Optimization of Fermentation Parameters for R3DSC5 and R3DPMP Strains for Ethanol Production

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Abstract

Two newly isolated strains of *Saccharomyces cerevisiae* - R3DPMP and R3DSC5 were subjected to optimization studies with varying inoculum size, initial glucose concentration, initial pH and temperature using yeast extract peptone dextrose medium. Both the strains accumulated peak ethanol early with higher inoculum density and exhibited similar pattern with changing initial pH. Though higher glucose tolerance was noticed, 20% g/v and 25% g/v glucose concentration were found to be optimum for R3DPMP and R3DSC5 respectively. Similar amount of ethanol was accumulated within 30-40^{0}C temperature by R3DPMP and 30-35^{0}C temperature by R3DSC5. However, based on higher temperature tolerance, sugar tolerance and peak ethanol level, R3DSC5 appears to be superior to R3DPMP. Therefore, R3DPMP strain is recommended for brewery industry whereas R3DSC5 for both brewery and very high gravity fermentation.

Keywords: *Saccharomyces cerevisiae,* ethanol, tolerance, Very High Gravity fermentation.

Biofuel research gained huge importance due to the projected rapid decrease in fossil fuel reserves because of increased global demand (Campbell and Laherrere, 1998). Use of ethanol as fuel is expected to reduce climatic change and global warming (Sheehan and Himmel, 1999) by bringing about 86% reduction in greenhouse gas emissions (Wang, 2005) and increased interest to develop rural economies by establishing agro-dependent industries (Oscar and Carlos, 2008).

Bioethanol is produced by the fermentation of sugars by microorganisms such as *Saccharomyces cerevisiae, Zymomonas mobilis* (Gi-Wook *et al.,* 2008)*, Mucor indicus* (Anna *et al.,* 2005), thermophillic bacteria like *Clostridium thermocellum* and *C. thermohydrosulfuricum* (Lovitt *et al.,* 1984), filamentous fungi-*Monilia*

sp., *Neocallimastix* sp., *Trichoderma reesei* and *Fusarium oxysporum* (Xu *et al.,* 2009); and *C. phytofermentans* has the ability use more number of carbohydrates (Cantarel *et al.,* 2009) and the feasibility of it's industrial use is under study (Christian *et al.,* 2010). However, bacteria produce less ethanol in large-scale fermentation, by-products, susceptible to high ethanol concentrations, can only grow at narrow and neutral pH range of 6.0 to 8.0 (Bothast *et al.,* 1999) and are prone to more viral infections (Jones *et al.,* 2000). *Zymomonas mobilis* isolate can only ferment glucose, fructose and sucrose. On the other hand, genetically engineered *S. cerevisiae* can consume more xylose than genetically engineered bacteria (Lau *et al.,* 2010).

Commercial ethanol production with engineered microorganisms has not succeeded so far (Laluce *et al.,* 2012). Among these, *S. cerevisiae* is the most preferred organism for industrial ethanol production. Therefore, though yeasts were isolated from number of sources, still search for new yeasts or *S. cerevisiae* strains is on involving various carbon sources of ethanol production such as fruit juices (low glucose), starch (high initial glucose and high ethanol), lignocelluloses (multiple sugars). Moreover, high initial sugar level is vital to get more ethanol accumulation and to reduce production costs (Gírio *et al.,* 2010). Therefore, industrial strains should possess characters such as high tolerance towards carbohydrate, ethanol and salt, be able to produce ethanol from various sugars, good yield and so on. Two newly isolated strains (RPDPMP and R3DSC5) of *S. cerevisiae* were subjected to experiments involving the effect of inoculum density, glucose concentration, initial pH and temperature on their ethanol producing efficiencies and to determine the optimum conditions for maximizing ethanol yields from these strains.

Materials and Methods

All the chemicals were purchased from Merck and Sigma. The two *S. cerevisiae* strains, RPDPMP and R3DSC5, were isolated from palm juice and sugar cane juice and identified in our laboratory.

Standard graphs

Determination of cell number using optical density was carried out following American Brewer's Guild http://www.abgbrew.com/pdf/haemocytometer.pdf) protocol and that of reducing sugar by dinitrosalicylate (DNS) method (Miller, 1959). Cell density was determined by measuring turbidity of small amount of the sample at 600 nm in a spectrophotometer. Three independent replications were used for each experiment.

Optimization

Inoculum of palm juice strain was developed on yeast extract peptone and dextrose (YEPD) medium (Leao and van Uden, 1982) slants. Three production flasks

were set up by taking 50ml of YEPD. These were inoculated with 5×10^8 cell/ml, 6×10^8 cells/ml, 7×10^8 cells/ml, 8×10^8 cells/ml and 9×10^8 cells/ml. For glucose concentration, the above medium was modified by taking 5, 10, 15, 20 and 25% of dextrose for RPDPMP whereas 5, 10, 15, 20, 25 and 30% of dextrose was used for R3DSC5 with an initial pH 5 (Ekunsanmi and Odunfa, 1990). For studies on the effect of initial pH, the above medium having 20% dextrose and initial pH values 3, 5, 7 and 9 were used for both the strains. For temperature studies, the above medium having 20% dextrose for R3DPMP strain and 30% glucose for R3DSC5 strain with initial pH 5 was taken into separate flasks and incubated at 30, 35, 40 and 45°C temperatures. Ethanol concentration in the samples was determined by Gas Chromatography (Agilent make GC equipped with flame ionization detector). The fermented samples were diluted with Dimethyl Sulfoxide (DMSO) at a concentration of 40mg/ml and analyzed using Agilent DB-624 column (length 30mts, film thickness 3µm, and diameter 0.53). The sample was introduced in to the column through head space technique. The oven was programmed to hold at 40°C for 12 min followed by gradual and uniform increase from 40°C to 220°C at the rate of 30°C per minute and hold for 5 min. Injector temperature was maintained at 180°C and detector temperature at 240°C. The carrier gas (Helium) was swept through the column with a flow rate of 3ml/min. Glucose concentration was estimated by DNS method (Miller, 1959).

Results and Discussion

Data on ethanol production and maximum growth noticed for different parameters are presented in Table 1. Significant variations were noticed with respect to glucose concentration and temperature and therefore, graphs (Figs. 1-4) for these two parameters are included for both the strains.

Effect of inoculum density

For R3DPMP, maximum growth was observed at 24 hours and a maximum cell number of 532.2×10^5 cells/ml was observed with the highest inoculum density of 158.8×10^5 cells/ml. Ethanol production was found to increase proportionately with inoculum level reaching peak ethanol concentration at 158×10^5 cells/ml inoculum. Peak accumulation of ethanol was also observed in less time with increase in inoculum densities. Therefore, it was planned to carry out ethanol production with higher inoculum densities and the results indicated that in general, increasing the initial inoculum density resulted in increased growth and ethanol production for both the strains. Simultaneously, for R3DPMP, glucose levels decreased to less than 1% at 9 hours in flasks with 7, 8 and 9×10^8 cells/ml of inocula and for R3DSC5, glucose was almost exhausted at all inoculum densities by 48 hours. Moreover, unutilized glucose in all the flasks was less than 0.5% at the end of fermentation and this is important because an efficient strain should be able to utilize most of the

glucose during fermentation otherwise, contamination problems will occur during storage of various brewery products. Maximum quantities of ethanol i.e., 8.3% g/v for R3DPMP and 13% g/v for R3DSC5 were noticed under these experimental conditions. As the above result suggested that maximum amount of ethanol was produced with inoculums of 7, 8 and 9×10^8 cells/ml, inoculum densities within this range were selected for further detailed studies with both the strains. Kazuyoshi *et al.,* 1993 also observed early accumulation of ethanol with higher inoculum.

Effect of Glucose concentration

For R3DPMP, maximum growth of 11.3×10^8 observed with 20% g/v glucose (Fig. 1.). Percentage yield of ethanol at 5, 10, 15, 20 and 25% glucose concentrations are 86, 93.8, 80.8, 81.2 and 80 respectively. Though the maximum yield was observed with 10% glucose concentration at 12 hours, 20% glucose concentration was selected since higher growth and early ethanol production were observed. At all glucose concentrations, residual glucose reached less than 0.33% which indicates that glucose was used up completely by this strain.

For R3DSC5, increase in cell number was observed (Fig. 2) at all other percentages except 5%. Maximum growth of 10×10^8 cells was observed at 10, 15 and 20% initial glucose concentrations. Yield of ethanol at 5, 10, 15, 20, 25 and 30% of glucose are 89.9, 97.7, 95.2, 92.3, 97.7 and 91.9 respectively. Although highest ethanol of 14.1% g/v (17.625 v/v) was produced by 60 hours with 30% glucose concentration, 25% initial glucose concentration was found to be the best one because maximum growth (11.8×10^8) and ethanol production of 12.5% g/v (15.6% v/v) were observed for this strain in comparison with lower glucose concentrations and early accumulation of ethanol than in 30% glucose. At each initial glucose concentration, almost complete utilization of glucose was observed at the end of fermentation.

In brewery industry, 18^0Plato (12g glucose/100ml) is desired to produce ethanol at low cost (Blieck *et al.,* 2007). In very high gravity (VHG) fermentation, pretreated mash having glucose concentration in the range of 15–32% g/v is used (Oscar and Sanchez, 2008). Therefore, R3DPMP strain giving 93.8% yield of ethanol at 10% g/v glucose can be recommended for brewery industry while R3DSC5 strain that gave consistently a minimum of 90% yield of ethanol (Table 1) from 5-30% g/v glucose concentration, could be used in both brewery and VHG fermentation. Moreover, R3DSC5 accumulated 12.5% g/v and 13% g/v of ethanol from 25% g/v and 30% g/v initial glucose concentrations respectively and these values are very close to the maximum ethanol of 13.2% g/v reported by Oscar and Sanchez, 2008 during VHG.

So far, high ethanol of 16% g/v was obtained within 3 to 5 days from raw ground corn with and without sucrose supplementation in the presence of a mutant,

Aspergillus awamori var. *kawachi,* which was found to produce high amount of raw starch-digestive amylase (Shinsaku *et al.,* 1982), more than 16% g/v ethanol during sake fermentation by sake yeasts (Hiroshi *et al.,* 2005) and highest final ethanol concentration of 17.04% g/v was obtained in 5 days using *S. cerevisiae* 1200 strain in the presence of fungal mycelium, *A. niger* 817, and intermittent addition of 64 g of sucrose. While in the absence of fungus it produced only 14.24% g/v ethanol in 6 days with 55 g of sucrose added intermittently and 16.32% and 16.8% g/v ethanol were obtained from chicory and dahlia inulin, respectively within 3 days using the above 5 day culture as source of inulinase (Kazuyoshi *et al.,* 1993). These reports further support our view that R3DSC5 strain can also be considered as a high ethanol producing strain. In addition, it accumulates maximum ethanol within two days which is earlier than those mentioned in the above reports and hence can further reduce the cost of ethanol production. It may produce more ethanol in analytical/large-scale fomenters where parameters can be more efficiently controlled compared to shake flask.

Effect of initial pH

Growth pattern was found to be similar at all initial pH values for both the strains. However, for R3DPMP strain growth is slightly less at initial pH 3 in comparison with other pH and ethanol production pattern is similar at initial pH 5, 7 and 9 whereas ethanol production at pH 3 is slightly less. For R3DSC5 strain, maximum growth was reached 24 hours earlier at initial pH 5 and 7 in comparison with other pH and eventually ethanol production pattern was also similar at initial pH 5 and 7 with slightly higher and lower ethanol levels at initial pH 5 and 3 respectively.

Effect of temperature

For R3DPMP strain, maximum growth was observed at 30°C at 24 hours whereas increase in growth was not observed at 45°C which indicates this strain cannot grow at this temperature (Fig. 3). Moreover, in another experiment, growth was not observed when this strain was subjected to heat treatment at 45°C for 48 hours and the temperature was later brought back to 30°C. Rate of glucose utilization is almost three times less at 40°C in comparison with 30 and 35°C. For R3DSC5 strain, maximum growth was observed at 35°C at 24 hours whereas increase in growth was not observed at 40 and 45°C (Fig. 4). Active bubbling was also not observed at 45°C during fermentation when compared with other temperatures. It indicates that this strain cannot grow at 45°C temperature. However, in another experiment, growth was observed within 9 hours at 30°C after subjecting this strain to heat treatment at 45°C for 48 hours. This indicates that this strain is superior to R3DPMP strain with respect to temperature tolerance. Ethanol production was found to be similar at 30 and 35°C temperatures whereas ethanol accumulation was about 3% less at 40°C in comparison to those at 30 and 35°C temperatures.

Consequently, glucose utilization was also almost complete and nearly same at 30 and 35°C temperatures whereas considerable amount of glucose was left unutilized at 40°C.

Hacking *et al.,* (1984) reported that six among the 55 isolated yeast strains were able to grow at 45°C. Szczodrak and Targonski 1988 and D'Amore *et al.,* 1989 reported thermotolerant strains of *Saccharomyces* that could grow at above 40°C and ferment sugars at 40°C. Sree *et al.,* 2000 reported *Saccharomyces* strains that tolerate 44°C but growth and ethanol yields were low. Optimal range of 30–35°C is required for fuel ethanol production (Bollók *et al.,* 2000; Lin and Tanaka 2006) but 40-50°C is desirable to decrease operation costs and moreover, ethanol was efficiently extracted at 40°C when compared to at 35°C (Babiker *et al.,* 2010). Unlike the statement of Cantarel *et al.,* 2009 that yeast ferments below 35°C, the above cited reports and the two strains of the present study are able to ferment at 40°C.

Though thermotolerant strain (Babiker *et al.,* 2010) or a metabolically altered thermophillic bacterium can be used (Shaw *et al.,* 2008) in simultaneous saccharification and fermentation (SSF), their ethanol tolerance is around 2% v/v which is very less compared to that of yeast. *Kluyveromyces marxianus* is thermo tolerant and natural xylose-fermenting yeast that could grow up to 52°C with 98% theoretical yield (Banat *et al.,* 1992) and could ferment at elevated temperatures above 40°C, however, like recombinant xylose-fermenting *S. cerevisiae* strains, it also accumulates xylitol during fermentation (Cantarel *et al.,* 2009). *Hansenula polymorpha* is also a naturally fermenting yeast which can ferment xylose at 48°C but produces low amount of ethanol.

Minor changes in physical or chemical parameters can affect fermentation efficiency of yeast strains leading to decreased ethanol yield. As the present two strains are showing nearly similar performance, they can be used for large-scale ethanol production. Among these two, R3DSC5 is better than R3DPMP due its ability to grow and produce ethanol at higher glucose level (30% g/v) with good yield and higher temperature tolerance. The optimum conditions identified are 7 to 9×10^8 cells/ml of inoculum density, 30 to 35 °C temperature and from 5-7 initial pH for both the strains whereas 20% and 25% (g/v) glucose concentrations for R3DPMP and R3DSC5 strains respectively.

Table 1: Comparative performance of the two strains with various parameters.

Parameter		Maximum growth in cell number× 10^8 (Time in hours)		Max. ethanol produced in % g/v (Time in hours)	
		R3DPMP	R3DSC5	R3DPMP	R3DSC5
Inoculum density in cell number× 10^8	5	9.1±0.4 (12)	10.9±0.8 (36)	6.9±0.2 (18)	9.8±0.2 (48)
	6	9.4±0.6 (09)	10.8±0.2 (36)	7.4±0.8 (12)	10.8±0.3 (48)
	7	9.5±0.3 (09)	10.9±0.7 (24)	8.4±0.2 (12)	12.9±0.14 (36)
	8	11.2±1 (18)	11.8±0.3 (24)	8.3±0.35 (12)	13.3±0.14 (36)
	9	11±0.4 (12)	11.4±0.5 (24)	8.4±0.2 (12)	13.1±0.14 (36)
% of glucose in g/v Concentration	5	7.9±0.8 (0 and 09)	8.8±2 (0)	2.2±0.9 (09)	2.3±0.8 (24)
	10	9 . 2 ± 0 . 0 8 (09)	9.9±1 (12)	4.8±0.9 (48)	5.0±0.6 (36)
	15	9.9±0.7 (12)	10±.9 (12)	6.2±0.5 (48)	7.3±0.6 (36)
	20	1 1 . 2 ± 0 . 1 (18)	9.9±0.1 (12)	8.3±0.3 (12)	9.5±0.8 (36)
	25	9.3±0.5 (09)	11.8±0.3 (24)	10.1±0.4 (24)	12.5±0.2 (36)
	30	-	11±0.4 (36)	-	13±0.5 (36)
Temperature in °C	30	11.3±2 (24)	11.4±0.1 (24)	8.3±0.34 (12)	12.2±0.8 (36)
	35	10.3±1 (12)	11.4±0.5 (24)	8.8±0.17 (24)	11.8±0.6 (24)
	40	8.5±0.7 (09)	8.8±0.5 (0 and 09)	8.7±0.28 (18)	8.7±0.5 (24)
	45	7.5±0.09 (0)	9.3±0.3 (0)	-	-
Initial pH of the medium	3	10±0.65 (09)	11.3±2 (48)	8.0±1.0 (15)	10.5±0.1 (48)
	5	1 0 . 8 ± 0 . 5 (06)	11.5±1.4 (24)	8.7±0.6 (12)	12.1±0.4 (36)
	7	11±0.61 (12)	11.6±1.1 (24)	8.3±0.3 (12)	11.4±0.1 (36)
	9	1 0 . 4 ± 0 . 4 1 (12)	11.4±0.6 (48)	8.7±0.56 (15)	11.6±0.1 (48)

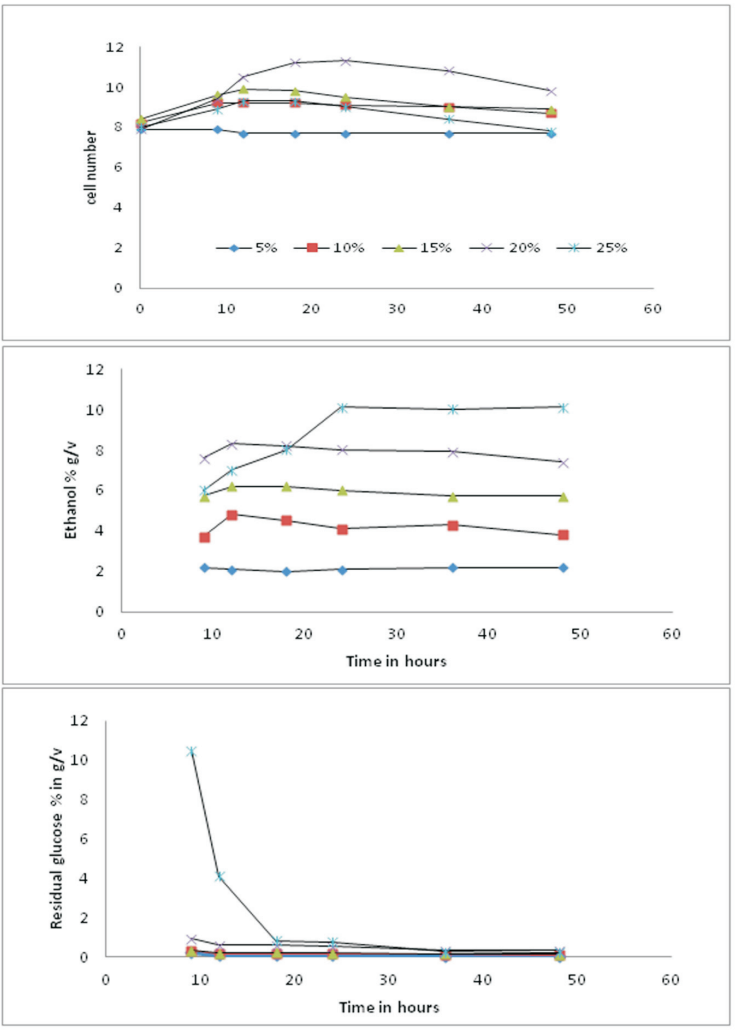

Fig. 1: Growth pattern, ethanol production and residual glucose at different glucose concentrations for R3DPMP strain.

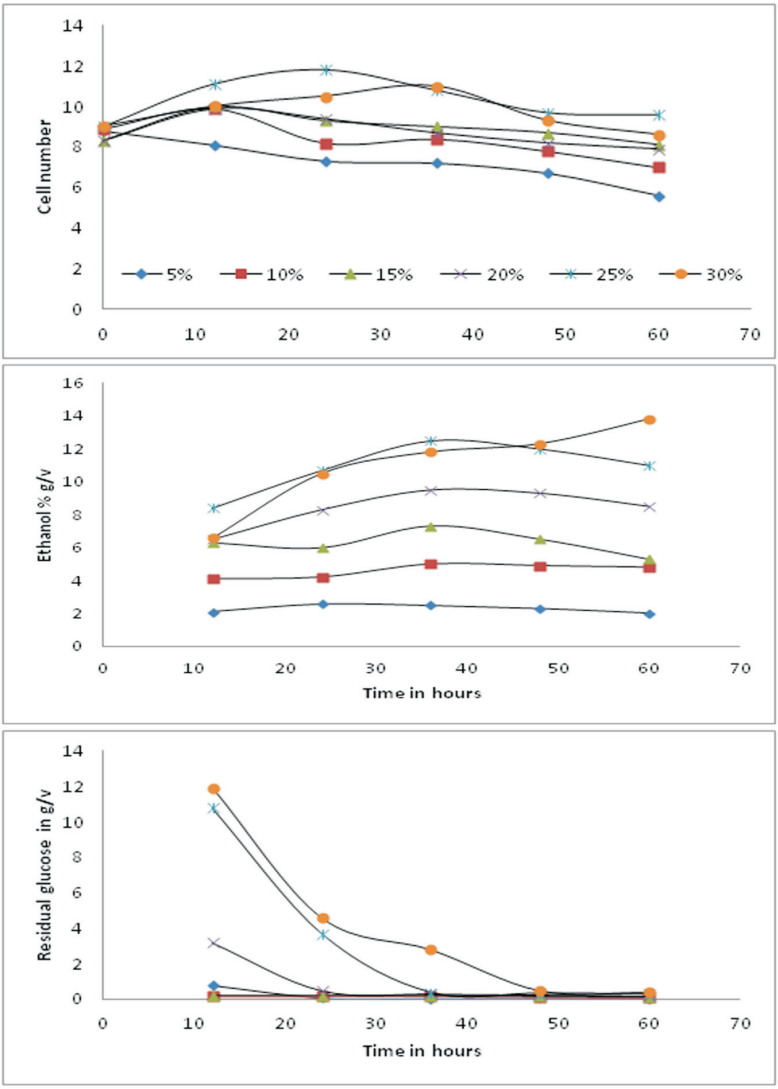

Fig. 2: Effect of glucose concentration on growth, ethanol and residual glucose for R3DSC5 strain.

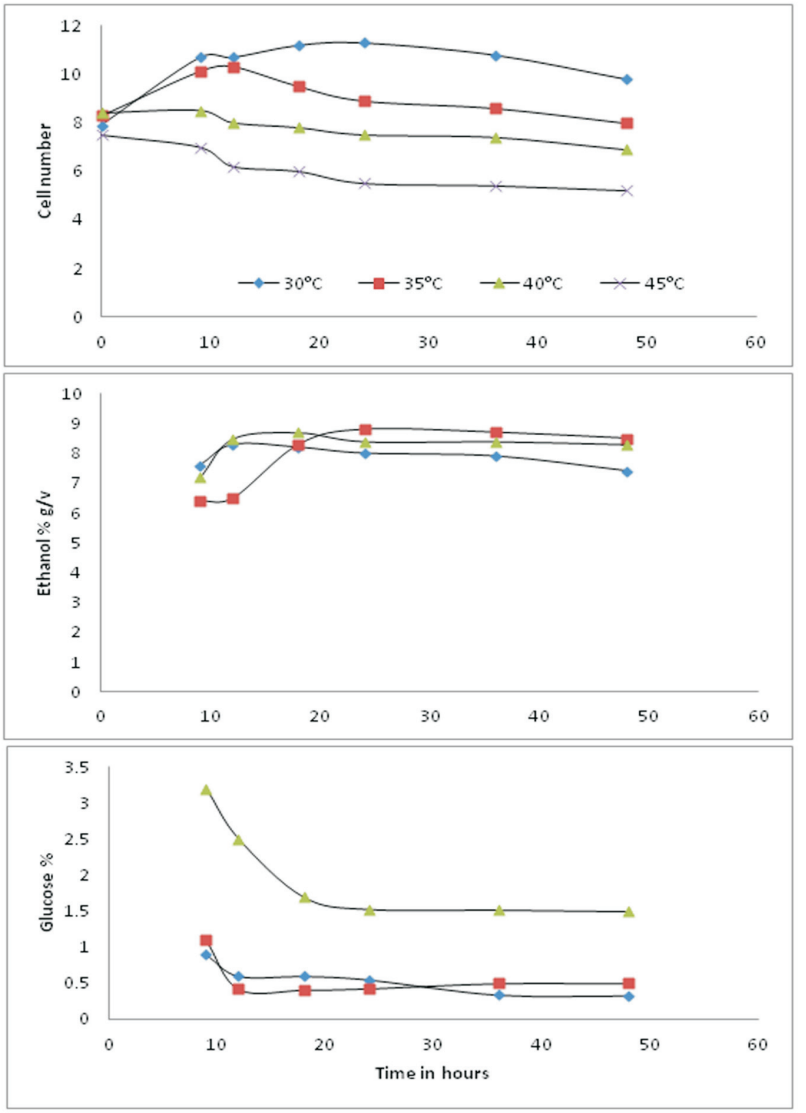

Fig. 3: Effect of temperature on cell number × 10^8, ethanol production and residual glucose for R3DPMP strain.

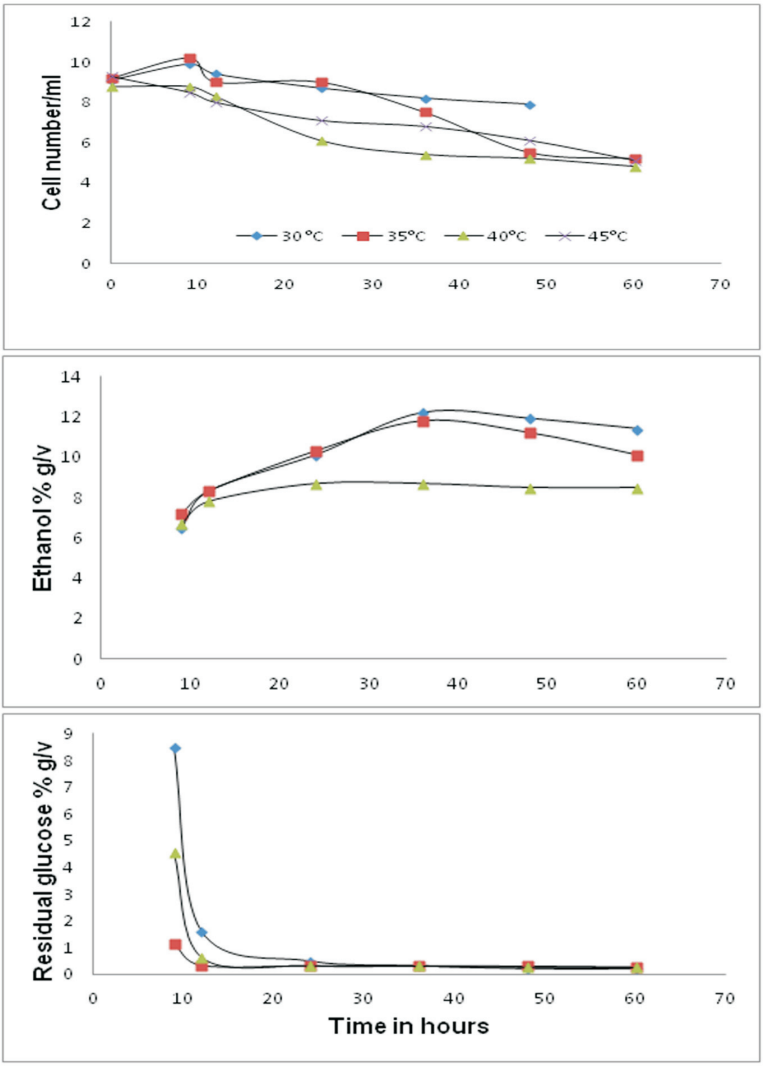

Fig. 4: Effect of temperature on growth, ethanol production and residual glucose for R3DSC5 strain.

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