Full Length Research Article

© 2024, Centre for Advanced Research in Agricultural Sciences
Research Journal of Agricultural Sciences
Volume 15; Issue 02 (Mar–Apr 2024); pp 331–335

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

Bio Ethanol Production from Sugarcane Bagasse Agriculture Waste using *Saccharomyces cerevisiae* and its Quantification by GC-MS

B. Ramalingappa[1], H. M. Siddesh[2] and G. L. Aruna*[3]

[1-2] Department of Studies and Research in Microbiology, Davangere University, Davangere - 577 002, Karnataka, India
[3] Post-Graduate Department of Microbiology, Maharani's Science College for Women (Autonomous), Mysore - 570 005, Karnataka, India

Received: 07 Dec 2023; Revised accepted: 18 Feb 2024; Published online: 06 Mar 2024

Abstract

The fossil fuels, coal and natural gases are used as energy sources for automobiles. These energy sources are not eco-friendly, non-renewable, and they could exhaust in near future. This energy crisis, repercussions and hazardous effects associated with fossil fuels necessitate discovering of novel idea of alternative, ecofriendly and renewable energy sources. Bio ethanol is an alternative renewable energy source made from various agricultural waste products by the fermentation activity of yeast. This work is an attempt to produce bio ethanol from sugarcane bagasse agro waste using highly potent sugarcane bagasse saccharifying fungus *Aspergillus niger* and potent fermentative yeast *Saccharomyces cerevisiae*. For high bioethanol yield, the fermentation parameters including substrate concentration, incubation time, incubation temperature and pH were optimized. The potassium dichromate method was used to determine the amount of bioethanol separated through fractional distillation and was confirmed by GC-MS analysis. It was found that 2.5g/100 ml concentration of sugarcane bagasse with pH of 5.5 after being incubated for 10 days at 30°C showed high yield of bioethanol. It was estimated to be 6.81 % (v/v) at optimum conditions. It was confirmed by GC-MS analysis which showed 9.99% (v/v) ethanol at the retention time 20.08 minutes and the peak value 207 indicates highest ethanol concentration. This study revealed that sugarcane bagasse is one of the cheap and abundantly available agricultural waste can be exploited for the production of bioethanol since it contains significant amount of sugar.

***Key words*:** *Aspergillus*, Ethanol, Fermentation, Potassium dichromate method, Retention time, Saccharification

The energy sources such as petrol, diesel, coal and natural gases are being used for automobiles. However, these energy sources are non-renewable and they might exhaust in coming years. Additionally, they contribute to environmental hazards such as global warming, smog and acid-rain. This energy crisis and dangerous consequences of fossil fuels necessitate the development of novel approaches for producing ecofriendly and renewable alternative energy sources [1]. The most promising eco-friendly and alternative renewable energy source for automobiles is bio ethanol. Despite having a 68 percent lower energy equivalent than fossil fuels, ethanol does not emit any harmful chemicals such as CO or CO_2 to the environment during its combustion [1-2]. Many researchers used cheap substances like oils and fatty acids, soy flour, waste food grain fungal mycelia, molasses, and vegetable and fruit pulp for bio ethanol production [3]. Sugarcane is the major crop is being used for production of ethanol around the world including India [4]. At present in India, sugarcane molasses is being for bioethanol production [5].

In industries, *Saccharomyces cerevisiae* is being commonly used for ethanol production [1]. High ethanol can be produced by concert saccharification and fermentation process (SSF). It is cost-effective strategy for bio ethanol production from agro wastes [6]. Bio ethanol production using *A. niger* and *S. cerevisiae* in concert saccharification and fermentation process [7]. The most widely used microorganism for producing bio ethanol is *Saccharomyces cerevisae*, which has yield maximum ethanol concentration of 18% in the fermentation broth. There are a variety of yeasts are available commercially including dry yeast and wet yeast. Commercial yeast strains, in comparison to natural yeast strains, have many advantages due to their lengthy shelf lives, high cell viability, efficiency, lack of microbial contamination, and low cost [8-9]. The pretreatment of raw material was carried out using various chemicals, among them, hydrochloric acid was most effective. Potassium dichromate method is used for estimation of concentration of ethanol production from raw materials [3]. The present work aimed to produce bioethanol from inexpensive substance sugarcane bagasse and it is widely available agriculture waste.

MATERIALS AND METHODS

Substrate preparation and its pre-treatment

For this study, agro waste sugarcane bagasse were brought from various agro waste lands of Davanagere, Sugarcane bagasse sample was thoroughly cleaned with tap water and dried. It was cut in to small pieces and dried in sun

***Correspondence to:** G. L. Aruna, E-mail: microarunagl@gmail.com; Tel: +91 9741404036

Citation: Ramalingappa B, Siddesh HM, Aruna GL. Bio ethanol production from sugarcane bagasse agriculture waste using *Saccharomyces cerevisiae* and its quantification by GC-MS. *Res. Jr. Agril. Sci.* 15(2): 331-335.

for 3 to 5 days based on water content. It was then cut into small particle size and it was soaked in 1% NaOH for two hours to remove lingo cellulose [10]. The powdered sugarcane bagasse was washed many times with distilled water to remove NaOH. The sample was autoclaved and filtered. It was then dried using hot air oven [11].

Isolation and identification of soil fungi

Soil samples were brought from Davangere university campus, Tholahunase, Davangere, collected at a depth of 10-20 cm and kept in sterile airtight containers. The soil samples were examined for microorganisms capable of hydrolyzing the sugarcane bagasse. *Aspergillus niger* was the most common fungal culture isolated and identified. The fungus was kept in the refrigerator on PDA slants until it was used [12].

Fermentation medium and fermentation

Fermentation was carried out in a medium containing yeast extract (0.2%), NH_4NO_3 (0.2%), $MgSO_4.7H_2O$. (1%), KH_2PO_4 (0.2%) and 5 gm sugarcane bagasse. A volume of 5% (V/V) *A. niger* inoculum and 10% (W/V) *S cerevisiae* inoculum were inoculated, and the pH was adjusted to 5.5. The fermentation culture medium was kept for incubation for ten days on a rotary shaker at 120 rpm. The fermentation culture broth was centrifuged at 6000 rpm for 10 minutes.

Estimation of reducing sugars

Reducing sugars of sugarcane bagasse were extracted from 100 mg of sugar bagasse treated with 10 ml of hot 80% ethyl alcohol twice; later extracted sugar solution was transferred to fresh tube and evaporated on a water bath at 80°C. A volume of 10 ml of water was transferred to supernatant to make the sugars to dissolve. The aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 ml of standard solution of concentration of 1mg/1ml were prepared in test tubes. The volume of all the tubes including blank and sample was made up to 2 ml with distilled water. A volume of 1ml alkaline copper tartarate reagent was added to all tubes and they were incubated for 10 min in boiling water bath. After cooling, 1ml of the arsenomolybdic acid reagent was added to each tube. At 660 nm, the optical densities of the standard and test samples were determined. A graph of OD versus concentration of sugar was plotted.

Estimation of protein

The protein concentration of sugarcane bagasse was determined by Bradford protein assay [13] using BSA as standard protein. The working solution of bovine serum albumin (1mg/1ml) aliquots of 0.2, 0.4, 0.6, 0.8, 1.0 ml were prepared in test tubes. The volume of all the tubes including sample and blank was made up to 3 ml using phosphate buffer. A volume of 5 ml Bradford reagent was transferred to all the tubes and they were incubated for 30 minutes at room temperature or till blue colour appears. The OD of the working standard and samples was taken at 595 nm.

Optimization of fermentation conditions for bioethanol production

The fermentation parameters such as substrate concentration, incubation time, incubation temperature and pH were optimized for high yield of bioethanol using chemically treated substrate [14].

Optimization of pH

A volume of 100 ml of fermentation broth supplemented with 5g of substrate (sugar bagasse) was taken in flasks and set with different pH (3.5, 4.5, 5.5, 6.5), then inoculated with 10% (v/v) *Saccharomyces cerevisiae* inoculum and 5% *A. niger* (w/v) and one flask kept as control without adding inoculum. All flasks were kept for incubation for 10 days on a shaker at 120 rpm at 30° C.

Optimization of temperature

A volume of 100 ml fermentation broth set with pH 5.5 was taken in flask supplemented with 5g of substrate (sugar bagasse) and inoculated with 10% (v/v) *Saccharomyces cerevisiae* inoculum and 5% *A. niger* (w/v). One flask was kept as control, with no inoculum added, and the flasks were kept for incubation at temperatures of 20 °C to 50 °C for 10 days.

Optimization of substrate concentration

The pH of 100 ml of fermentation broth supplemented with different concentrations of sugar bagasse (2.5g, 3.0g, 3.5g, 4.0 g, 4.5 g, and 5g), was set to 5.5 and then inoculated with 10% (v/v) *Saccharomyces cerevisiae* inoculum and 5% *A. niger* (w/v). One flask kept as control and all the flasks were incubated at 30°C on rotary shaker for 10 days at 120 rpm.

Optimization of incubation time

The flasks containing 100ml of fermentation broth supplemented with 5g of substrate (sugar bagasse) set with pH 5.5 were inoculated with 10% (v/v) *Saccharomyces cerevisiae* and 5% *Aspergillus niger* (w/v) inoculums except one flask which kept as control. The flasks were incubated on rotary shaker at 120 rpm for different incubation time (2, 4, 6, 8 and 10 days) at 30° C.

Separation of ethanol by distillation method

Fractional distillation was used to separate the ethanol from water produced in the fermentation broth.

Estimation of bioethanol

Potassium dichromate method was used estimate the bioethanol separated by fractional distillation as reported by Caputi *et al*. [15]. The OD of colour developed was taken in UV -Visible spectrophotometer at 660 nm.

Quantitative estimation of ethanol by GC-MS method

Gas chromatography Mass Spectroscopy (GC-MS) was used for quantitative estimation of ethanol in fermentation broth. The compounds were determined by their relative retention times and quantified based on a calibration curve plotted with standard sugars and fermentation products.

RESULTS AND DISCUSSION

Isolation and Identification of soil fungi

The predominant sacchrifying fungal culture isolated from soil samples was *Aspergillus niger*. It was identified by wet mount method using lacto phenol cotton blue staining.

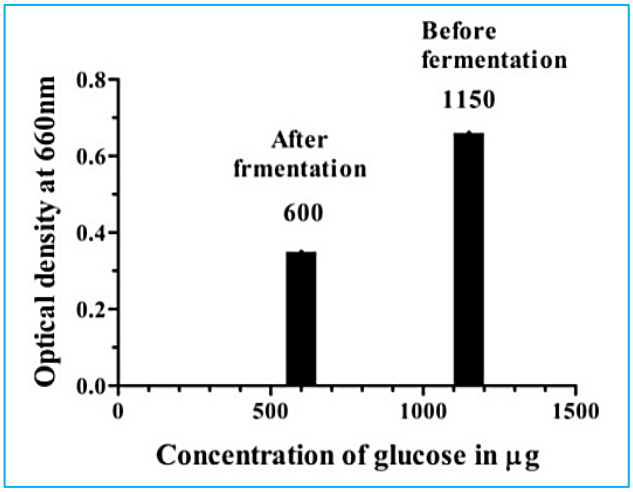
Estimation of reducing sugars

The maximum amount of sugar produced from saccharification of sugarcane bagasse by *A. Niger*, it was found to be 1150 µg/ml The sugar concentration reduced to 600µg/ml after 10 days of fermentation, (Fig 1a). This revealed that the sugar was fermented into bioethanol by the yeast added to the sugarcane bagasse.

Estimation of protein

The protein content of substrate is essential biomass for the production of bio ethanol. In our study, the protein concentration in sugarcane bagasse prior to the addition of *A.*

niger was 600 µg/ml and after the addition of *A. niger*, it was 1090 µg/ml following the addition of *A. niger* (Fig 1b). It was evident from this that *A. niger* increased the biomass needed to produce bioethanol.



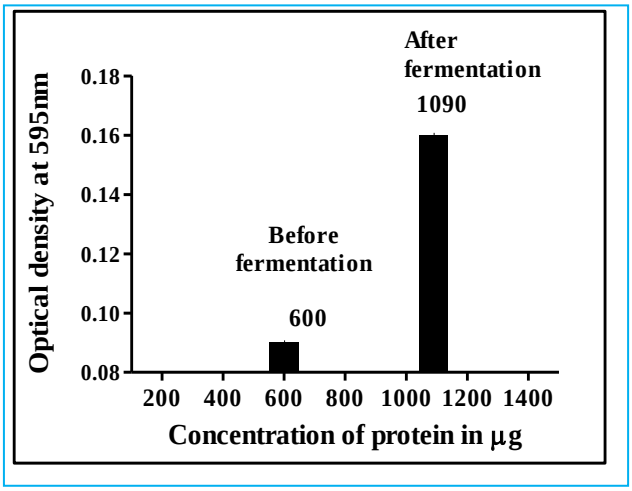


Fig 1(a) Graph showing concentration of glucose before and after fermentation. The glucose concentration before fermentation was 1150 µg/ml and after fermentation it was 600 µg/ml

(b) Graph showing concentration of protein in sugarcane bagasse before the addition of *A. niger* was 600 µg/ml and after the addition of *A. niger*, it was 1090 µg/ml

Optimization of fermentation conditions

The fermentation parameters such as substrate concentration, incubation time, incubation temperature and pH were optimized for high yield of bioethanol [14]. The present work showed that sustainable waste sugarcane bagasse at low concentration yielded maximum percentage of alcohol and it was estimated to be 6.81%. The percentage of bioethanol production varied in different environmental factors such as pH, substrate concentration and incubation time. This was consistent with the findings of earlier researchers [5].

Optimization of pH

The pH is one of the important factors which influence the enzyme activity and there by alcohol fermentation. As *S. cerevisiae* is acidophilic, maximum ethanol percentage was observed at acidic pH. Estimation of percentage of bioethanol at different pH (3.5 to 7.5) showed that at pH 5.5, it was maximum (6.3%). This showed that pH 5.5 is optimum for high yield of bioethanol from sugarcane bagasse by *S. cerevisiae* (Fig 2). This pH not only favoured *Saccharomyces cerevisiae* for ethanol production but also prevented the bacterial contamination during fermentation. As the pH decreases, the fermenting broth became more acidic, thus changing the metabolic activities of the yeast for increased ethanol production. Previously, several workers have studied the effect of pH on sugarcane bagasse fermentation by *S. cerevisiae*. Our result was in concordance with previous workers who reported that high ethanol production was obtained at pH 5.0 to 6.0 [16-17]. Baz *et al*. [17] reported the increased ethanol production between pH 4.0 to 7.0 and then decreased marginally below this value. As *S. cerevisiae* is acidophilic and mesophilic in nature and hence more biomass production and maximum fermentation activity and high ethanol production was found at acidic pH and at moderate temperature [17]. Turhan *et al*. [16] who revealed the production of high ethanol at pH 5.0 - 6.0 [16]. The suitable pH for the production of ethanol by yeast was 4.0 to 4.6 [18].

Optimization of Temperature

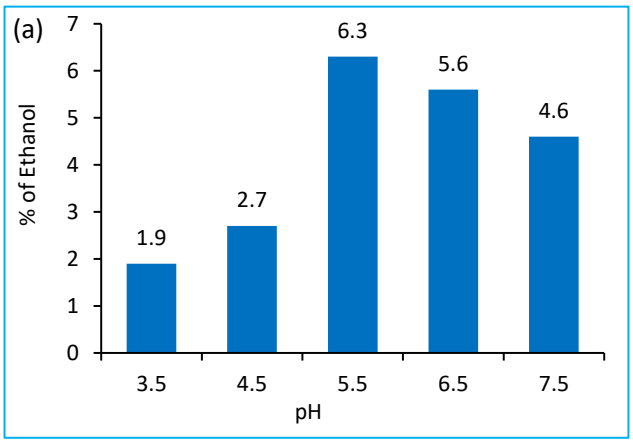
The temperature is one of the important factors which affects alcohol fermentation. The alcohol production was increased with increase in temperature till 40°C. Further increase in temperature does not increase alcohol production. Estimates of bioethanol percentage at various temperatures revealed that it was highest of 6.3 percent at 30 °C. This indicated that 30°C is optimum temperature for high production of bioethanol from sugarcane bagasse by *S. cerevisiae*. Our findings were in in agreement with those of Baz *et al*. [17] who claimed that bioethanol production peaked at 30 °C and dropped as temperature increased. *S. cerevisiae* produces bioethanol best at a temperature of 32 °C from fruit wastes fermentation [19]. The high yield of 7.9% (v/v) bioethanol produced by *S. cerevisiae* at 30 °C with a fermentation efficiency of 87.85 [18]. The higher temperatures inactivate the fermentation enzymes of *S. cerevisiae* and consequently produce less bioethanol. Therefore, one of the crucial factors that must be regulated during the manufacturing of bioethanol is temperature.

Optimization of substrate concentration

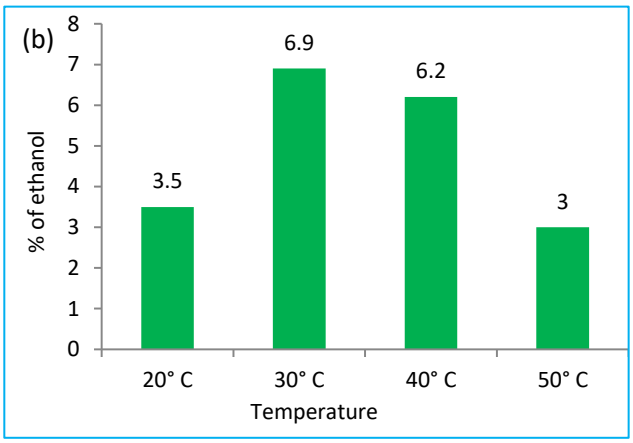
Substrate concentration and fermentation time are two additional parameters that affect the production of bioethanol [19]. Estimation of percentage of bioethanol at various substrate concentrations (sugarcane bagasse) revealed that it was highest at 2.5g/100 ml. This demonstrated that 2.5g/100 ml of sugarcane bagasse was ideal for S. cerevisiae to produce bioethanol.

Optimization of incubation time

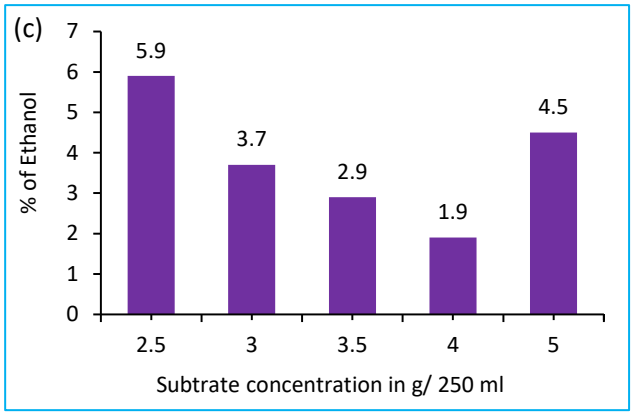
Estimation of bioethanol % at varied incubation times (2, 4, 6, 8, and 10 days) revealed that it was highest at 10 days. This demonstrated that incubation for 10 days was the best time for *Saccharomyces cerevisiae* to produce bioethanol in sugarcane bagasse. The optimal period is required for yeast growth followed by sugar fermentation. The maximum percentage of ethanol, 3.5, was obtained after 10 days of incubation in our investigation. The ethanol content was determined using the potassium dichromate method after the manufacture of bioethanol from sugarcane bagasse and distillation. The bioethanol yield from sugarcane bagasse fermentation by *Saccharomyces cerevisiae* is shown in (Fig 2). After ten days of incubation, it was assessed to be 6.81 percent.



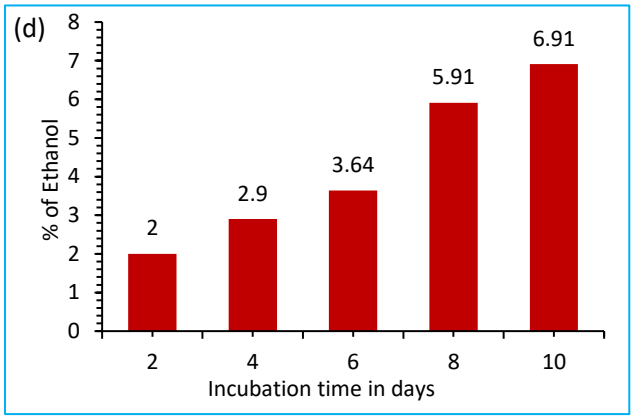
(a) Effect of pH



(b) Effect of temperature



(c) Effect of substrate concentration



(d) Effect of incubation period

Fig 2 Graph showing effect of fermentation conditions on bioethanol production

Estimation of ethanol

After the production of bioethanol from sugarcane bagasse and distillation, the ethanol concentration was estimated by potassium dichromate method. (Fig 3) shows the bioethanol yield from fermentation of sugarcane bagasse by *S. cerevisiae*. It was estimated to be 6.81% after 10 days of incubation.

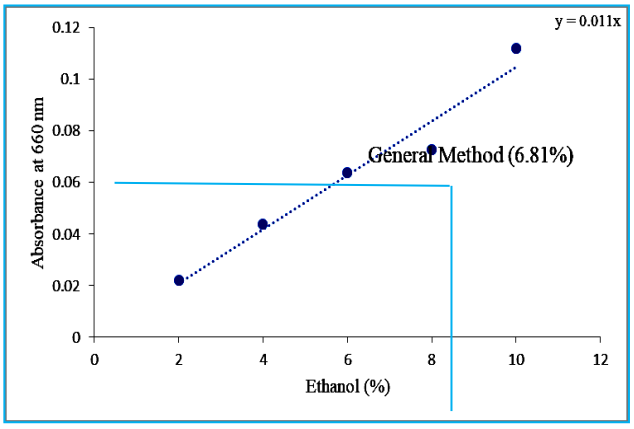


Fig 3 Graph showing percentage of ethanol estimated by potassium dichromate method. It was 6.81% after 10 days of incubation

Quantification of bio ethanol by GC-MS method

The bioethanol yield from sugarcane bagasse was 6.81 percent (v/v) as estimated by the potassium dichromate process, which was confirmed by GC-MS analysis. It produced 9.99% (v/v), had 20.08-minute retention time and had a peak of 207 (Fig 4) showed the highest ethanol concentration. Previously, researchers used a potassium dichromate technique to estimate bioethanol generated from diverse substrates, which was then validated using gas chromatography [4].

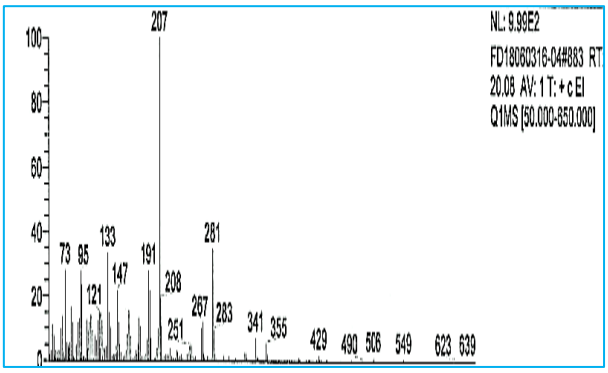


Fig 4 Graph showing quantification of bio ethanol by GC-MS Method. It produced 9.99% (v/v), had a 20.08-minute retention duration, and a peak of 207 shows highest ethanol concentration

CONCLUSION

The results of this study revealed that sugarcane bagasse agro waste is a suitable substrate for bioethanol production. This study implies that agricultural wastes can be used to manufacture ethanol in a cost-effective and efficient manner. The development of an environmentally friendly technique for pre-treating agricultural wastes, as well as the screening of highly efficient microorganism for the production of fermentable sugars conversion from pre-treated waste and potential microorganism that ferment sugar into ethanol, should be prioritized. Bioethanol has a lot of advantages when it comes

to safeguarding the environment from pollution, supporting rural agriculture, and assuring fuel security.

Acknowledgments

The authors are thankful to KSCST for providing Project (No.7.1.01/SPP/08) fund to carry out research work on Bio ethanol Production from Sugarcane bagasse agro waste using Saccharomyces cerevisiae and Quantification by GC-MS at Davangere University, Tholahunse, Davangere.

Conflict of interest

The authors declare that there is no conflict of interest.

LITERATURE CITED

1. Lebaka VR, Ryu H, Wee H. 2014. Effect of fruit pulp supplementation on rapid and enhanced ethanol production in very high gravity (VHG) fermentation. *Bioresour. Bioprocess* 1: 22-28.
2. Vohra M, Manwar J, Manmode R, Padgilwar S, Patil S. 2014. Bioethanol production: Feedstock and current technologies. *Jr. Environ. Chem. Engineering* 2: 573-584.
3. Kulkarni SJ, Shinde NL, Goswami A. 2015. A review on ethanol production from agricultural waste raw material. *Int. Jr. Sci. Res. Sci. Eng. Technology* 1: 231-233.
4. Mushimiyimana, Tallapragada. 2016. Bioethonol production from Agro wastes by acid hydrolysis and fermentation process. *Jr. Sci. Ind. Research* 75: 383-388.
5. Singh S, Adak A, Saritha M, Sharma S, Tiwari R, Rana S, Arora A, Nain L. 2017. Bioethanol production scenario in India: Potential and Policy Perspective. *In*: (Eds) Chandel A, Sukumaran R. Sustainable Biofuels Development in India. Springer, Cham. pp 87-133.
6. Sheikh RA, Al-Bar OA, Soliman YMA. 2016. Biochemical studies on the production of biofuel (bioethanol) from potato peels wastes by *Saccharomyces cerevisiae*: Effects of fermentation periods and nitrogen source concentration. *Biotechnology Biotechnol. Equip*. 30: 497-505.
7. Zhang Q, Weng C, Huang H, Achal V, Wang D. 2016. Optimization of bioethanol production using whole plant of water hyacinth as substrate in simultaneous saccharification and fermentation process. *Front. Microbiology* 6: 1-9.
8. Rose AH, Harrison JS. 2012. *The Yeasts: Yeast Technology*. Academic Press, London. pp 394.
9. Jayusa B, Nurhayati B, Mayzuhroha A, Arindhania S, Caroenchaic C. 2016. International Conference on Food, Agriculture and Natural Resources, IC-FAN Res. Studies on Bioethanol Production of Commercial Baker's and Alcohol Yeast under Aerated Culture Using Sugarcane Molasses as The Media. *Agriculture and Agricultural Science Procedia* 9: 493-499.
10. Ali MN, Mohd MK, Majid M. 2011. Ethanol fuel production through microbial extracellular enzymatic hydrolysis and fermentation from renewable agro based cellulosic wastes. *Int. Jr. Pharma Bio Science* 2: 331-334.
11. Irfan M, Gulsher M, Abbas S, Syed Q, Nadeem M, Baig S. 2013. Effect of various pre-treatment conditions on enzymatic saccharification. *Songklanakarin Jr. Sci. Technology* 33: 397-404.
12. Ajani AO, Agarry SE, Agbede OO. 2011. A comparative kinetic study of acidic hydrolysis of wastes cellulose from agricultural derived biomass. *Jr. Appl. Sci. Environ. Management* 15: 531-537.
13. Bradford MM. 1976. A dye binding assay for protein. *Anal. Biochemistry* 72: 248-254.
14. Duhan JS, Kumar A, Tanwar SK. 2013. Bioethanol production from starchy part of tuberous plant (potato) using *Saccharomyces cerevisiae* MTCC-170. *Afr. Jr. Microbiol. Research* 7: 5253-5260.
15. Caputi A, Ueda M, Brown T. 1968. Spectrophotometric determination of ethanol in wine. *American Journal of Enol. Vitic.* 19: 160-165.
16. Turhan I, Bialka KL, Demirci A, Karhan M. 2010. Ethanol production from carob extract by using *Saccharomyces cerevisiae*. *Bioresource Technology* 101: 5290- 5296.
17. Baz WA, Nawar LS, Aly MM. 2017. Production of biofuel from sugarcane bagasse wastes using *Saccharomyces cerevisiae. Jr. Exp. Biol. Agric. Sciences* 5: 871-877.
18. Khoja AH, Ali E, Zafar K, Ansari AA, Nawar A, Qayyum M. 2015. Comparative study of bioethanol production from sugarcane molasses by using *Zymomonas mobilis* and *Saccharomyces cerevisiae. Afr. Jr. Biotechnology* 14: 455-2462.
19. Karma VH, Kumar A, Singh J, Dwivedi S, Kumar M. 2014. Production of ethanol from fruit wastes by using *Saccharomyces cerevisiae. Int. Jr. Renew. Energy Technology* 3: 1-5.