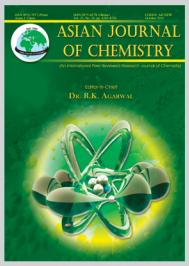
Asian Journal of Chemistry; Vol. 24, No. 3 (2012), 1162-1166

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in



Simultaneous Saccharification and Fermentation of Sorghum Bicolour Grains by Ethanol and Sugar Tolerated *Saccharomyces cerevisiae*

ZAHID QURESHI[1], SAJID MEHMOOD[2], MUKHTAR UL HASSAN[3], TANZEEL-UR-RAHMAN[1,*], MUHAMMAD ZEESHAN HYDER[4], MUHAMMAD FAHEEM MALIK[5], MUHAMMAD GULFRAZ[6] and NAVEEDA AKHTAR[7]

[1]Department of Chemistry, Government College University, Lahore, Pakistan
[2]University of Gujrat, Hafiz Hayat Campus, Gujrat, Pakistan
[3]Department of Chemistry, University of the Punjab, 54000 Lahore, Pakistan
[4]Department of Biochemistry, PMAS Arid Agriculture University, Rawalpindi, Pakistan
[5]Arid Agriculture University, Murree Road, Rawalpindi, Pakistan
[6]Department of Biosciences, Comsats Institute of information Technology, Islamabad, Pakistan
[7]Quaid-i-Azam University, Islamabad, Pakistan

*Corresponding author: E-mail: tanzeel_u@yahoo.com

(*Received*: 26 February 2011; *Accepted*: 11 November 2011) AJC-10615

Saccharomyces cerevisiae was used to evaluate sugar tolerance and efficient production of ethanol from Sorghum bicolour grains. The results revealed that the strain tolerates ethanol up to 11 % (v/v) and glucose level up to 25 % (w/v). Simultaneous saccharification and fermentation was used to produce ethanol from grain starch of different Sorghum bicolour varieties by utilizing and yeast strain *S. cerevisiae*. The samples (20 %, w/v) were liquefied at different temperatures (85-100 ºC) for complete conversion of starch into oligosaccharide and saccharification. Separate hydrolysis and fermentation results showed that 70.84 to 82.12 gL^{-1} ethanol was produced with the highest 95 % fermentation efficiency at 60 ºC. Simultaneous saccharification and fermentation resulted in a slightly higher concentration of ethanol. It was observed that sugar produced from starch was promptly used up for fermentation in both separate hydrolysis and fermentation and simultaneous saccharification and fermentation.

Key Words: α-Amylase, Glucoamylase, Saccharification, Fermentation, Ethanol.

INTRODUCTION

Energy consumption is increasing steadily with the population growth and industrial development. Due to diminishing conventional energy resources it is difficult to cope up with fuel demand and its balanced providence. Therefore, to maintain sustainable growth of the society and to explore alternative energy sources is getting a great concern. Furthermore, burning of fossil fuels causes a release of pollutants that increase global climate change, acid rain and ozone problem[1]. Due to this increasing demand of energy and truly increased environmental problems, people are focusing on some renewable and environmentally friendly energy sources. Therefore, during last two decades most of the research was focused on developing an economical and environmentally friendly ethanol production process. Currently, crop grains are the main feedstock used for ethanol production. Brazil is the largest ethanol producer with a capacity of 15.5 Giga-L in 2004 and uses sugar cane as feedstock, while the USA is seated (12.9 Giga-L), uses corn as feedstock[2].

Grain based ethanol production requires a feedstock with a high level of degradable starch[3]. Milling and grinding, liquefaction, saccharification and fermentation are the main steps involved in the starch hydrolysis and fermentation. Many microorganisms play key role in fermentation[4]. Yeast, bacteria and fungi have been used as such or genetically modified to achieve the goals[5-9]. There are many factors like larger sizes, thicker cell walls, better growth at low pH, less stringent nutritional requirements and greater resistance to contamination that give advantage to yeast over bacteria for commercial fermentation[10]. *Saccharomyces cerevisiae* is extensively used in ethanol production due to its high yielding ethanol capability, ethanol tolerance and least oxygen requirement since thousands of years[11].

Ethanol is used in vehicles either as a sole fuel or blended with gasoline. Ethanol is an oxygenated compound that provides additional oxygen in combustion, thus offers better and complete combustion with reduced emission of carbon monoxide and hydrocarbons is reduced by 32.5 and 14.5 % respectively[12].

Sorghum (*Sorghum bicolour* L.) is drought resistant low input cereal crop grown throughout the world[13] that is utilized for the production of bio ethanol. *Sorghum bicolour* is also

best known as a grain crop. The seeds and stalks are rich source of sugar and thus can be processed for the production of ethanol. *Sorghum* has remarkable resistance and tolerance to drought, salinity and alkalinity of soils and water logging[14]. It is expected that in future, *Sorghum* will be the primary source for the production of biofuel (ethanol) because it has advantage over other crops due to high starch contents (53-68 %) and it can be grown throughout the year (both in winter and summer seasons) in several Asian and African countries. Approximately 7,000 L of ethanol per hectare can be produced from *Sorghum* that makes it highly attractive for developing countries like, China, India, Pakistan, *etc*.[15].

Starch liquefaction is usually carried out in pressure cooking at high temperatures such as 90-95 ºC carried out ethanol production from low grade wheat flour[16]. 100 U-α-amylase/g-flour (commercial, non thermostable α-amylase) were used for liquefaction at 55. For one variety the (LG1) maltose production was 0.273 g-maltose/g flour whereas for the other (LG2) it was found 0.019 g-maltose/g of flour. In the cooked, starch the slurry is treated with viscosity reducing enzymes during liquefaction. The production of ethanol was 5.99 and 6.10 % (v/v) from cooked and uncooked starch respectively. Further for damaged grains of wheat and *Sorghum*, as substrate, utilizing crude amylase preparation from *B. subtilis* VB_2, the concentration was 25 % higher than optimum[17].

Saccharomyces cerevisiae is widely used for ethanol production but it is nonamylolytic thus cannot be used for starch hydrolysis. Therefore starch is first hydrolyzed by enzymes into monosaccharides, which can be then used by the fermenting microorganism into ethanol. Saccharification is the process by which starch is converted into monosaccharides (alcohol) while fermentation is the distillation in which glucose or maltose is converted into alcohol. Fermentation of alcohol is mostly carried out by *Saccharomyces cerevisiae* yeast. Pretorius[18] and Walker and Dijck[19] reported that fermentation depends upon yeast nutrition (oxygen, nitrogen, sulphur, phosphorus, mineral elements, vitamins and amino acids) and stress factors (physical: temperature, osmotic shock, dehydration, radiation; chemical: ethanol and other metabolism toxicity, nutrients limitation, oxidative stress, pH shock, metal ion stress, chemical mutagenesis and biological: cellular ageing, genotypic changes, competition from other organisms).

EXPERIMENTAL

Collection and preparation of samples: Grain of five different *Sorghum bicolour* varieties (84-Y01, 86-G-87, Mr. Buster, RARI S-3 and YSS 9) were obtained from Millet Research Station Rawalpindi, Pakistan. The varieties were grown during July-October, 2006. After harvesting, the biomass of all varieties was air dried in the field for one week. Dried grains were prepared according to the methodology described by the laboratory analytical procedure[20] and stored at -20 ºC for further analysis.

Biochemical analysis: Wet and dry weight analysis was intended to determine the amount of total solids remained after 45 and 105 ºC[21]. For starch analysis AOAC method 996.11 and megazyme (total starch assay procedure) was adopted with minor modifications. Glucose was analyzed by Glucose Kit (AMP, Austria). Protein content (nitrogen × 6.25) was determined by micro-Kjeldahl nitrogen analysis by the AOAC methods, 1990 (979.09 and 920.87). The other nutritional components such as fat, fiber and ash were also analyzed by AOAC 920.85, 962.09E and 923.03 methods respectively. A macro and micro elements study was carried out by wet digestion method using HNO_3-$HClO_4$ as described in soil and plant analysis laboratory manual[22]. Sodium and potassium were analyzed by flame photometer (Jenway PFP-7) while the remaining elements (magnesium, calcium, zinc, copper, iron and manganese) were analyzed by atomic absorption spectrophotometer (AAS) (GBC 932 plus, Australia).

Yeast strain and preparation of inoculum: The yeast strain *Saccharomyces cerevisiae* was used in this study. The strain was maintained in glycerol vials at -20 ºC as working stock. This stock solution was incubated in defined yeast medium gL^{-1} [$(NH_4)_2SO_4$ 5.0, $MgSO_4 \cdot 7H_2O$ 0.5 and KH_2PO_4 3.0] with addition of ergosterol/tween 80 solution, vitamins $g\ L^{-1}$ (Ca-pantothenate 1.0, nicotinic acid 1.0, myo-inositol 25, Thiamin-HCl 1.0, Pyridoxin-HCl 1.0 and *p*-aminobenzoic acid 0.2), trace metals gL^{-1} ($ZnSO_4 \cdot 7H_2O$ 4.5, $MnCl_2 \cdot 2H_2O$ 0.84, $CoCl_2 \cdot 6H_2O$ 0.3, $CuSO_4 \cdot 5H_2O$ 0.3, $Na_2MoO_4 \cdot 2H_2O$ 0.4, $CaCl_2 \cdot 2H_2O$ 4.5, $FeSO_4 \cdot 7H_2O$ 3.0, H_3BO_3 1.0 and KI 0.1) and glucose 200 gL^{-1}. Cultures were incubated for 24 h at different temperatures (28-35 ºC) and at different agitation rate (120-160 rpm) to optimize the culture conditions. After optimization, cultures was incubated and used as seed culture for fermentation.

Ethanol and sugar tolerance of *Saccharomyces cerevisiae*: Ethanol and sugar tolerance of *Saccharomyces cerevisiae* was checked according to the methodology described by Novek *et al*.[23-25]. Malt yeast peptone glucose (MYPG) broth and saboroud dextrose broth was used as a medium which was sterilized at 121 ºC for 15 min and cooled. Required quantity of absolute ethanol and glucose was then added to different flask of the same medium to constitute varying percentages of ethanol differing by 1 % (v/v) from one flask to another and 5 % (w/v) for glucose. Initial and final optical density (OD) was taken at 600 nm against the blank. The inoculated flask was maintained at 150 rpm at 30 ºC for at least 72 h. Optical density was directly proportional to the growth of yeast and optical density in a flask was recorded at different interval as evidence of growth.

Simultaneous Saccharification and Fermentation: Liquefaction of the grain samples was performed with final dry matter content of 20 % (w/w). The pH was adjusted to 5.4 with HCl and NaOH and the slurry was heated to 85 ºC under agitation. The α-amylase (termamyl SC, novozymes, Bagsvaerd, Denmark; declared activity 120 kilo novo units-S/g) was added at a dose of 1 µL/g of dry matter. After 0.5 h the slurry was heated at boiling temperature (100 ºC) for 5 min and subsequently cooled to 85 ºC. A second dose of termamyl SC (2 µL/g DM) was added and slurry was agitated for 1.5 h[26].

Simultaneous saccharification and fermentation (SSF) was done just after liquefaction with out prior long saccharification. Spirizyme fuel tech enzymes was used simultaneously at the rate of 1 µL/g of dry matter with *S. cerevisiae* BF001.

Fermentation was carried out for 72 h and samples were with drawn at different intervals to check the efficiency for the production of ethanol.

Analytical methods: Sugars (glucose), end-fermentation product (ethanol) were determined by HPLC (Agilent Technologies, 1200 system) equipped with an Aminex HPX-87H organic acid analysis column (Bio-Rad) at 60 ºC. The eluent was 4 mM H_2SO_4 at a flow rate of 0.6 mL/min with detection on a refractive index detector. Prior to HPLC analysis, 1 mL samples were acidified with 10 µL of 20 % H_2SO_4 and centrifuged at 14000 rev./min for 10 min, followed by filtration through a 0.45 µm membrane filter.

Statistical analysis: Randomized complete block design (RCBD) and Duncan's multiple range test (DMRT) test was used to analyze the data. The values were expressed in the form of average ± standard deviation, wherever applicable.

RESULTS AND DISCUSSION

Ethanol and sugar tolerance: Fig. 1 shows tolerance pattern of *S. cerevisiae*. The yeast has tolerated the ethanol up to 6 % with the same normal pattern and up to 9 % with delay. This yeast strain was not capable to tolerate the ethanol when growth started with 10 % ethanol. After careful evaluation of the results, we have then adopted a slightly different strategy and growth was started at 7 % ethanol concentration. After 24 h when enough cell density was achieved, fed batch incorporation of ethanol @ 1 % (v/v) started and went up to 11 % (v/v) at 60 h. With this pattern, the optimum growth was achieved by the same yeast strain (Fig. 2).

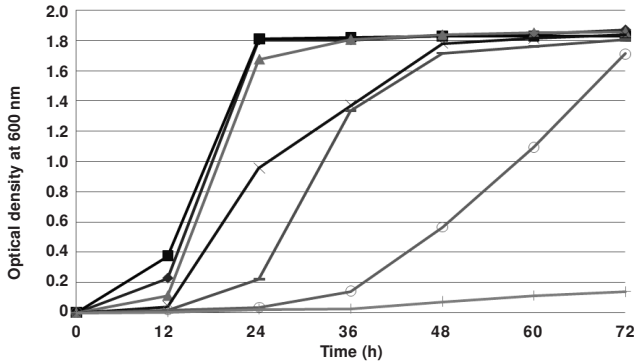


Fig. 1. Growth curve of *Saccharomyces cerevisiae* BF001 in increasing ethanol concentration. Growth conditions were, pH 4.5, 150 rpm at different ethanol concentration (v/v), 4 % (•), 5 % (■), 6 % (▲), 7 % (×), 8 % (-), 9 % (O) and 10 % (+).

Mitochondrial DNA damage[27], degradation of cellular membranes[28-31], inactivation of some enzymes such as hexokinase and dehydrogenase[32] and ultimately an inability to metabolize[33] are the factors, which influence the inhibition of micro organism under high ethanol concentrations. It has also been reported that the resistant strain to ethanol stress have other abilities like resistant to osmotic pressure, oxidative and heat. This observation is in agreement with present results. *S. cerevisiae* BF001 has tolerated 25 % (w/v) glucose level (Fig. 3). At all concentration up to 25 % sugar the yeast growth pattern was normal. Ethanol tolerance is very important as it can be hardly avoided during fermentation because substrate inhibition can be overcome to stepwise addition of substrate to the fermentation medium. Multiple stress tolerance of *S. cerevisiae* is under the control of multiple loci widely distributed throughout the genome of yeast cells and many of these genes are not characterized[34,35]. In order to attempt multiple stress tolerance having no clearly defined genetic basis by rational approaches based on DNA technologies has met with stern barrier.

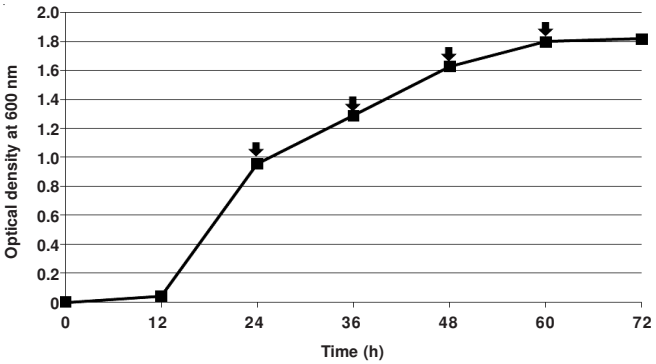


Fig. 2. Growth curve of *Saccharomyces cerevisiae* BF001 in fed batch increasing ethanol concentration. Incubation was started from 7 %. Arrows indicate the fed batch increase in ethanol concentration of 1 % each time

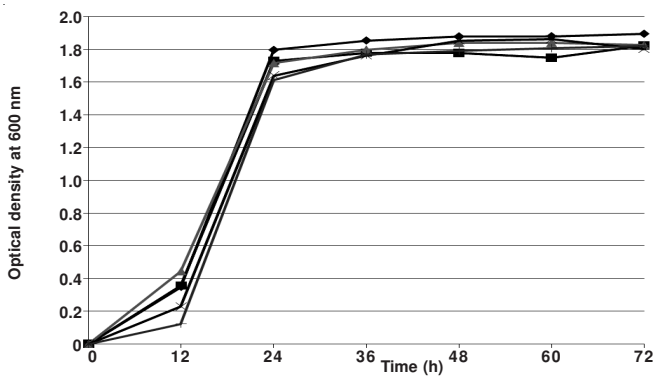


Fig. 3. Growth curve of *Saccharomyces cerevisiae* BF001 in increasing glucose concentration. Growth conditions were, pH 4.5, 150 rpm at different glucose concentration (w/v), 5 % (•), 10 % (■), 15 % (▲), 20 % (×) and 25 % (+).

Effect of temperature on liquefaction: Liquefaction of starch is the first enzymatic step during transformation of starch into ethanol. During the process, squashed starch granules are expanded and opened to allow enzymatic breakdown to soluble dextrins. These dextrins are then saccharified into fermentable sugars, which yeast can use to produce ethanol. Starch liquefaction is usually carried out in pressure-cooking. α-amylase enzyme is used to gelatinize the grain slurry under high temperature and pressure[36].

Table-1 shows the liquefaction of grains of five different *S. bicolour* varieties, carried out at high temperature. The degree of starch hydrolysis was quantitatively determined by the iodine affinity method. Almost all starch was liquefied during 2 h at 85 ºC and 96.43 to 98.82 % hydrolyzed starch was achieved.

Liquefaction basically comprises of two steps *i.e.* gelatinization and dextrinization. Gelatinization was achieved by high temperature firstly with 95 ºC having the heat stable enzyme termamyl SC, concentration of 1µL/g of dry matter.

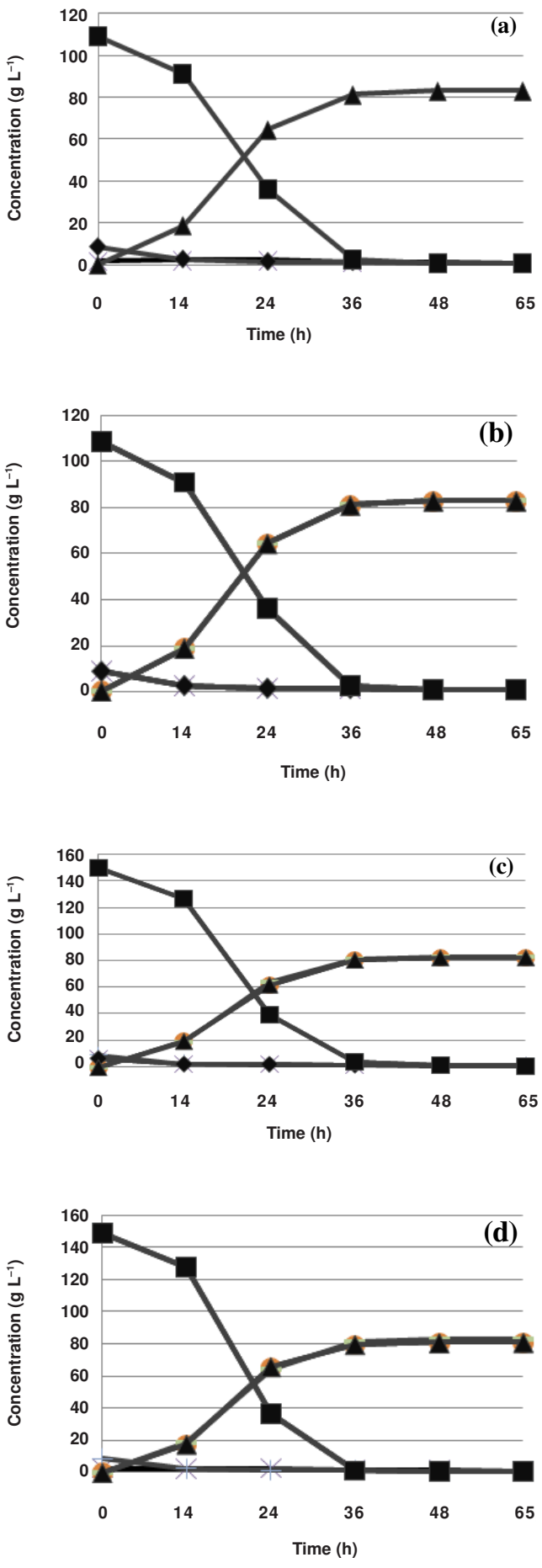
After 0.5 h the slurry was heated at boiling temperature (100 ºC) for 5 min and subsequently cooled to 85 ºC. This step is referred as pre liquefaction. In post liquefaction, a second dose of termamyl SC (2 µL/g DM) was added and slurry was agitated for 1.5 h. During the pre liquefaction under high temperature, gelatinization causes the starch grains to swell and open up enough for the α-amylase to hydrolyze the long chains into shorter dextrins. Once the gelatinization achieved, dextrinization of starch grains starts. There are many factors which influences the dextrinization step during the liquefaction. Dextrinization must be followed after liquefaction without any delay. Delay in dextrinization after gelatinization may results in the recrystallization of starch in a process called retrogradation. Retrograded starch is a highly stable crystalline material that cannot be degraded by α-amylase and will pass undegraded through the fermenter that results poor yield of ethanol. The temperature sequence adopted in this study during pre and post liquefaction does not allow the starch to become crystallize. High temperature would also cause the inhibition of any unwanted microorganism, competing for substrate utilization, which might be resulted into poor final product yield.

TABLE-1
DEGREE OF STARCH HYDROLYSIS AFTER LIQUEFACTION AND PRODUCTION OF FREE SUGARS AFTER SACCHARIFICATION DURING SEPARATE HYDROLYSIS AND FERMENTATION OF *Sorghum bicolour* GRAINS

Sorghum Varieties	Degree of Starch Hydrolysis (%)	Sugar produced gL^{-1}		
		Glucose	Maltose	Total DE*
86-G-87	98.82	150.14	9.52	154.90
84-Y-01	95.23	120.99	6.58	124.28
Mr. Buster	98.44	153.10	4.51	155.36
RARI S3	97.39	149.53	7.56	153.31
YSS 9	96.43	118.46	6.24	121.58

*Dextrose equivalent. The calculation of DE is based on reducing value *i.e.* 1 for glucose and 0.50 for maltose

Simultaneous saccharification and fermentation: Fig. 4 expresses simultaneous saccharification and fermentation trial. The results show slightly higher concentration of ethanol. It has been observed in both separate hydrolysis and fermentation and simultaneous saccharification and fermentation cases that sugar produced from the starch was promptly used for fermentation. The earlier and higher concentration of ethanol production could be due to the physical factors that decrease the osmotic pressure or by chemical factor like polysaccharides, proteins and fatty acids present in *Sorghum* grains. These factors may help the yeast cells viable for longer period of time and producing such high concentration of ethanol in short duration. It may also be assumed that these compounds may also reduce the osmotic stress during the fermentation. Fujii *et al.*[37] observed early extinction of glucose during the fermentation. Difference between the optimum temperature of amylogulcosidase activity (60 ºC) and yeast growth (30-34 ºC) might be reason of his findings. Lower temperatures are preferred because the metabolic activity of the yeast is increased that results faster completion of fermentation[38]. Zhan *et al.*[39] investigated the impact of genotype and growth environment on the fermentation quality of eight *Sorghum* hybrids grown at two different



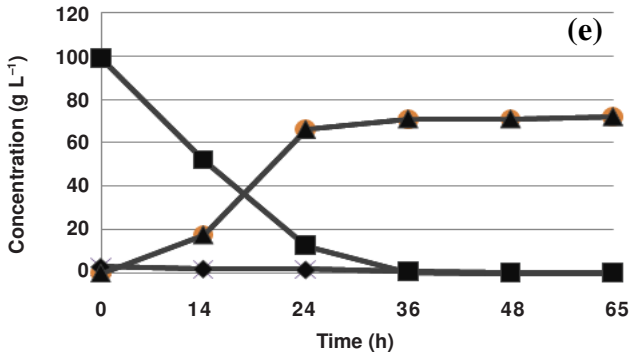


Fig. 4. Time course for simultaneous Saccharification and fermentation with Saccharomyces cerevisae BF 001 of different *Sorghum bicolour* varieties; (a) 86-G-87; (b) 84-Y-01; (c) Mr. Buster; (d) RARI S3; (e) YSS 9 maltose (◆), glucose (■) and ethanol (▲)

locations for the production of ethanol. Zhan's methodology included heating with thermostable α-amylase at 95 ºC and then 80 ºC (liquefaction), incubation with amyloglucosidase at 60 ºC (saccharification), inoculation with *S. cerevisiae* and fermentation for 72 h at 30 ºC. It was also found that ethanol concentrations varied relatively narrowly (about 5 %) across the 16 samples and that significant genotype and environment interactions existed. Corredor *et al.*,[40] reported that improvement in breeding techniques, fermentation quality and pre-processing of *Sorghum* grain can be used to improve ethanol yields and process efficiency. Sree *et al*.[41] conducted simultaneous saccharification and fermentation of damaged grains of wheat and *Sorghum* by using α-amylase from *B. subtilis* VB_2 and *S. cerevisiae*. Their study revealed that cheap substrates like damaged grains of wheat and *Sorghum* can be utilized in uncooked form more effectively to obtain high yields of ethanol than cooked form. Rice starch gave more ethanol at 37 ºC (10 g) than the amount of ethanol produced at 42 ºC (3.5 g), but it was less than ethanol produced from sweet *Sorghum* (7.5 g) at 42 ºC. The amount of left over sugar present in 96 h sample was more in case of rice starch (1 g at 37 ºC and 5g 42 ºC) than in case of sweet *Sorghum* (0.25 g at 37 ºC and 0.45g at 42 ºC). Hence sweet *Sorghum* is a better substrate for production of ethanol at higher temperatures as confirmed by Sree *et al.*[41].

REFERENCES

1. M.G. Barker and K.A. Smart, *J. Am. Soc. Brew. Chem.*, **54**, 121 (1996).
2. F. Rosillo-Calle and A. Walter, *Ener. Sustain. Dev.*, **10**, 18 (2006).
3. W.M. Ingledew, in ed.: A.H. Rose and J.S. Harrison, Yeasts for Production of Fuel Alcohol, The Yeasts, Academic Press: New York. Vol. 5, edn. 2, pp. 245-291 (1993).
4. R. Purwadi, Ph.D. Thesis, Continuous Ethanol Production from Dilute-acid Hydrolyzates: Detoxification and Fermentation Strategy, Chalmers University of Technology, Goteborg, Sweden (2006).
5. M. Abbi, R.C. Kuhad and A. Singh, *Process Biochem.*, **31**, 555 (1996).
6. L.O. Ingram, H.C. Aldrich, A.C.C. Borges, T.B. Causey, Alfredo Martinez, F. Morales, A. Saleh, S.A. Underwood, L.P. Yomano, S.W. York, J. Zaldivar and S. Zhou, *Biotechnol. Prog.*, **15**, 855 (1999).
7. R. Millati, C. Niklassonp and M.J. Taherzadeh, *Process Biochem.*, **38**, 512 (2002).
8. J.N. Nigam, *J. Biotechnol.*, **80**, 189 (2000).
9. L. Olsson and B. Hahn-Hagerdal, *Enzyme Microb. Technol*., **18**, 312 (1996).
10. T.W. Jeffries and Y.S. Jin, *Adv. Appl. Microbiol.*, **47**, 221 (2000).
11. L. Olsson and B. Hahn-Hägerdal, *Process Biochem.*, **28**, 249 (1993).
12. T.V. Rasskazchikova, V.M. Kapustin and S.A. Karpov, *Chem. Technol. Fuels Oils*, **40**, 203 (2004).
13. S. Mehmood, I. Orhan, Z. Ahsan, S. Aslan and M. Gulfraz, *Food Chem.*, **109**, 855 (2008).
14. L. Miranda, *J. Agric. Food Chem.*, **1**, 90 (1992).
15. FAO, Sweet *sorghum* in China. Agriculture Department, Food and Agriculture Organization of the United Nations. Available online at URL: http://www.fao.org/Ag/magazine/pdf/ag06.pdf. (2002).
16. A.N. Marcos, T. Kimura, N. Shimizuand and K. Shiiba, *Brazil. Arch. Biol. Tech.*, **49**, 481 (2006).
17. K. Suresh, N. Kiransree and L.V. Rao, *J. Bioproc. Eng.*, **21**, 165 (1999).
18. I.S. Pretorius, *Yeast*, **16**, 675 (2000).
19. G.M. Walker and P.V. Dijck, Physiological and Molecular Responses of Yeasts to the Environment. Yeasts in Food and Beverages, Springer Berlin Heidelberg, pp. 111-152 (2006).
20. B. Hames, Preparation of Samples for Compositional Analysis, Biomass Analysis Technology Team, Laboratory Analytical Procedure, National Renewable Energy Laboratory (NREL), Version 2004, pp. 1-9 (2004).
21. Standard Method for Determination of Total Solids in Biomass, Biomass Analysis Technology Team, Laboratory Analytical Procedure, National Renewable Energy Laboratory (NREL), pp. 1-7 (1994).
22. J. Ryan, G. Estefenm and A. Rashid, Soil and Plant Analysis Laboratory Manual, International Center for Agricultural Research in the Dry Areas (ICARDA) and National Agricultural Research Center (NARC) edn. 2 (2001).
23. M. Novak, P. Stretiajano, M. Moreno and G. Goma, *Biotechnol. Bioeng.*, **23**, 201 (1981).
24. A. Osho, *Afr. J. Biotech.*, **4**, 660 (2005).
25. T.J. Ekunsanmi and S.A. Odunfa, *J. Appl. Bact.*, **69**, 672 (1990).
26. H. Tsuge, E. Tatsumi, N. Ohtani and A. Nakazima, *Starch/Starke*, **44**, 29 (1992).
27. J.I. Ibeas and J. Jimenez, *Appl. Environ. Microbiol.*, **63**, 7 (1997).
28. H. Alexandre, I. Rousseaux and C. Charpentier, *FEMS Microbiol. Lett.*, **124**, 17 (1994).
29. C. Leao and N. van Uden, *Biochim. Biophys. Acta*, **774**, 43 (1984).
30. T.M. Swan and K. Watson, *Can. J. Microbiol.*, **43**, 70 (1997).
31. K.M. You, C.L. Rosenfield and D.C. Knipple, *Appl. Environ. Microbiol.*, **69**, 1499 (2003).
32. C. Pascual, A. Alonso, I. Garcia, C. Romay and A. Kotyk, *Biotechnol. Bioeng.*, **32**, 374 (1988).
33. J. Santos, M.J. Sousa, H. Cardoso, J. Inacio, S. Silva, I. Spencer-Martins and C. Leao, *Microbiology*, **154**, 422 (2008).
34. A. Ando, F. Tanaka, Y. Murata, H. Takagi and J. Shima, *FEMS Yeast Res.*, **6**, 249 (2006).
35. P. Wei, L. Zilong, L. Yuping, H. Peng and J. Ning, *Biotech. Lett.*, **29**, 1501 (2007).
36. K.C. Thomas, A. Dhas, B.G. Rossnagel and W.M. Ingledew, *Cereal Chem.*, **72**, 360 (1995).
37. N. Fujii, A. Sakurai, S. Suye and M. Sakakibara, *J. Ind. Microbiol. Biotechnol.*, **27**, 52 (2001).
38. K.C. Thomas, S.H. Hynes, A.M. Jones and W.M. Ingledew, *Appl. Biochem. Biotechnol.*, **43**, 211 (1993).
39. D.W. Zhan, M.R. Tuinstra, S. Bean, P.A. Seib and X.S. Sun, *Ind. Crops Prod.*, **18,** 245 (2003).
40. D.Y. Corredor, S.R. Bean, T. Schober and D. Wang, *Cereal Chem.*, **83**, 17 (2006).
41. N.K. Sree, M. Sridhar, K. Suresh and L.V. Rao, *Biopro. Eng.*, **20**, 561 (1999).