OPTIMIZATION OF INITIAL PH AND INITIAL GLUCOSE CONCENTRATION FOR MAXIMUM ETHANOL PRODUCTION WITH RESPECT TO DIFFERENT FERMENTATION KINETIC PARAMETERS BY USING *S.CEREVISAE* AND CHEMICALLY DEFINED MEDIUM.

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Abstract—**This study presents the work regarding optimization of initial pH and initial Glucose concentration (% W/V) for maximum Ethanol production with respect to different fermentation kinetic parameters by using S.cerevisae and chemically defined medium. Ethanol is growth associated by-product synthesized by S.cerevisae and its yield (g/g) is very sensitive to the changes in the initial pH and initial Glucose concentration (g/L).Hence batch experiments were designed to optimize initial pH and initial Glucose concentration mainly with respect to maximum Ethanol yield (g/g), maximum glucose utilization rate (%) and specific growth rate (hr^{-1}).Other kinetic parameters like Biomass productivity (g/L-hr),Biomass yield (g/g),Ethanol productivity (g/L-hr) and fermentation efficiency (%) are also studied. Random experiments were designed to optimize one parameter at a time with different values. The initial pH values selected were 4.0,4.5,4.75 and 5.0.The initial Glucose concentration (% W/V) selected were 5,10,15 and 20.The optimum pH for maximum ethanol yield (0.453g/g), fermentation efficiency (88.63 %) and maximum ethanol production(39.73 g) was found to be 4.5 and it is used for the optimization of initial glucose concentration in later studies. The optimum initial glucose conc.(% (W/V)) for maximum ethanol yield (0.487g/g), fermentation efficiency (43.42 %) and maximum ethanol production(39.73 g) was found to be 10 % (W/V) .Glucose concentration is monitored with DNS assay, yeast biomass was analysed by absorbance at 525 nm and ethanol production is monitored by alcoholmeter after applying temperature correction factors.**

***Index Terms*—S.*cerevisae*, Ethanol, yield, alcoholmeter and kinetic parameters.**

INTRODUCTION

Bio-ethanol is an eco-friendly fuel that can be used in unmodified petrol engines. Combustion of ethanol results in relatively low emission of volatile organic compounds, carbon monoxide and nitrogen oxides. The emission and toxicity of ethanol are lower than those of fossil fuels such as petroleum, diesel etc. *Saccharomyces cerevisiae* is the cheapest strain used for bio-ethanol production from sugar molasses. *S.cerevisae* is capable of very rapid rates of ethanol production under optimal conditions.[1] The largest single use of ethanol is as a motor fuel and fuel additive.[2]

S.cerevisae is highly sensitive to initial pH and initial sugar concentration. At higher pH and sugar concentration it favours acid production and decrease in ethanol production respectively.[1] Ethanol production by *S.cerevisae* is influenced by various factors like initial pH, aeration rate, and temperature and sugar concentration. [3] The use of bioethanol as gasoline oxygenate is beneficial in terms of

higher oxygen content, octane number and reduction of CO emission.[4] Fed batch system for fermentation has advantages over batch processes like higher productivity, higher dissolved oxygen in the medium, decreased fermentation time and reduced toxic effects of the medium components, which are present at high concentrations.[5] Wild type *S. cerevisiae* has limitation being unable to ferment pentoses and hard efforts have been made to design a suitable engineered *S. Cerevisiae*.[6]

Ethanol production by *S.cerevisae* is very sensitive to initial pH and sugar concentration. This research aims at finding optimum pH and sugar concentration with respect to high ethanol yield, fermentation efficiency, sugar utilization rate, biomass yield and productivity etc.

MATERIALS AND METHODS

Revival of dry Baker's yeast powder:

0.5 % (W/V) of dry Baker's yeast powder is suspended in 50 ml of sterile distilled water in 100 ml sterile conical flasks and kept in orbital shaker incubator at 30°C and at 150 rpm for 15 minutes.

Maintenance of Baker's yeast culture:

1 ml of revived yeast culture is transferred to sterile YEPD broth(yeastextract-10g/L,Peptone-20g/L,Glucose-100g/L,pH-4.5) and kept for incubation for 3 days. After sufficient growth, the broths were preserved in refrigerator for further use. One loop full culture is streaked on YEPD agar slants and incubated at 30 °C for 3 days. After sufficient growth, the broths were preserved in refrigerator for further use. Aseptic conditions were maintained.

Determination of Glucose Concentration:

Glucose concentration is determined by DNS assay. Glucose standard stock (1 g/L) is prepared in distilled water and is used to prepare glucose solutions with different concentrations .Optical density is determined by using Spectrophotometer Elico SL-159 adjusted at 550 nm. Standard graph is obtained by plotting Concentration of Glucose (g/L) on X axis and corresponding optical density at 550 nm on Y axis. The Glucose concentration of suitably diluted fermented broth samples was estimated by using this curve. 1 ml of previously centrifuged (without Biomass) fermented broth sample is suitably diluted to obtain OD within calibration of standard glucose curve.3 ml of this diluted broth sample is mixed with 3 ml of DNS reagent (Dinitrosalicyli acid-10 g,Phenol-2 ml,Sodium sulfite-0.5 g and Sodium hydroxide (0.4 M)-10 ml,make up the volume to 1000 ml with distilled water.) and heated in boiling water bath for 15 minutes till red brick colour developed.1 ml of Rochelle salt was added and allowed to cool. OD is recorded at 550 nm. The residual sugar concentration is then determined by standard glucose curve .Distilled water and DNS is taken as blank. [7]

Determination of Biomass Concentration:

50 ml of fermented broth samples were periodically and aseptically removed. The fermented broth sample is centrifuged at 7000 rpm for 10 minutes . The pellet is separated from supernatant and dried in incubator till constant weight is obtained. The supernatant is stored and analyzed for determination of sugar concentration and ethanol production. The dry yeast biomass weight is then diluted to with distilled water and OD is recorded at 525 nm. By using standard yeast dry weight graph, actual biomass concentration was determined and is then expressed in g/L. [7]

Determination of Ethanol Concentrations:

Approximately 40 ml of fermented broth is centrifuged at 7000 rpm and supernatant is taken in 50 ml measuring cylinder and the room temperature is noted down. The Alcoholmeter was allowed to dip freely without touching the inner walls of measuring cylinder. The readings on Alcoholmeter were noted down and the correction factor was applied to measure % (V/V) alcohol content of broth. The corrected % (V/V) alcohol content value is then multiplied with density of ethanol (at temperature at which the readings were taken) to give % (W/V) of ethanol. This value is then expressed in terms of ethanol concentration (g/L).Ethanol content of final samples was confirmed by distillation at 76^0C estimated by using alcoholmeter. Ethanol is estimated by Gay Lussac Alcoholmeter. [7]

Determination of fermentation kinetic parameters:

The values of fermentation kinetic parameters can be determined by using following formulas or Method,

Biomass productivity (g/L-hr**):** Slope of the graph obtained by plotting Biomass concentration (g/L) against time (hr).

Biomass yield (g/g) = Biomass dry weight/Mass of Glucose utilized

Determination specific growth rate μ (hr^{-1}): Slope of curve obtained by plotting lnX (g/L) against time (hr).

Determination of Ethanol productivity (g/L-hr): Ethanol productivity (g/L-hr): Slope of the graph obtained by plotting Ethanol concentration
(g/L) against time (hr).

Ethanol yield (g/g): Weight of ethanol produced/ Mass of Glucose utilized.

% sugar Conversion: [(Initial sugar Conc. – Final sugar Conc.) / Initial Sugar Conc.] * 100.

Sugar Utilization rate (g/hr): Slope of graph obtained by plotting sugar utilized (g) against time (hr).

Fermentation Efficiency (%): (Ethanol yield/Max. possible true yield for Ethanol) * 100.

Maximum Biomass Conc. (g/L**):** Highest value of Biomass Conc. (g/L).

Maximum Ethanol Conc. (g/L): Highest value of Ethanol Conc. (g/L).

Optimization of initial pH value:

Initial pH optimization is carried out by using defined medium-Normal strength working nutrient medium with composition ($MgCL_2.6H_2O$- 0.52 g/L, $(NH4)_2SO_4$- 12.0 g/L, $H3PO_4$ (85%)-1.6 mL/L,KCl-0.12 g/L,$CaCl_2.2H_2O$-0.2 g/L, NaCl-0.06 g/L,$MnSO_4.H_2O$-0.024 g/L,$CaSO_4.5H_2O$-0.0005 g/L,H_3BO_3-0.0005g/L,$Na_2MoO_4.2H_2O$-0.002g/ L,NiCl-0.0025mg/L,ZnSO4.$7H_2O$-0.012g/L,Co $SO_4.7H_2O$-0.0023mg/L,KI-0.0001g/L,$FeSO_4(NH4)_2SO_4.6H_2O$-0.035g/L,Myo-Inositol-0.125 g/L,Pyridoxine-HCL (Vit- B6)-0.00625 g/L,Ca-n-Pantothenate-0.00625 g/L, Thiamine-HCL (Vitamin B1)-0.005 g/L, Nicotinic Acid-0.005 g/L,D-Biotin (Vitamin H0-0.000125 g/L,EDTA- 0.1 g/L,Glucose-50 g/L).2 L of Normal strength working nutrient medium is prepared out of which 400 ml is distributed in 4 separate 500 ml sterile flaks and pH is adjusted accordingly (pH-4.0,4.5,4.75,5.0) and were labelled carefully. The media were autoclaved at 15 psi for 15 min and allowed to cool at room temperature. Meanwhile the preserved Baker's yeast culture flask was removed and kept in room temperature.20 ml inoculums from this is transferred aseptically to each of above flasks. The flasks were incubated at 30°C in an orbital shaker incubator for 72 hr. The samples were aseptically removed from the fermentation flasks and analyzed for yeast biomass dry weight (g/L), Glucose concentration and for estimation of Ethanol produced (g/L).The initial pH value which gives better ethanol production is selected for further experimentation.

Optimization of Sugar Concentration:

Sugar concentration optimization is carried out by using defined medium-Normal strength Working nutrient medium at pH 4.5 and different sugar concentrations.2 L of Normal strength working nutrient medium is prepared and pH was adjusted to 4.5, out of which 400 ml is distributed in 4 separate 500 ml sterile flaks and Sugar conc. is adjusted accordingly (sugar concentrations % (W/V):5,10,15,20) and were labelled carefully. The media were autoclaved at 15 psi for 15 min and allowed to cool at room temperature. Meanwhile the preserved Baker's yeast flask was removed and kept in room temperature. 20 ml (inoculum) from this is transferred aseptically to each of above flasks. The flasks were incubated at 30°C in an orbital shaker incubator for 72 hr. The samples were aseptically removed from the fermentation flasks and analyzed for yeast biomass dry weight (g/L), Glucose concentration and for estimation of Ethanol produced (g/L).The initial sugar concentration value which gives better

ethanol production is selected for further experimentation.

OBSERVATIONS

Observations For initial pH:

Table 1:

Biomass concentration (g/L) against time (hr) at different initial pH values. [Temperature 30°C, fermentation time 72 hr, Glucose Concentration 10% (W/V)].

Time (hr)	pH-4.0	pH-4.5	pH-4.75	pH- 5.0
0	0.4	0.4	0.4	0.4
6	0.6	0.8	0.44	0.62
24	2.6	0.9	0.84	2.04
30	4	4.6	1.04	3.8
48	5.8	7	3.8	5
54	8.6	9.8	5.98	5.8
72	10.02	11	10.2	8.2

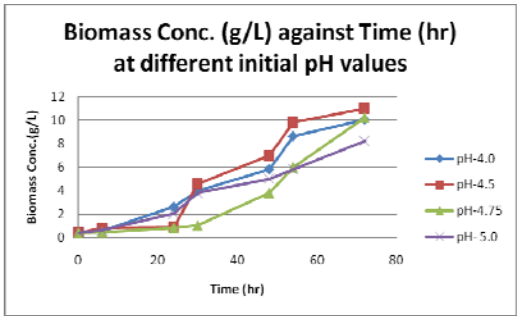

Fig.1 Biomass concentration (g/L) against time (hr) at different initial pH values.

Table 2

Residual Glucose concentration present in fermentation broth (g/L) against time (hr) at different initial pH values. [Temperature 30°C, fermentation time 72 hr, Glucose Concentration 10% (W/V)].

Time (hr)	pH-4.0	pH-4.5	pH-4.75	pH- 5.0
0	100	100	100	100
6	95	89	93	97
24	72	65	76	79
30	65	59	69	73
48	51	30	31	52
54	45	23	22	51
72	20	13	15	32

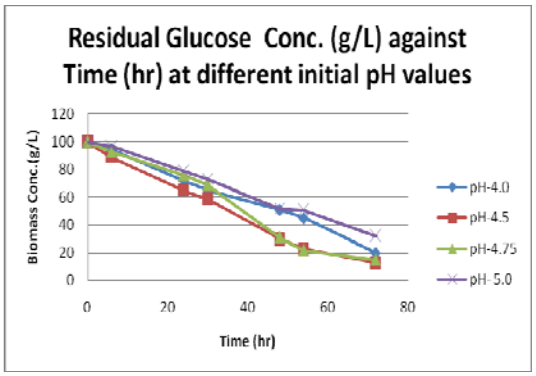

Fig.2 Residual Glucose concentration present in fermentation broth (g/L) against time (hr) at different initial pH values.

Table 3. Ethanol produced (g/L) against time (hr) at different initial pH values. [Temperature 30°C, fermentation time 72 hr, Glucose Concentration 10% (W/V)].

Time (hr)	pH-4.0	pH-4.5	pH-4.75	pH- 5.0
0	0	0	0	0
6	0	0	0	0
24	0	0	0	0
30	0	19.736	0	0
48	15.789	27.631	22.894	19.736
54	23.684	35.525	27.631	23.684
72	27.631	39.473	35.525	27.631

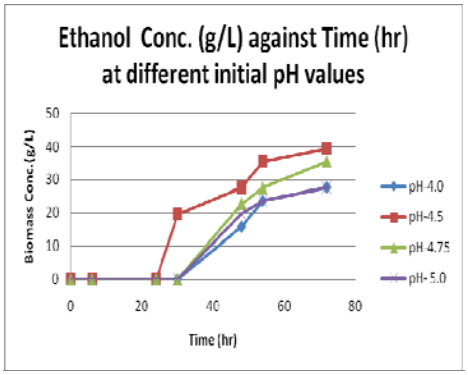

Fig.3 Ethanol produced (g/L) against time (hr) at different initial pH values.

Observations For initial Glucose conc.:

Table 4.

Biomass concentration (g/L) against time (hr) at different initial Glucose concentration (%W/V). [Temperature 30°C, fermentation time 72 hr, pH 4.5]

Time (hr)	Glucose 5%	Glucose 10 %	Glucose 15 %	Glucose 20 %
0	0.4	0.4	0.4	0.4
6	0.6	0.9	0.44	0.67
24	2.8	2.5	2.73	1.7
30	3	3.9	2.98	2.2
48	4.5	5.2	3.97	3.9
54	5.2	5.9	4.5	4.2
72	5.8	11.5	8.5	4.9

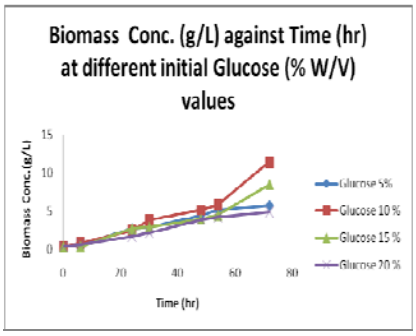

Fig.4 Biomass concentration (g/L) against time (hr) at different initial Glucose concentration(%W/V).

Table 5. Residual Glucose concentration present in fermentation broth (g/L) against time (hr) at different initial Glucose concentration. [Temperature 30°C, fermentation time 72 hr, pH 4.5].

Time (hr)	Glucose 5%	Glucose 10 %	Glucose 15 %	Glucose 20 %
0	50	100	150	200
6	48	85	142.5	196
24	40.5	63	130.5	178
30	34	55	91.5	154
48	22	31	70.5	126
54	14	22	79.5	118
72	7	11	61.5	110

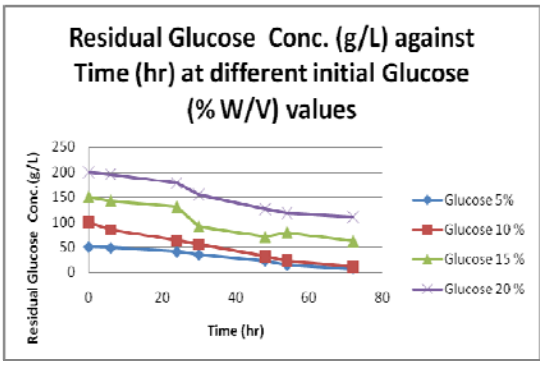

Fig. 5 .Residual Glucose concentration present in fermentation broth (g/L) against time (hr) at different initial sugar concentration.

Table 6.Ethanol produced (g/L) against time (hr) at different initial Glucose concentration. [Temperature 30°C, fermentation time 72 hr, pH 4.5].

Time (hr)	Glucose 5%	Glucose 10 %	Glucose 15 %	Glucose 20 %
0	0	0	0	0
6	0	0	0	0
24	0	0	0	0
30	0	23.648	0	0
48	0	27.631	0	0
54	0	35.525	27.631	23.648
72	15.789	43.42	35.525	31.578

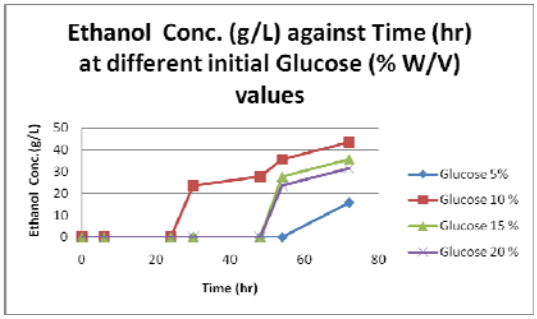

Fig.6. Ethanol produced (g/L) against time (hr) at different initial Glucose concentration.

RESULTS AND DISCUSSION:

Table 7. comparison of different kinetic parameters at different initial pH values Glucose conc.-100g/L,Temp.-30 ^{0}C, at different initial pH values):

Sr. No.	Parameter	pH-4.0	pH-4.5	pH-4.75	pH-5.0
1	Biomass Productivity (g/L-hr)	0.141	0.163	0.13	0.108
2	Biomass yield (g/g)	0.125	0.126	0.12	0.12
3	Specific growth Rate (hr^{-1})	0.046	0.048	0.049	0.042
4	Ethanol productivity (g/L-hr)	0.435	0.632	0.555	0.449
5	Ethanol yield (g/g)	0.345	0.453	0.417	0.406
6	Maximum Ethanol Conc. (g/L)	27.631	39.473	35.525	27.631
7	% Glucose utilized	80	87	85	68
8	Glucose Utilization rate (g/hr)	1.077	1.267	1.31	0.966
9	% Fermentation Efficiency	67.5	88.63	81.6	79.49

10	Maximum Biomass Conc. (g/L)	10.02	11	10.2	8.2

Maximum Biomass productivity is observed at initial pH of 4.5 which corresponds to 0.163 g/L-hr. Maximum Biomass yield is observed at initial pH of 4.5 which corresponds to 0.126 g/g. Maximum Specific growth rate is observed at initial pH of 4.75 which corresponds to 0.049 hr^{-1}.Maximum Ethanol productivity is observed at initial pH of 4.5 which corresponds to 0.632 g/L-hr. Maximum Ethanol yield is observed at initial pH of 4.5 which corresponds to 0.453 g/g. Maximum Ethanol Conc. is observed at initial pH of 4.5 which corresponds to 39.473 g/L. Maximum % Glucose utilization is observed at initial pH of 4.5 which corresponds to 87 %. Maximum Glucose utilization rate is observed at initial pH of 4.5 which corresponds to 1.267 g/hr. Maximum fermentation efficiency is observed at initial pH of 4.5 which correspond to 88.63 %. Maximum Biomass Conc. is observed at initial pH of 4.5 which corresponds to 11 g/L.

Table 8 Comparison of different kinetic parameters at different initial Glucose concentration:

Sr.No.	Parameter	Glucose 5%	Glucose 10 %	Glucose 15 %	Glucose 20 %
1	Biomass Productivity (g/L-hr)	0.08	0.138	0.101	0.067
2	Biomass yield (g/g)	0.135	0.129	0.096	0.054
3	Specific growth Rate (hr^{-1})	0.037	0.042	0.042	0.035
4	Ethanol productivity (g/L-hr)	0.148	0.666	0.473	0.416
5	Ethanol yield (g/g)	0.367	0.487	0.401	0.35
6	Maximum Ethanol Conc. (g/L)	15.789	43.42	35.525	31.578
7	% Glucose utilized	86	89	59	45
8	Glucose Utilization rate (g/hr)	0.637	1.252	1.326	1.407
9	% Fermentation Efficiency	71.8	95.3	78.47	68.49
10	Maximum Biomass Conc. (g/L)	5.8	11.5	8.5	4.9

Maximum Biomass productivity is observed at initial Glucose Conc. Of 10 % W/V which corresponds to 0.138 g/L-hr. Maximum Biomass yield is observed at initial Glucose Conc. Of 10 % W/V which corresponds to 0.129 g/g. Maximum Specific growth rate is observed at initial Glucose Conc. Of 10 % and 15 % W/V which corresponds to 0.042 hr^{-1}.Maximum Ethanol productivity is observed at initial Glucose Conc. Of 10 % W/V which corresponds to 0.666 g/L-hr. Maximum Ethanol yield is observed at initial Glucose Conc. Of 10 % W/V which corresponds to 0.487 g/g. Maximum Ethanol Conc. is observed at initial Glucose Conc. Of 10 % W/V which corresponds to 43.42 g/L. Maximum % Glucose utilization is observed at initial Glucose Conc. Of 10 % W/V which corresponds to 89 %. Maximum Glucose utilization rate is observed at initial Glucose Conc. Of 20 % W/V which corresponds to 1.407 g/hr. Maximum fermentation efficiency is observed at initial Glucose Conc. Of 10 % W/V which correspond to 95.3 %.Maximum Biomass Conc. is observed at initial Glucose Conc. Of 10 % W/V which corresponds to 11.5 g/L.

CONCLUSION

The fermentation kinetic parameters are very sensitive to initial pH and Sugar concentration. We need to select the experimental conditions depending on the product we are interested in. No single experimental condition is ideal to get all the kinetic parameters at optimum value.

REFERENCES

[1] Arifa T., Madiha A. & Tasnim F.," Effect of cultural conditions on ethanol production by locally isolated *saccharomyces cerevisae* BIO-07," J App Pharm,Vol 3,(2),pp 72-78 ,2010.

[2] Janani K., Ketzi M., Megavathi S., Dr.Vinothkumar D., Dr. Ramesh Babu N.G.," I J of Inno. Res. in Sci., J. Engg. and Tech.," Vol. 2, Issue 12, December 2013.

[3] Yan L.,Wei Z.,Chunjie l.,Kei S.,Shuzo T.,Hainan K.," Factors affecting ethanol fermentation using *Saccharomyces cerevisiae*

hBY4742," Biomass and Bioenergy,vol xxx ,1,7,September 2012.

[4] Farah A., Ahmad J., Mohd K. and Maizirwan M., Study of growth kinetic and modeling of ethanol production by *Saccharomyces cerevisae"* African Journal of Biotechnology Vol. 16(81), pp. 18842-18846, 16 December, 2011.

[5] Zarifa R., Dr. Babar S., Md. Enayetul, Akbor Md. A.," Process Optimization for Batch Culture of *Saccharomyces cerevisiae*," Sch. Acad. J. Biosci., 1(7):363-373,2013.

[6] Udhayaraja P. and Narayanan S.," Optimization for production of bioethanol using sorghum stovar by *Saccharomyces cerevisiae,*" Int. J. of Res. in Pure and App. Microbiology,vol 2,4,pp 64-67,October 2012.

[7] Chavan Ram.,Saxena K.,Manoj K.,"Comparison of biomass and ethanol production kinetics of commercial baker's yeast (*S.cerevisae*) in a chemically defined medium against cane molasses,waste banana pulp and waste potato flour," International J. of App. Engg. Research,vol 8,15, pp 1853 – 1860,2013.