Journal of Metabolomics and Systems Biology. Vol. 2(1), pp. 10-14, April 2011
Available online http://www.academicjournals.org/jmsb

Full Length Research Paper

Determination and detoxification of cyanide content in sorghum for ethanol production using *Saccharomyces cerevisiae* strain

S. Prasad* and M. S. Dhanya

Division of Environmental Sciences, Indian Agricultural Research Institute, New Delhi 110012, India.

Accepted 30 March, 2011

Sorghum is a promising crop for the animal feed and fuel ethanol production. Presence of cyanide in its biomass, particularly in the tips of young leaves, carries some risk of toxicity to livestock and microbes, especially when at an immature stage of growth, its application may be restricted. The study was carried out at Indian Agricultural Research Institute, New Delhi with nine forage sorghum cultivars to determine cyanide content and its detoxification and fermentation. The cyanide content in sorghum biomass varied with the development stages and plant height. It ranged from 5.41 to 6.65 mg/100g at heading (50 to 82 cm height) and gradually decreased at flowering stage (82 to 175 cm height) from 2.75 to 3.46 mg/100 g. The minimum cyanide content was observed at milking (1.68 to 2.66 mg/100g) and dough stage (1.49 to 2.75 mg/100g) when the height was 175 to 220 cm. *Saccharomyces cerevisiae* strain, NCIM, 3186 was used to carry out fermentation of sorghum juice and found capable of detoxification and reduction of the cyanide content up to 84.6% improving the ethanol yield and fermentation efficiency by 91.8%. Thus, the utilization of sorghum cultivars at milking and dough stage could be considered safe to be for feed and fuel ethanol production.

Key words: Cyanide, detoxification, sorghum, *Saccharomyces cerevisiae*, ethanol yield.

INTRODUCTION

Sorghum plays a significant role in providing nutritious feed to dairy and farm animals. In recent years, the increased realization of the finite nature of the world's oil supplies and vagaries in oil prices have rekindled interest in ethanol production by fermentation of carbohydrate containing sorghum [*Sorghum bicolor* (L.) *Moench*] cultivars (Prasad et al., 2007). Because of its rapid growth rate, early maturity (90-180 days), high biomass yield and wide adaptability, sorghum has frequently been suggested as a good source of ethanol production (Hill et al., 1990; Prasad et al., 2007; Dimple et al., 2010). However, important crops like sorghum, cassava, lima beans, white clover, rubber and stone fruits contain cyanogenic β-glucosides and corresponding β-glucosidases. Upon damage to tissues, the enzyme and its substrate, which are compartmentalized in intact tissues come into contact and release a toxic aglycone or a derivative e.g. HCN or cyanide (Selmar, 1993). Sorghum tissue contains different levels of hydrocyanic acid (HCN) at different stages of maturity with leaves and stalks contribute most of the cyanide content in the sorghum plants at all stages of maturity.

Dhurrin is the major cyanogenic glucoside in sorghum, representing 30% of the dry weight of shoot tips of seedlings. Sorghum has two isozymes of the cyanogenic β-glucosidase dhurrinase namely dhurrinase-1 (*Dhr1*) and dhurrinase-2 (*Dhr2*). Dhurrin is hydrolyzed by the endogenous β-glucosidase (dhurrinase) to produce *p*-hydroxymandelonitrile which subsequently disassociates to free HCN and *p*-hydroxybenzaldehyde (Figure 1), (Muzaffer and Asim, 1998). Cyanogenic β-glucosides have long been known to be involved in the defense against some pathogens and herbivores releasing the respiratory poison, HCN upon hydrolysis by β-glucosidase (Poulton, 1993). In young sorghum leaves, dhurrin is localized in vacuoles of epidermal cells while

*Corresponding author. E-mail: shiv_drprasad@yahoo.co.in. Tel: +91-11-25841490. Fax: +91-11-25841866.

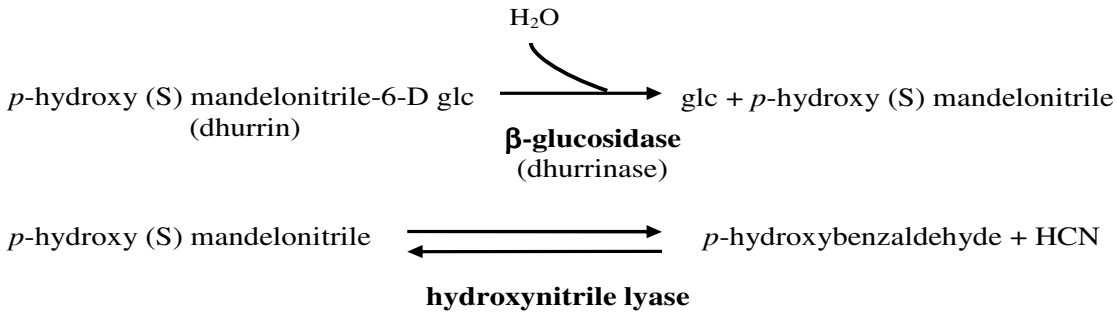

Figure 1. Hydrolysis of dhurrin by β-glucosidase (dhurrinase) and the production of HCN.

the enzymes responsible for the release of HCN from dhurrin are located in mesophyll cells. This separation of enzyme and substrate probably accounts for the fact that in healthy intact leaf tissue little, if any, free cyanide is present. Dhurrin concentration of sorghum leaves is highest at the seedling stage and decreases as the plant matures (Kojima et al., 1979). Naturally, occurring hydroxycinnamates may interfere with the fermentation of some of the yeast strains (Baranowsky et al., 1980). Microbial fermentation has also been reported as an effective means of cyanogens removal from cassava by-products (Padmaja et al., 1993; Bokanga et al., 1995). The objective of this study was to determine and detoxify the cyanide content in forage sorghum and its utilization for ethanol production using *S. cerevisiae* strain NCIM 3186.

MATERIALS AND METHODS

Details of field experiments

The field experiments were conducted during the Kharif (June to September) season at Pusa-farm, Indian Agricultural Research Institute, New Delhi. The site is located in the Indo-Gangetic alluvial tract at 28°40'N and 77°12'E, at an altitude of 228 m above the mean sea level. The climate is subtropical semi arid. The mean maximum and minimum temperatures during (kharif) rainy season are 35 and 18°C respectively with an annual rainfall of 750 mm. Nine sorghum cultivars such as PC1, PC6, PC9, PC23, PC121, PC129, PC601, PCH109, and SSG610 generally grown for forage were cultivated on sandy loam soil in a completely randomized block design with three replications in a plot of size 5 x 5 m., with a spacing of 45 x 15 cm. The plot was ploughed thoroughly and farmyard manure was applied uniformly at 10 t ha^{-1} before sowing. N:P:K at 60:40:30 kg ha^{-1} was applied as per normal recommended dose. Irrigation was given at seedling, primordial and flowering stages.

Sample collection

The fresh plant samples of each sorghum cultivars were collected from all replications in triplicates (108 samples) at four growth stages that is, Heading, flowering, milking, and dough stage, for determining the cyanide contents. All samples were packed individually in ice and transported to the laboratory. Samples were kept frozen at -4°C till further analysis. The average length of the plant was measured and recorded before chopped into small pieces. After harvesting the mature crop was defoliated and the stalks were cut at the nodes. The juice was extracted with a horizontal 3-roller power mill and the juice was immediately frozen at -20°C till further use.

Quantification of cyanide content in fresh green sorghum stalk

Cyanide content in sorghum stalk was estimated by the method described by Al-Sultan (2003). The sorghum tissue was first thoroughly chilled and 100 g of the finely minced tissue along with 300 ml cold water, 2 ml mineral oil and 10 ml of 5% tartaric acid was placed in 500 ml flask. The flask was connected to a vertical, water-cooled condenser, which in turn was connected to three absorption tubes (air sampling train) with 10 ml 0.1N NaOH connected in a series and a water aspirator was connected to the side arm of the last absorption tube. The flask was then heated in a water bath. The suction was regulated so that the contents of the flask were well agitated. And the distillation into first absorption tube was proceeded at the rate of about 5 drops per minute. Any cyanide content from the first absorption tube proceeded for two hours. Finally the contents of the three absorption tubes were transferred to a 100 ml volumetric flask and then made up the volume. 2 ml concentrated ammonium hydroxide (NH_4OH) and 1.0 ml 10% potassium iodide (KI) solution was added into it after thorough mixing. The cyanide was titrated against 0.005 N silver nitrate solutions. The bluish white opalescence caused by the formation of silver iodide (AgI) was the end point. One ml 0.005 N silver nitrate (AgNO3) is equivalent to 0.266 mg HCN.

Microorganism and Inoculum production

A non-pathogenic and saprophytic aerobic yeast strain of *S. cerevisiae,* NCIM 3186, (National Collection of Industrial Microorganisms*)* was procured from the National Chemical Laboratory, Pune, India. Pure culture of yeast strain was maintained in malt-extract glucose yeast-extract peptone (MGYP)-agar slants Yeast growth from 24 h Malt-extract glucose yeast-extract peptone (MGYP) agar was scrapped into 10 ml MGYP broth was used as the inoculum for growing the strains (Prakasham et al., 1999). Yeast was grown for 24 h in Malt-extract glucose yeast-extract peptone (MGYP) agar slant and loopful of the strain was transferred into 10 ml MGYP broth for inoculum preparation (Prakasham et al., 1999). The inoculum was grown at 30ºC in gyratory shaker (150 rpm) for 24 h and this inoculum was

used to inoculate 100 ml MGYP broth in 250 ml Erlenmeyer flask, for the fermentation and detoxification study in subsequent experimentation.

Fermentation and detoxification of sorghum juice

The experiment had two sets of treatments: (1) the clear sorghum juice containing 14.7% total sugar and 47 ppm cyanide as independent variables, (2) equivalent amount of sugar and cyanide as present in the juice were dissolved in distilled water to be used as synthetic media. Each experiment was performed in triplicates.

Clear sorghum juice and synthetic media solution of 250 ml taken in 500 ml Erlenmeyer flasks were treated with H_2SO_4 to adjust the pH to 4.5. The flasks were inoculated with *S. cerevisiae* NCIM 3186 innoculum at10% (v/v). All the flasks were then incubated at 30°C for 75 h under non-shaking conditions. The samples for total residual sugar, ethanol and cyanide analysis were taken throughout the course of the fermentation for 75 h at an interval of 10 h.

Quantification of cyanide content in sorghum juice and fermented supernatant

Cyanide determination is based on the principle that in the presence of a β-glucosidase and on hydrolysis, cyanide (HCN) is released from cyanoglycosidase and is trapped by sodium picrate solution. The absorbance of the resulting orange-yellow colour was determined spectophotometrically at 540 nm.

About 50 µl sorghum juice sample and fermented supernatant was dissolved in 2 ml 200 mM acetate buffer, pH 5.0 (containing 50 mM Na acetate and 50 µM $NaNO_3$) and decanted into outer section of a Warburg flask. 300 µl sodium picrate solution was pipetted into the inner well of the flask. 1 ml β-glucosidase was added to the sample-acetate buffer mixture. The flasks were sealed tightly and incubated at 40°C for four hours. Standards were prepared by adding 0-200 µl KCN in outer section 3 ml of acetate buffer and sodium picrate was added to the inner well. The flask was then sealed and incubated as described for the samples. After incubation, the flasks were cooled and 1ml 1 M HCl was added to the outer section. The flasks were sealed again and incubated at 60°C for four hours.

After incubation, 150 µl sodium picrate from the original 300 µl in central well was removed and added to a test tube containing 900 µl Sodium acetate buffer. The contents were thoroughly mixed and analyzed in spectrophotometer at 540 nm. Concentration of cyanide in the sample was then calculated against the standards taking into account that only 150 µl of the 300 µl Na picrate in the central well was used in the assay (Eustace and Dorothy, 2000).

Estimation of ethanol yield

The fermented liquor was centrifuged at 13,000 rpm for 10 min at a temperature of -20°C. The supernatant was analyzed for total sugars by a phenol-sulfuric acid method (Mecozzi, 2005). Ethanol concentration was analyzed by GC with flame ionization detector (Shimadzu GC-14B, Japan, Solid phase: polyethylene glycol PEG-20M, carrier gas: nitrogen, 90°C isothermal packed column, injection temperature 160°C, flame ionization detector temperature 230°C; and isopropanol as an internal standard). Percentage of conversion efficiency or yield efficiency (Ey) was calculated as Ey = Yps*100/0.51 where Yps is ethanol yield expressed as g ethanol per g sugar utilized (g g^{-1}) and 0.51 is the maximum theoretical ethanol yield of glucose consumption (Prasad et al., 2009).

Statistical methods

The results of the three replicates were pooled and expressed as mean (n = 3), and ± standard deviations. Statistical analysis of the data was carried using SPSS package v.10, significance was accepted at $P \geq 0.05$).

RESULTS

HCN content in different sorghum varieties

The cyanide content in biomass (mg/100 g) of sorghum cultivars varied with different growing stages and plant height and was highest at heading at 50 to 82 cm length. It gradually decreased with increasing height, that is, 82-175 cm length at flowering and found minimum at the milking followed by dough stages, at 175-220 cm length (Table 1). Mean of the cyanide content among various stages was significantly higher at heading followed by flowering stage; while lower at milking and dough stage (Table 1) and had no variation. In the present study, results are parallel with that recorded by Ibrahim et al. (1969) and Al-Sultan (2003).

The cyanide content among the cultivars at heading stage was significantly higher in PC601 followed by PC9 and PCH109. The cyanide content at flowering stage was significantly higher in PC121 and PC129 followed by PC6. The cultivar PC129 recorded maximum followed by PC6 and PC23 cyanide content at milking as well as dough stages followed by PC6 and PC23. The variety PC1 recorded minimum cyanide content at heading, flowering, milking and dough stages (Table 1).

Effect of fermentation by *S. cerevisiae*, NCIM 3186 on cyanide reduction and ethanol yield

The cyanide content, ethanol yield and fermentation efficiency in the fermented supernatant by *S. cerevisiae,* NCIM 3186 is shown in Figure 2. The result showed that the *S. cerevisiae* NCIM 3186 was able to reduce cyanide in the sorghum juice from 47 to 7.71 ppm and in the solution-media to 7.14 ppm. The reduction in cyanide content was because of increase in β-glucosidase activities in juice during the fermentation (Bokanga, 1995).

The analysis of fermentation data indicated that the ethanol yield was 6.9% w/v from 14.7% sugar present in juice. Ethanol yield coefficients (Yps) were 0.46 g ethanol g^{-1} sugar utilized. During the initial period of incubation up to 25 h, slight changes were noticed but after that no significant difference was found in fermentation efficiency (Ey) of both sorghum juice and synthetic media. These changes indicate that the *S. cerevisiae,* NCIM 3186 showed great effect on the detoxification of cyanide content in forage sorghum varieties juice during the fermentation and taken for the study and its utilization for ethanol production.

Table 1. HCN content at different growth stages and heights in nine sorghum cultivars. Results are expressed as the mean (n = 3) and ± standard deviations are given in parentheses. Mean with the same letter (s) along same row and column are not significantly different (P= 0.05).

Sorghum cultivars	HCN (mg/100 g biomass)			
	Heading (50-82) cm	Flowering (82-175) cm	Milking (175-220) cm	Dough (175-220) cm
PC1	5.05c ±0.27	2.93c ±0.53	1.68c ±0.15	1.49c ±0.37
PC6	5.76c ±0.21	3.37b ±0.18	2.57b ±0.15	2.43b ±0.54
PC9	6.38b ±0.53	3.20c ±0.27	2.21c ±0.55	2.07b ±0.11
PC 23	5.58c ±0.27	3.02c ±0.36	2.48b ±0.18	2.37b ±0.21
PC121	5.67c ±0.18	3.46a ±0.27	2.31b ±0.18	1.98b ±0.24
PC129	6.03c ±0.32	3.46a ±0.30	2.66a ±0.43	2.75a ±0.10
PC601	6.65a ±0.27	2.75c ±0.41	2.04c ±0.41	1.81c ±0.25
PCH109	6.12b ±0.80	3.0c ±0.15	1.68c ±0.15	1.61c ±0.33
SSG610	5.41c ±0.67	3.19c ±0.27	2.04c ±0.15	1.86c ±0.26
LSD (P=0.05) within varieties	0.77	0.49	0.53	0.44
Mean within stages	5.85a	3.15b	2.19c	2.18c
LSD (P=0.05) within growth stages	0.25			

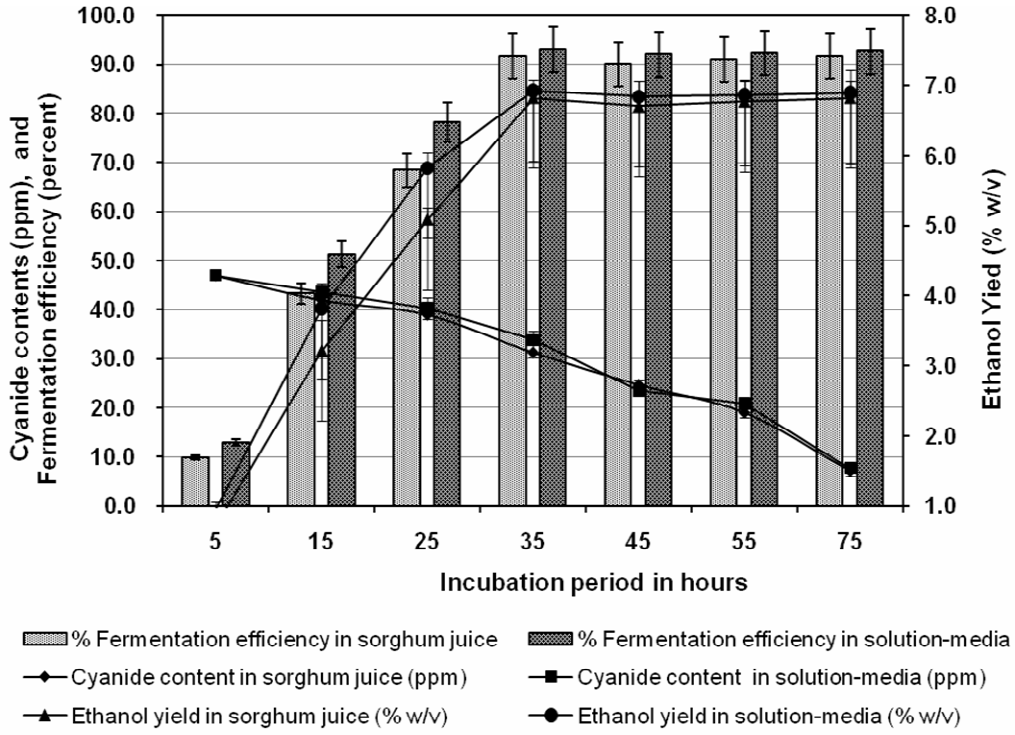

Figure 2. Changes in ethanol yield, fermentation efficiency and Cyanide contents following batch fermentation with NCIM 3186. Error bars are standard deviations over triplicate samples.

DISCUSSION

The cyanide content of sorghum increases rapidly during early growth stage, after which it declines with plant age (Busk and Møller, 2002). When measured per milligram of fresh biomass in the nine sorghum varieties, the cyanide potential peak at the heading stage with 50 to 82 cm length, which is also in agreement with findings of Ibrahim et al. (1969) where the maximum cyanide content reaches at 78 cm of plant height (corn). The reports by Akazawa et al. (1960) and Halkier and Møller (1989) confirmed the cyanogenic glucoside content in the tip of

young seedlings of *Sorghum bicolor* which reaches 6% of the dry weight. The cyanide content was found gradually declining with increasing height of sorghum varieties, that is, 82-175 cm length at flowering and found minimum at the milking followed by dough stages, at 175-220 cm length (Table 1). This is also seen parallel with those of Ibrahim et al. (1969) where the cyanide content start declining at 102 cm length and it gradually decreased to 0.19 mg/lb at 180 cm length. The cyanide content varied in all the nine sorghum varieties with higher PC601 followed by PC9 and PCH109. The concentration of cyanide also differs with the growth stages of all the varieties. This shows that cyanide content in sorghum depends highly on growth conditions and genetic background (Nelson, 1953; Gorz et al. 1987).

S. cerevisiae, NCIM 3186 was capable of detoxifying the cyanide content in the sorghum juice and solution media by 84.58 and 85.72 % respectively (Figure 2). The reduction in cyanide content was because of increase in β-glucosidase activities in juice during the fermentation (Bokanga, 1995). Padmaja et al (1993) reported the efficacy of a mixed culture inoculum in detoxifying intact cassava tuber and peel pieces and approximately 24–26% and 15–33% of total cyanide only remained in bound form in the fermented tuber and peel pieces respectively after 72 h fermentation. Eustace et al. (2000) also reported similar cyanide reduction of 76.69% in cassava peels by *S. cerevisiae.* Factors such as changes in texture in the plant tissue during the growth stages, increase β-glucosidase activity and the utilization of the cyanogenic glucosides and their products of fermentation breakdown by the micro organisms explains the reduction in cyanide levels by the microorganisms. The ethanol yield results found similar to that of the study of Laopaiboon et al. (2000), indicating sweet sorghum juice as one of the most promising raw materials for ethanol production with ethanol yield of 0.46 g ethanol g^{-1} sugar utilized. This is higher than that of other potential sugary raw materials such as sugar beet juice (*Yp/s* = 0.42, Ogbonna et al., 2001) and soybean molasses (*Yp/s* = 0.25, Siqueira et al., 2008).

The study concluded that utilization of sorghum varieties at milking and dough stage could be considered safe as feedstock for fuel ethanol production. The *S. cerevisiae* NCIM 3186 was capable for fermentation of sorghum stalks juice for ethanol production in terms of cyanide reduction, fermentation efficiency and ethanol yield.

REFERENCES

Akazawa T, Miljanich P, Conn EE (1960). Studies on cyanogenic glycosides of *Sorghum vulgare*. Plant Physiol., 35: 535–538.

Al-Sultan SI (2003). Sorghum Halepenses and its Cyanide Content. Pak. J. Nutr., 2: 123-124.

Baranowsky JD, Davidson PM, Nagel CW, Branen AL (1980). Inhibition of *S. cerevisiae* by naturally occurring hydroxycinnamates. J. Food Sci., 45: 592–594.

Bokanga M (1995). Biotechnology and Cassava processing in Africa. Food Tech., 49: 86-90.

Busk PK, Møller BL (2002). Dhurrin synthesis in sorghum Is Regulated at the transcriptional Level and induced by nitrogen fertilization in Older Plant. Plant Physiol., 129: 1222–1231.

Dimple KK, Danielle DB, Raymond LH, Mark RW, Claypool PL (2010). influence of temperature, pH and yeast on in-field production of ethanol from unsterilized sweet sorghum juice. Biomass Bioenergy, 34: 1481-1486.

Eustace AI, Dorothy ML (2000). Cyanide detoxification in cassava by-products by fungal solid state fermentation. J. Food Technol. Africa, 5: 48-51.

Gorz HJ, Haskins FA, Morris R, Johnson BE (1987). Identification of chromosomes that condition dhurrin content in sorghum seedlings. Crop Sci., 27: 201–203.

Halkier BA, Møller BL (1989). Biosynthesis of the cyanogenic glucoside dhurrin in seedlings of *Sorghum bicolor* (L.) Moench and partial purification of the enzyme system involved. Plant Physiol., 90: 1552–1559.

Hill FJ, Levellen RT, Skoyen IO (1990). Sweet sorghum cultivars for alcohol production. California Agric., 44: 14-16.

Ibrahim TM, Shaker M, Kamel SH (1969). Cyanide content in growing corn. Vet. Med. J. Cairo Univ. Egypt, 96: 127-134.

Kojima M, Poulton JE, Thayer SS, Conn EE (1979). Tissue distributions of therein and of enzymes involved in its metabolism in leaves of *Sorghum bicolor*. Plant Physiol., 63: 1022–1028.

Laopaiboon L, Thanonkeo P, Jaisil P, Laopaiboon P (2007). Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by *S.cerevisiae.* World J. Microbiol. Biotechnol., 23: 1497–1501.

Mecozzi M, (2005). Estimation of total carbohydrate amount in environmental samples by the phenol-sulphuric acid method assisted by multivariate calibration. Chemom. Intell. Lab. Syst., 79: 84–90

Muzaffer C, Asim E (1998). Structure and Expression of a Dhurrinase (b-Glucosidase) from Sorghum. Plant Physiol., 116: 1469–1478.

Nelson CE (1953). Hydrocyanic acid content of certain sorghums under irrigation as affected by nitrogen fertilizer and soil moisture stress. Agron J., 45: 615–617.

Ogbonna JC, Mashima H. Tanaga, H (2001). Scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized bioreactor. Bioresour. Technol., 76(1): 1-8.

Padmaja G, George AM, Moorthy SN (1993). Detoxification of cassava during fermentation with a mixed culture inoculums. J. Sci. Food Agric., 63: 473-841.

Poulton JE, (1993). Enzymology of cyanogenesis in rosaceous stone fruits. In A Esen, ed, b-Glucosidases: Biochem. Mol. Biol., ACS Symp. Ser. ACS, Washington, DC, 533: 170–190.

Prakasham RS, Kurikose B, Ramakrishna SV (1999). The influence of inert solids on ethanol production by *S. cerevisiae*. Appl. Biochem. Biotechnol., 82: 127-134.

Prasad S, Lata, Joshi HC, Pathak H (2009). Selection of efficient *S. cerevisiae* strain for ethanol production from sorghum stalk juice. Curr. Adv. Agril. Sci., 1: 70-72.

Prasad S, Singh A, Jain N, Joshi HC (2007). Ethanol production from sweet sorghum syrup for utilization as automotive fuel in India. Energy Fuels, 21: 2415 -2420.

Selmar D (1993). Apoplastic occurrence of cyanogenic bglucosidases and consequences for the metabolism of cyanogenic glucosides. In A Esen, ed, b-Glucosidases: Biochem. Mol. Biol., ACS Symp. Ser., ACS, Washington, DC, 533: 191–204.

Siqueira PF, Karp SG, Carvalho JC, Sturm W, Rodriguez-Leon JA, Tholozan JL, Singhania RR, Pandey A, Saccol CR (2008). Production of bio-ethanol from soybean molasses by *S. cerevisiae* at laboratory, pilot and industrial scales. Bioresour. Technol., 99(17): 8156-8163.