/L within 96 hrs. Hence PDB was further selected single parameter optimization studies.

3.3.1. Effect of valine concentration on isobutanol production by *C. glabrata*

There was a substantial change in production of isobutanol with change in valine concentration. According to the published reports based on the studies conducted in *Saccharomyces cerevisiae,* it is expected to produce isobutanol by valine degradation pathway [19, 24]. Since there are no reports on isobutanol production by *Candida glabrata* which is closely related to *Saccharomyces cerevisiae,* similar pathway is being anticipated for *C. glabrata* too. Isobutanol formation in *S. cerevisiae* results from valine degradation in the cytosol and the valine is synthesized from pyruvate in mitochondria during normal metabolic cycle. This spatial separation into two different cell compartments is one of the limiting factors for higher isobutanol production in yeast [25]. Hence if the valine is provided externally, it would be uptake directly to the cytosol and on its degradation, isobutanol is

produced. Our experiment showed a positive relation between valine and isobutanol concentration (Fig. 2).

3.3.2. Effect of pH on isobutanol production by *C. glabrata*: For the identification of optimum pH for the production of isobutanol, experiments were conducted at different pH ranges from 4.0 to 9.0, *Candida glabrata* was able to grow and produce isobutanol in all the tested ranges. The isobutanol production varied from 0.34 g/L to 0.49 g/L and maximum production was obtained at pH 5.0 (Fig. 3). The sensitivity of yeast cells to alcohol depends on the pH of the medium and earlier studies reported that sensitivity of the yeast cells to alcohol (ethanol) was marginally increased on decreasing the pH from 6.0 to 3.0 [26]. Our studies also indicate the similar pH pattern as there is low pH, the organism is able to produce more isobutanol and its tolerance.

3.3.3. Effect of Inoculum age on isobutanol production by *C. glabrata*: Inoculum age is one of the important parameters in microbial fermentation which determine the yield of final product. Optimization of inoculums age results the shortest lag phase during fermentation process. Inoculum age has a significant role in isobutanol production. Among the selected ages; 24 hrs old seed medium was found to be more productive than others as shown in Fig. 4. Maximum production of 0.6 g/L was obtained for 24 hrs old cultures.

3.3.4. Effect of various nitrogen source on isobutanol production by *C. glabrata*: To understand the effect of nitrogen source on isobutanol production, four different source like ammonium sulphate, ammonium chloride, yeast extract and peptone were evaluated. Inorganic nitrogen source has less effect as compared to organic source. Among the organic source peptone serves as an effective media component for isobutanol production (Fig. 5). The concentration of peptone was further optimized by RSM.

3.3.5. Effect of carbon concentration on isobutanol production by *C. glabrata*: Carbohydrate is an inevitable energy source of microorganism for their growth and development. Different concentrations of glucose were selected in the present study to understand the effect of carbon source on isobutanol production. With increase in concentration of glucose from 2% to 4% there was a rise in isobutanol production. Increasing the concentration beyond 6% showed a reduction in isobutanol production (Fig. 6).

3.4. Statistical analysis using design of experiment

The key components that affect isobutanol production are glucose, valine and peptone. So in order to study the suitable levels as well as to elucidate the combinational effect of all these components central composite design was done. The design matrix along with isobutanol yield is shown Table 3. This composed of independent variables of three level low (-1), medium (0) and high (+1). Isobutanol production ranges from 0.3 g/L to 0.96 g/L. Based on the result second order polynomial equation was generated by multiple regression analysis using Minitab17 software. The following regression equation presented below shows isobutanol concentration (Y) as a function of test variables X_1, X_2, X_3 where it represents glucose, peptone and valine respectively.

$$Y = 0.269 + 0.1420\ X_1 - 0.239\ X_2 + 0.1486\ X_3 - 0.00907\ X_1^2 + 0.0307\ X_2^2 - 0.0274\ X_3^2 - 0.0061\ X_1*X_2 - 0.00036\ X_1*X_3 + 0.0071\ X_2*X_3$$

Analysis of variance (ANOVA) for the quadratic equation was evaluated (Table 4). The model F value implies the model is significant. P value corresponds to the pattern of mutual interactions between the variables. P value less than 0.05 indicate that the model is significant. The coefficient of determination R^2 is 0.92 which implies 92.0% of the variability in the response could be explained by the model.

The effects of interaction between the variables were explained by the contour plot. Contour plot are represented in Fig 7A-C. Fig. 7A depicts the interaction between valine and glucose. Low level of both valine and glucose showed less isobutanol production. Increasing the glucose level in between 6.5% and 8% showed improved isobutanol production. In case of valine optimum level of 2% to 3.5 % showed more isobutanol production. Fig. 7B shows the relationship between peptone and glucose. Lower peptone level and higher glucose level of 5% to 9% showed higher isobutanol production. Fig.7C represents the interaction between valine and peptone. In this study also optimum valine ranges from 2% to 3.5% and too low level of peptone showed maximum isobutanol production.

There are no reports on the production of isobutanol by native *Candida* sp. Several studies have been done on the production of isobutanol by genetically modified strains of *Saccharomyces* [25, 27–29] . The native biosynthesis level of isobutanol in yeast is very low and the current maximum isobutanol production with *S. cerevisiae* is still far below the theoretical yields of 410 mg/g glucose [25, 30].Hence there is a need for genetic modification approaches to improve the butanol isomers [31–34]. Like *Saccharomyces*, *Candida* could also be a potent producer of isobutanol through genetic engineering techniques. The major advantage of using *Candida* for isobutanol production could be in the purview of sustainable utilization of agricultural residue because *Candida* is a xylose-utilizing yeast species with a high-capacity xylose transport system [35]. This makes an added advantage over *Saccharomyces* as it cannot utilize xylose, where as *Candida* can utilize both glucose and xylose and hence maximum sugar derived from the lignocelluloisc biomass could be utilized. This makes the process more viable with single-pot utilization of biomass derived sugars and hence it improves the isobutanol production. In this context, the present study would be highly significant as this organism could be genetically modified to relocate of all enzymes involved in anabolic and catabolic reactions of valine metabolism in cytosol so that overall

production of isobutanol could be improved. Once the strain is developed, this strain could be used for the production of isobutanol using lignocellulosic biomass derived hydrolysate where there is presence of mixed C_5 and C_6 sugars.

4. Conclusion

The present study reports the production of isobutanol by a wild type strain of *C. glabrata* through submerged fermentation approach. This organism is not yet reported for the production of isobutanol and addition of external sources of valine improves the isobutanol yield. Optimization of fermentation condition for isobutanol production was studied through single parameter optimization and response surface methodology. Initial production of 0.2 g/L was further increased to 0.9 g/L by our optimization studies. The isobutanol yield is higher compared to the other native strains and there are no reports of native yeast strains, without any genetic manipulation, producing this level of isobutanol production. Further deciphering the isobutanol pathway in *C. glabrata* and genetic manipulations may lead to higher rate of production.

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Table1: Design matrix for RSM for isobuatnol production by *Candida glabrata*

Run Order	Glucose (%)	Peptone (%)	Valine (%)
1	5.25	2.47	2
2	8	0.6	1
3	8	2	1
4	5.25	1.3	2
5	2.5	2	1
6	0.62	1.3	2
7	2.5	0.6	3
8	5.25	1.3	2
9	5.25	1.3	2
10	5.25	1.3	3.6
11	5.25	1.3	2
12	5.25	0.12	2
13	9.87	1.3	2
14	5.25	1.3	0.31
15	2.5	2	3
16	5.25	1.3	2
17	8	2	3
18	2.5	0.6	1
19	8	0.6	3
20	5.25	1.3	2

Table 2: Isobutanol production profile by positive isolates from different samples

Sample type	Positive isolate	Isobutanol production (g/L)
Yeast purchased from local market	2	0.06 g/L, 0.08 g/L
Marcha sample	1	0.19 g/L

Table 3: Design matrix with corresponding isobutanol production

Run Order	Glucose (%)	Peptone (%)	Valine (%)	Isobutanol (g/L)
1	5.3	2.5	2	0.531
2	8	0.6	1	0.794
3	8	2	1	0.507
4	5.25	1.3	2	0.626
5	2.5	2	1	0.320
6	0.63	1.3	2	0.370
7	2.5	0.6	3	0.578
8	5.25	1.3	2	0.674
9	5.25	1.3	2	0.609
10	5.25	1.3	3.7	0.745
11	5.25	1.3	2	0.612
12	5.25	0.1	2	0.963
13	9.9	1.3	2	0.622
14	5.25	1.3	0.3	0.486
15	2.5	2	3	0.367
16	5.25	1.3	2	0.669
17	8	2	3	0.585
18	2.5	0.6	1	0.529
19	8	0.6	3	0.779
20	5.25	1.3	2	0.643

Table 4: Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	0.471851	0.052428	13.48	0.000
Linear	3	0.388330	0.129443	33.29	0.000
Glucose	1	0.149647	0.149647	38.48	0.000
Peptone	1	0.209310	0.209310	53.82	0.000
Valine	1	0.029373	0.029373	7.55	0.021
Square	3	0.082208	0.027403	7.05	0.008
Glucose*Glucose	1	0.067798	0.067798	17.43	0.002
Peptone*Peptone	1	0.003254	0.003254	0.84	0.382
Valine*Valine	1	0.010820	0.010820	2.78	0.126
2-Way Interaction	3	0.001313	0.000438	0.11	0.951
Glucose*Peptone	1	0.001105	0.001105	0.28	0.606
Glucose*Valine	1	0.000008	0.000008	0.00	0.965
Peptone*Valine	1	0.000200	0.000200	0.05	0.825
Error	10	0.038889	0.003889		
Lack-of-Fit	5	0.024000	0.004800	1.61	0.307
Pure Error	5	0.014890	0.002978		
Total	19	0.510740			

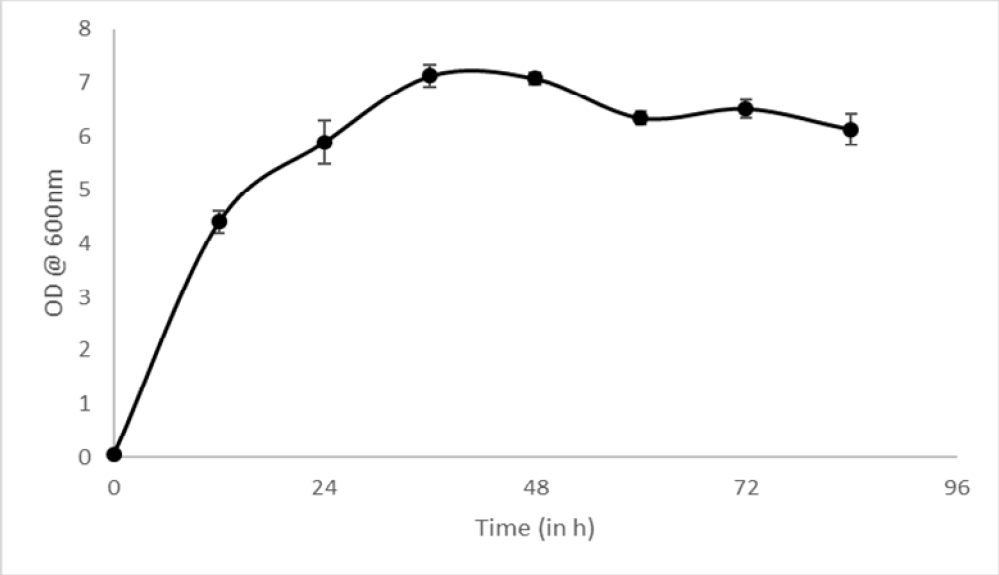


Fig. 1

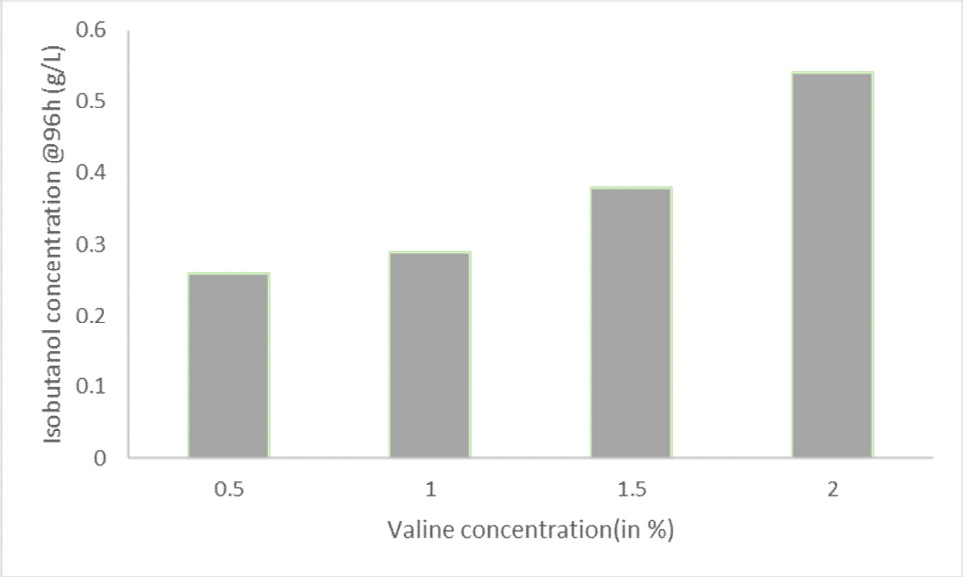

Isobutanol concentration @96h (g/L)
0.6
0.5
0.4
0.3
0.2
0.1
0
0.5
1
1.5
2
Valine concentration(in %)

Fig. 2

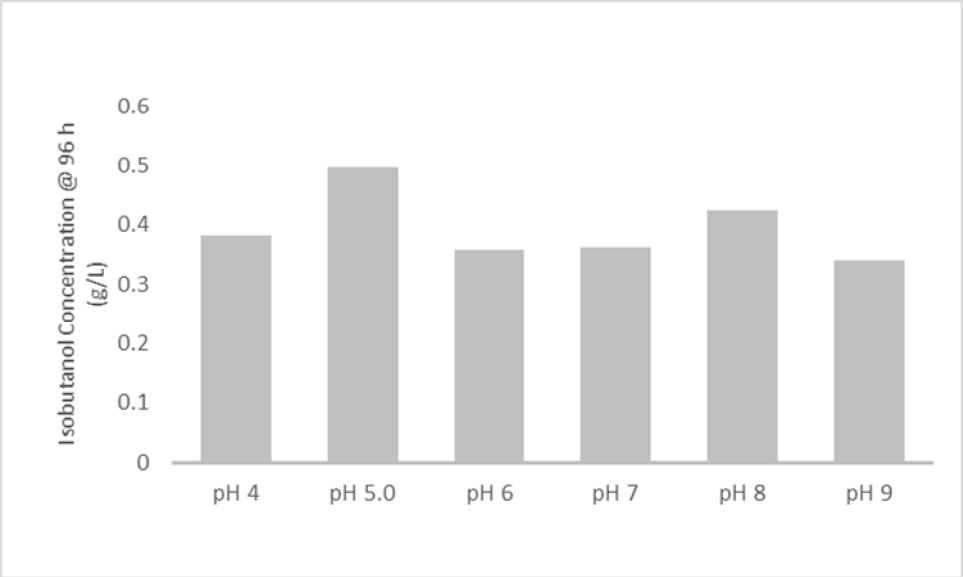

Isobutanol Concentration @ 96 h (g/L)
0.6
0.5
0.4
0.3
0.2
0.1
0
pH 4
pH 5.0
pH 6
pH 7
pH 8
pH 9

Fig. 3

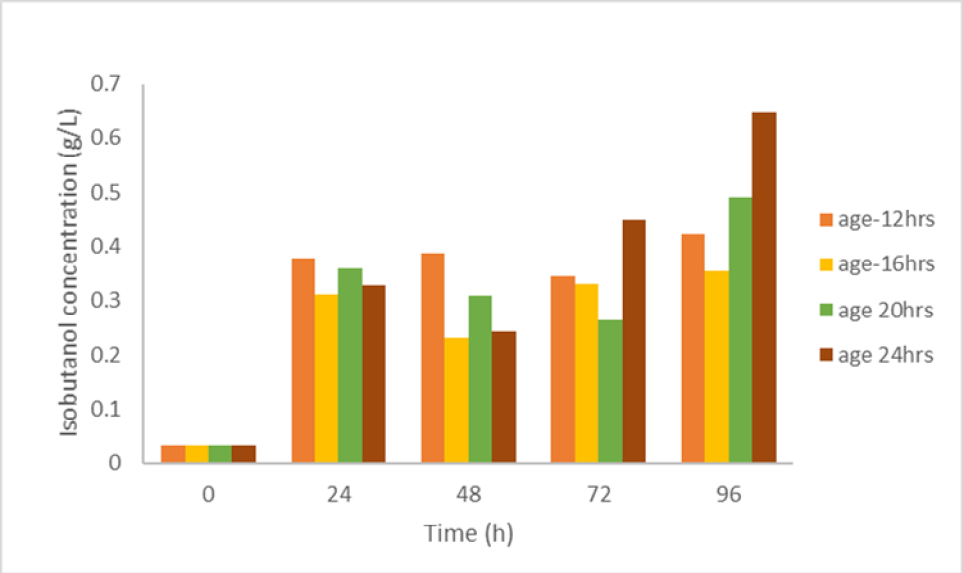

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Isobutanol concentration (g/L)
0
24
48
72
96
Time (h)
age-12hrs
age-16hrs
age 20hrs
age 24hrs

Fig. 4

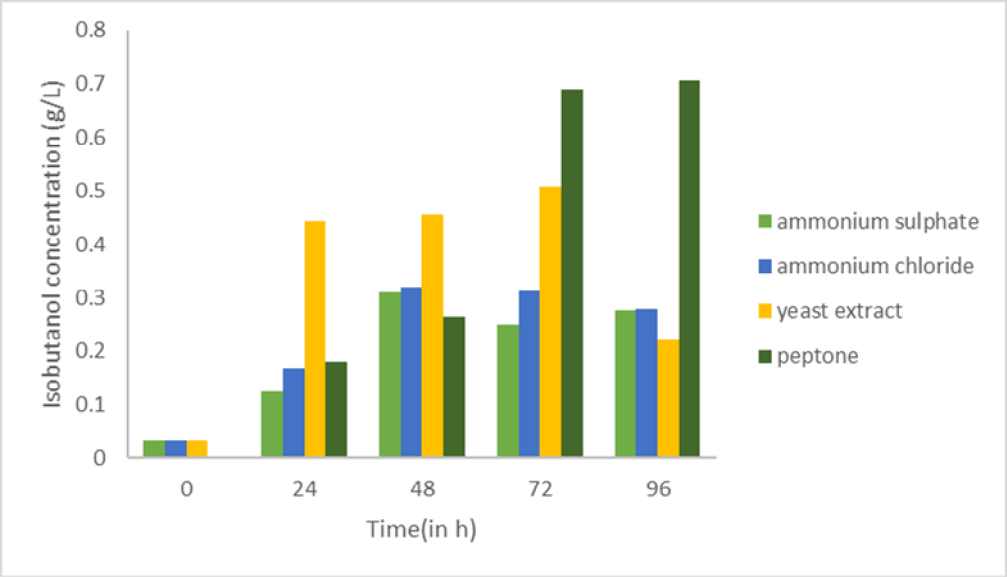

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Isobutanol concentration (g/L)
0
24
48
72
96
Time(in h)
ammonium sulphate
ammonium chloride
yeast extract
peptone

Fig. 5

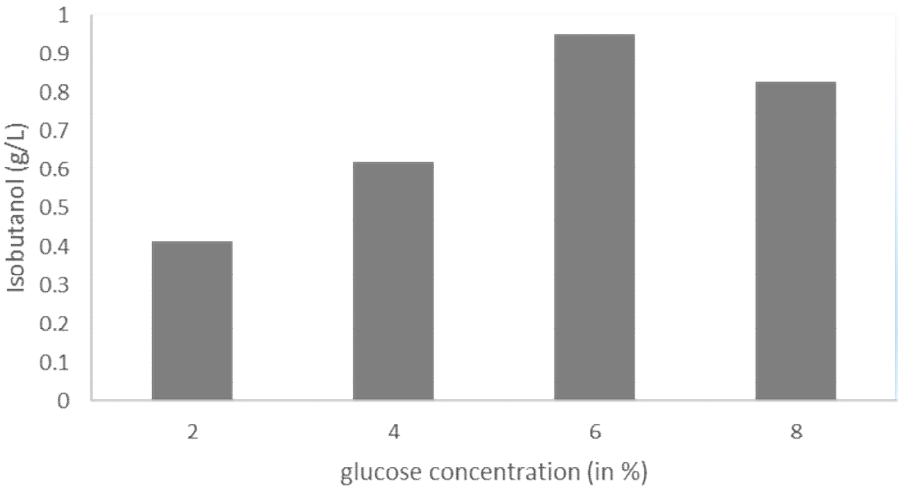

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Isobutanol (g/L)
2
4
6
8
glucose concentration (in %)

Fig.6.

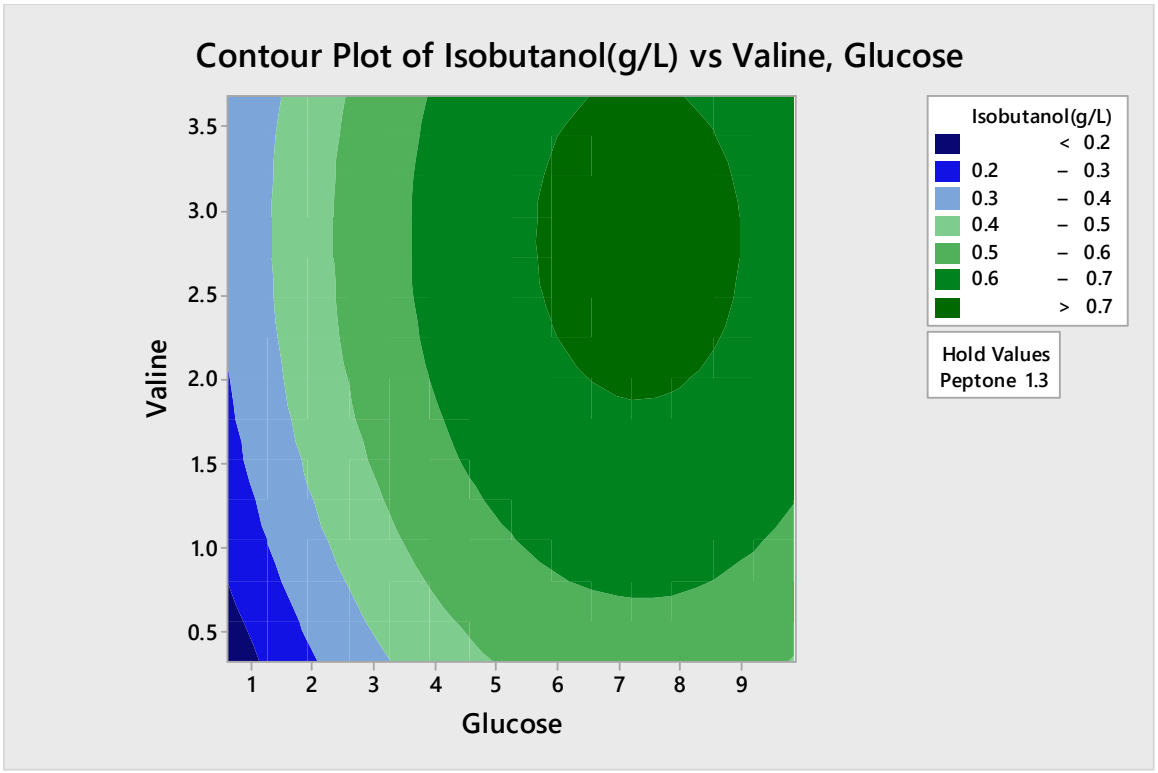

Contour Plot of Isobutanol(g/L) vs Valine, Glucose
Valine
Glucose
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1
2
3
4
5
6
7
8
9
Isobutanol(g/L)
< 0.2
0.2 – 0.3
0.3 – 0.4
0.4 – 0.5
0.5 – 0.6
0.6 – 0.7
> 0.7
Hold Values
Peptone 1.3

Fig. 7A

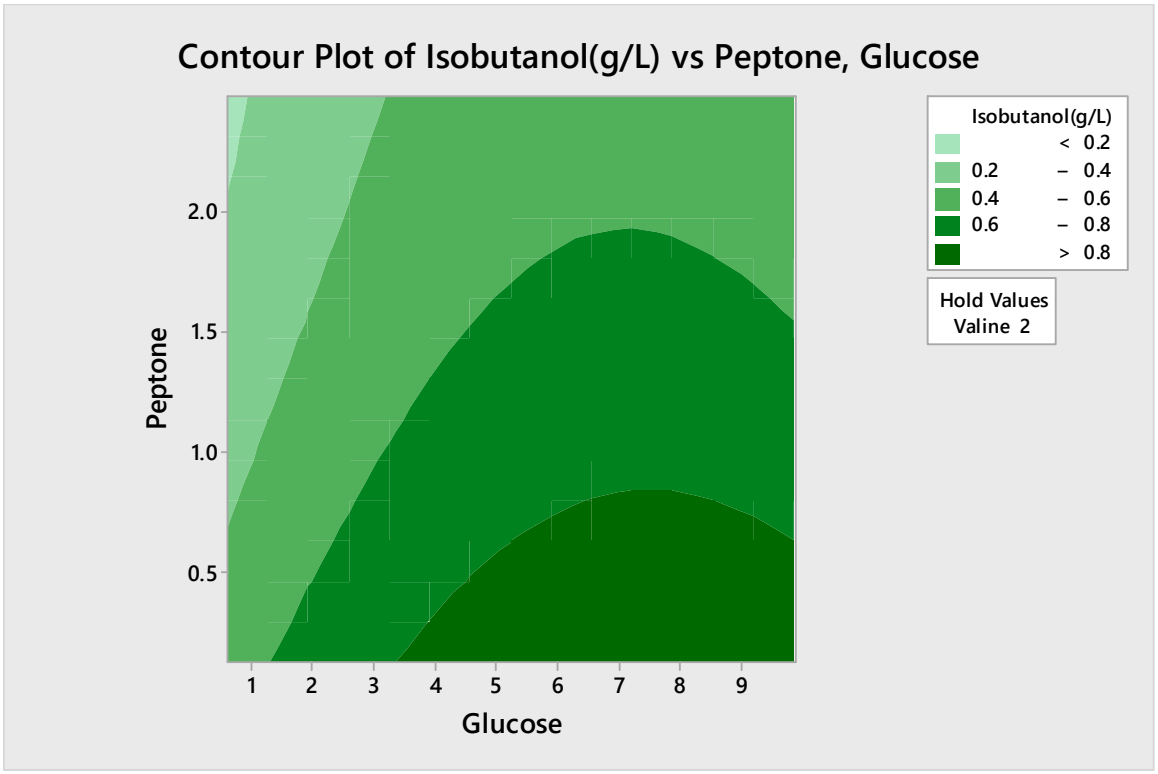

Contour Plot of Isobutanol(g/L) vs Peptone, Glucose
Peptone
Glucose
2.0
1.5
1.0
0.5
1
2
3
4
5
6
7
8
9
Isobutanol(g/L)
< 0.2
0.2 – 0.4
0.4 – 0.6
0.6 – 0.8
> 0.8
Hold Values
Valine 2

Fig. 7B

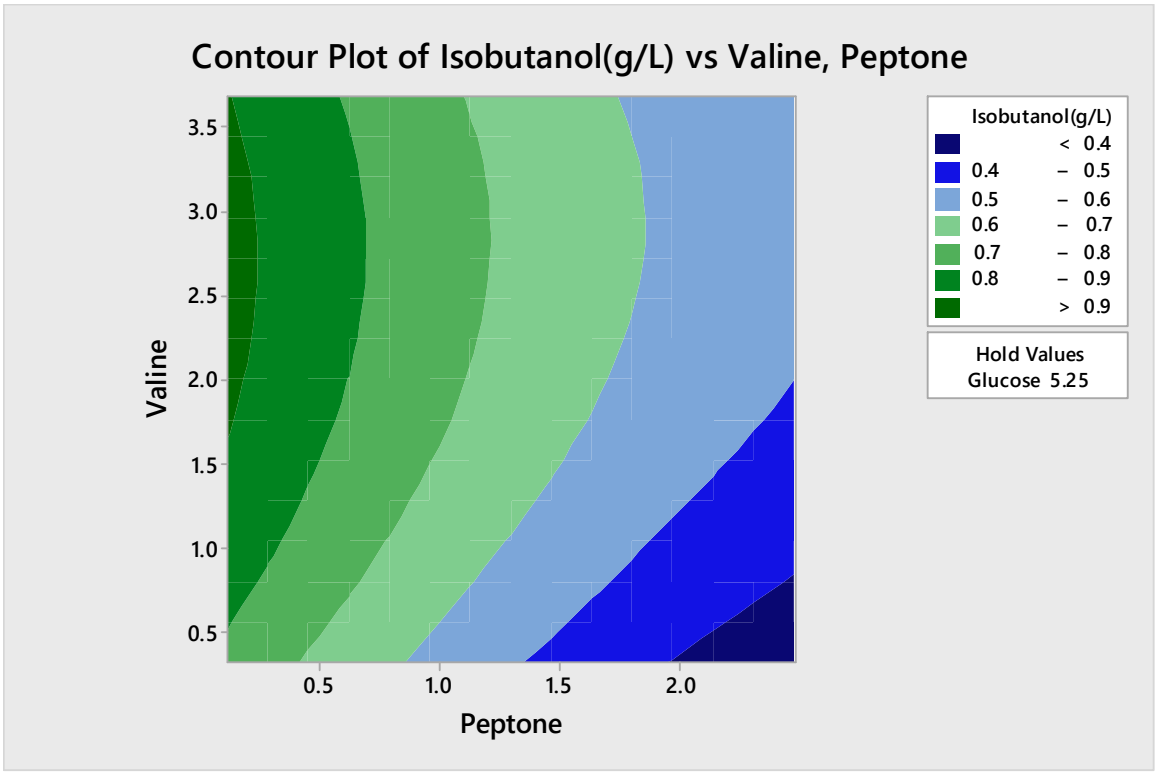
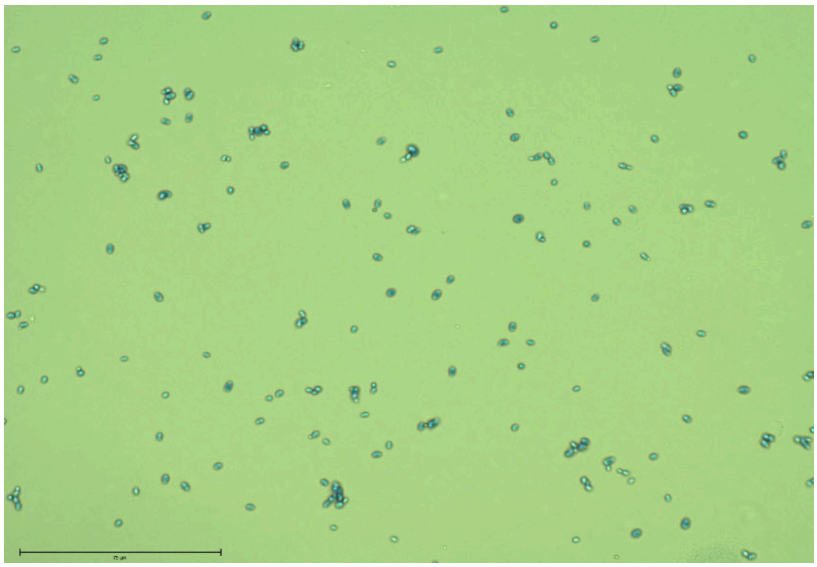
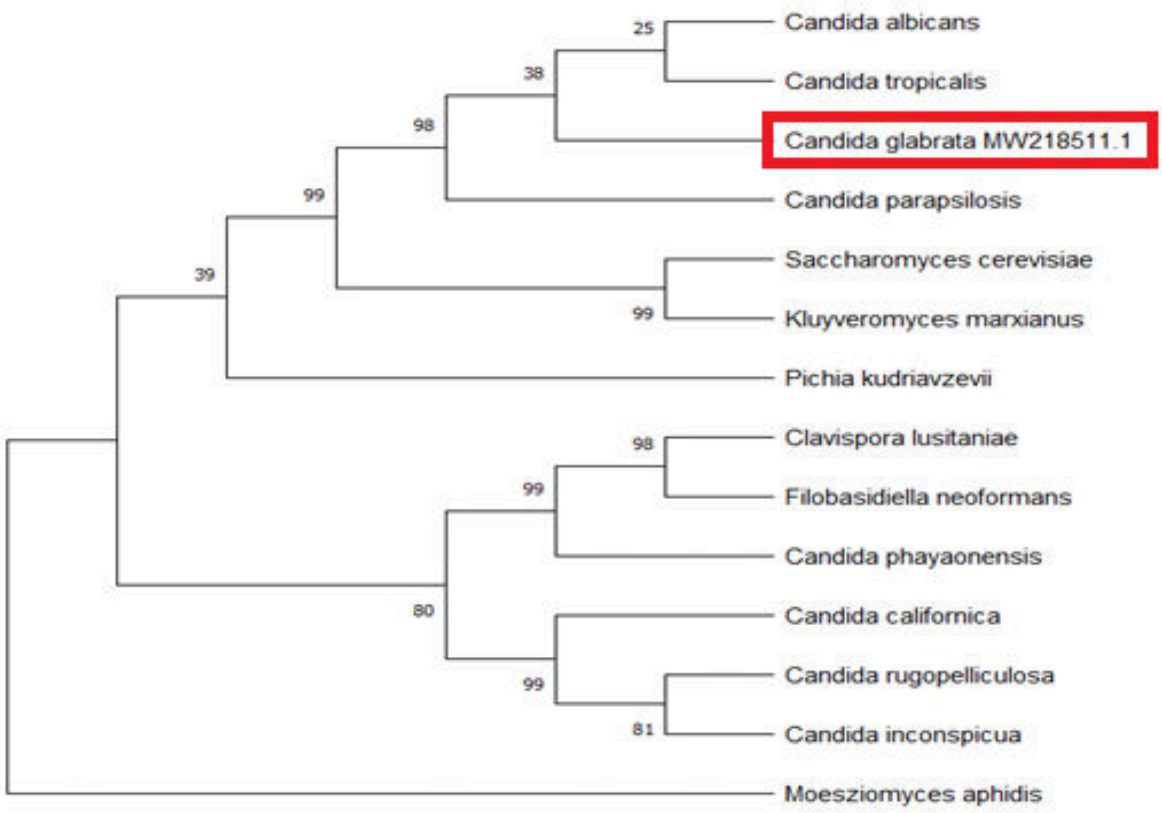

Contour Plot of Isobutanol(g/L) vs Valine, Peptone
Valine
Peptone
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.5
1.0
1.5
2.0
Isobutanol(g/L)
< 0.4
0.4 – 0.5
0.5 – 0.6
0.6 – 0.7
0.7 – 0.8
0.8 – 0.9
> 0.9
Hold Values
Glucose 5.25

Fig. 7 C



Supplementary Fig.1.



Supplementary Fig.2.