ORIGINAL RESEARCH PAPER | Science | Volume : 6 | Issue : 8 | August 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Enhancing Ethanol Yield From Sugar Cane Molasses Fermentation by Addition of Depolymerising Enzymes

KEYWORDS: sugar cane molasses, ethanol, unfermentable sugars, enzymes

Fadel, M	Abdel Nasser A. Zohri	Adel M..Kamal El-Dean
Microbial Chemistry Department, National Research Center, Cairo, Egypt	Botany and Microbiology Department, Faculty of Science, Assiut University, Egypt	The Dean of Sugar Technology and Research Institute, Assiut University, Egypt

A.H. Auob	B. Deiab
Chemicals Factories, Sugar and Integrated Company , El- Hawamdia Giza, Egypt	Chemicals Factories, Sugar and Integrated Company , El- Hawamdia Giza, Egypt

ABSTRACT *The demand of energy specially an eco-friendly clean biofuel is increased due to shortage of resources which leads to search for alternate fuel to replace fossil fuels.Sugarcane molasses (the by-product of sugar industry from sugarcane) is a main raw material for bioethanol production in many countries. The cost of molasses in increasing sothere is a need to improve the ethanol production from cane molasses. Molasses nearly involved about 10 % unfermentable sugars relative to the fermentable sugars. as alpha amylase, glucoamylase, dextranase and cellulase were applied to break down the biopolymer such as starch,dextran and cellulose to monosacchadides can be fermented to ethanol by Saccharomyces cerevisiaeduring the process of fermentation. Enzymes level addition of alpha amylase, glucoamylase,dextranase and cellulase to fermentation molasses medium were optimized to be 4,6,3 and 2 mg /100g unfermentable sugars, respectively.The fermentation applied in sugar cane molasses Brix 21 contained 16.6 % total sugars involved 1.8 % unfermentable sugars. The fermentation was carried out for 36 hrs at 35oC by S. cerevisiae F-514 giving ethanol yield 8.89% v/v compared to 8.17% v/v in untreated enzyme molasses.The obtained ethanol yield increase resample 8.81% v/v.*

Introduction

The demand of energy specially an eco-friendly clean biofuel is increased due to shortage of resources which leads to search for alternate fuel to replace fossil fuels.Sugarcane molasses (the byproduct of sugarindustryfrom sugar cane) is a main raw material for bioethanol production in many countries. The cost of molasses in increasing so there is a need to improve the ethanol production from cane molasses. Molasses nearly involved about 10 % unfermentable sugars relative to the fermentable sugars. Depolymerising enzymes such as alpha amylase, glucoamylase, dextranase and cellulase were applied to break down the biopolymer such as starch; dextran and cellulose to monosaccharides can be fermented to bio-ethanol by ***S. cerevisiae*** during the process of fermentation. Alpha-amylase acting at random locations along the starch chain breaks down, yielding maltotriose and maltose from amylose, or .maltose, glucose and "limit dextrin" from amylopectin (Ghalanbor,2008 andKadziola et al.,1998). Glucose only instead of other product can be ingest by ***S.cerevisiae*** and excrete alcohol in the fermentation medium Glucoamylase is an enzyme which decomposes the above smaller fragments into glucose by tearing-off glucose units from the non-reduced end. The liberated glucose fermented by ***S. cerevisiae*** to ethanol yield, and thus added value to ethanol production from molasses.

Molasses contains biopolymer dextran which produced from sucrose by microorganisms.The most prevalent of these is ***Leuconostocrnesenteroides***which is generally the source of the dextran in sugarcane products. Dextran cannot crystallize during sugar manufacture. So go to loss in molasses. Dextranase hydrolyzes the biopolymer dextran to glucose units.The glucose units increased the fermentable sugars concentration in the fermentation medium led to more ethanol yield by yeast.

Cellulase contains three types responsible for saccharification of cellulose polymer to glucose (Worthington Biochemical Corporation,2014),1-Endocellulase which break down of the non covalent interactions present in the amorphous structure of cellulose; 2-Exocellulase which hydrolyze of cellulose chain from reducing ends to break the polymer into smaller sugars (both endocellulase and exocellulase resulting disaccharide cellobiose or tetrasaccharides); and 3-Beta-glucosidase responsible for hydrolysis of disaccharides and tetrasaccharides into glucose (Zverlov et al., 2005 and Telke, et al., 2013).

In Egypt about 400,000 tons annually of sugar cane molasses used for ethanol production in Egyptian distillery factories. This amount contains about 40,000 ton unfermentable sugars can be hydrolyzed by enzymes to added value of about 24,000 cubic meter of ethanol annually.

The aim of the present study was using enzymes to hydrolyze the unfermentable sugars in cane molasses to added value to ethanol production from sugars in cane molasses.

MATERIALS and METHODS

Sugarcane Molasses:

Molasses sample (Brix.79. total sugars 55.2%, fermentable sugars 50.1% and unfermentable sugars 5.1%) was supplied by Egyptian sugar and integrated industries Company (ESIIC)

Yeast Strain:

Saccharomyces cerevisiae F-514, which are already .applied for ethanol production in Egyptian distillation factories supplied by Microbial Chemistry Lab .National Research Centre, Dokki, Cairo Egypt

Volume : 6 | Issue : 8 | August 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Depolymerising enzymes
Enzymes such as alpha amylase, glucoamylase, cellulase, and dextranase were gained from commercially available sources and were of industrial grade from Stern Enzym GmbH and Co.KG

Table (1): commercial depolymeryzing enzymes

Enzyme	Product name	Activity U/g	source
Alpha amylase	SternEnzymC21032	60000	Bacteria
Glucoamylase	SternEnzym GA14400L	5000	Fungi
Dextranase	SternEnzymDX1oL	700	Fungi
Cellulase	SternEnzym	2800	Fungi

Individual enzymes were assayed by routine biochemistry before the start of the experiments as follows**:**

Alpha-amylase assay
Alpha-amylase activity was determined by measuring the amount of starch hydrolyzed in the reaction mixture by the iodine method (Manning and Campbell 1961). One unit of enzyme activity has been defined as the amount of enzyme that hydrolyses one mg of starch min^{-1} under assay conditions.

Glucoamylase assay
Glucoamylase activity was determined according to the method reported by Miller (1959), by incubating 1 % (w/v) maltose with 0.9 mL sodium citrate buffer (0.05 M, pH 5.0) and 0.1 mL of diluted enzyme solution at 35°C for 30 min. The reaction was terminated by placing tubes in a boiling water bath for 10 min. The released reducing sugars were measured with DNS reagent using glucose as a standard. Glucoamylase activity unit (U) was expressed as the amount of enzyme releasing 1µ mol of glucose per min under assay conditions.

Dextranase assay
Dextranase activity was analyzed with the 3,5-dinitrosalicylic acid (DNS) method by assaying the reducing sugars released during a 25 min reaction (1% (w/v) dextran, 0.05 M acetate buffer, pH 5.0, 50°C). The absorbance was read at 550 nm using a spectrophotometer. The amount of reducing sugar was calculated from the standard curve based on the equivalent glucose. One enzyme activity unit (U) was defined as the amount of enzyme that liberates one micromole of glucose per min reaction under assay conditions (Miller 1959). The results of the analysis are the mean values of duplicate separate experiments

Cellulase assays
Endo cllulase and exocellulase) activities were assayed according to the method described by Mandels et al.(1974). Endo cellulase activity was determined in assay mixture contained 50 mg of Whatman No.1 strip (1 × 6 cm) in 1 ml of 0.05 M citrate buffer (pH 5) and 0.5 ml of diluted crude enzyme. The mixture was incubated at 35 °C for 30 min. Exocellulase activity was determined in the assay mixture contained 0.5 ml of 1 % of carboxyl methyl cellulose (CMC) in 0.05M citrate buffer (pH 5.0) and 0.5 ml of diluted crude enzyme. The mixture was incubated at 35 °C for 30 min. β-glucosidase activity was estimated using p-nitro phenol-β-D-glucoside as substrate (Kubik, 1981).

Inoculum Preparation:
Sterilized 500 ml capacity conical flasks each contained 200 ml of medium containing (g /L) malt extract,3,yeast extract,3, peptone ,5 and sucrose,30 was steam sterilized at 121°C for 15 minutes, cooled to room temperature, then inoculated with a loop of yeast strain ***S. cerevisiae***(F-514) and incubated statically at 34°C for 24 hrs, then transferred to flat round bottom flasks of 2L capacity each containing 1L sterilized molasses diluted to 4-5% sugar content supplemented with 0,4% DAP and 0,2% yeast extract. The inoculated flat round bottom flasks incubated statically at 34°C for 24 hrs. (Fadel et al., 2013)

Preparation of molasses medium
The sugar cane molasses was diluted with water to 21 Brix gave total sugars involved1.8 % unfermentable sugars and the rest 14.8 was fermentable sugars. The previous diluted molasses supplemented with 4 g/l urea and4 g/l diammomium phosphate as a source for nitrogen and phosphorus and0.5 g/l magnesium sulfate.Molasses medium was dispensed into 500 mL Erlenmeyer flasks contained 200 ml.

Fermentation and enzyme addition
The molasses fermentation was carried out for 36 hrs at 35 °C under anaerobic conditions using ***S. cerevisiae*** F-514 (0.5% v/v) inoculum. Fermentation of molasses was carried out with the addition of carbohydrate depolymerising enzymes as follow:

1. Alpha amylase was added at various levels such as 1,2,3,4 and 5mg/100 g US to fermentation medium such as Enzyme unit /100 g US in the beginning of the fermentation and let to ferment for 36 hrs. At the end of fermentation time both ethanol and US were determined.
2. Second trail was carried out by adding the optimum level of alpha amylase 3 mg /100g US and after 30 minutes different levels such as 2, 4, 6 and 8 mg /100g US of glucoamylase were added and let to ferment for36 hrs. At the end of fermentation time both ethanol and US were determined.
3. Third trail: was carried out by adding the optimum level of alpha amylase 3mg /100g US and after 30 minutes optimum level of glucoamylase gained from Second trail 6mg /100g US and different levels of dextranase such as 1, 2, 3, 4 and 5mg/100 g US were added and let to ferment for 36 hrs. At the end of fermentation time both ethanol and US were determined
4. Fourth trail: was carried out by adding the optimum level of alpha amylase 3 mg /100g US and after 30 minutes optimum level of glucoamylase gained from Second trail(6mg /100g US) , optimum level of dextranase gained from third trail (3mg /100g US) and different levels of cellulase i.e. 1, 1.5, 2 and 2.5 mg /100g US were added and let to ferment for 36 hrs. At the end of fermentation time both ethanol and US were determined

Analytical determination
Determination of unfermentable sugar (US) as residual sugarsin fermented wash: The sugar concentration was determined by Fehling's titrimetric method (Lane and Eynon,1923). The 20 ml of fermented sample was taken and completed to 70 ml of distilled water and mixed with 5 ml of conc. HCL acid and heated at 70°C for a period of 10 min. The obtained sample was neutralized by adding NaOH (6 N) and it was prepared to 100 ml with tap water and taken into burette solution. The 5 ml of Fehling A and 5 ml of Fehling B were taken and mixed with 10 to 15 ml of distilled water in a conical flask and boil this solution,once boiling, add 3-4 drops of methylene blue indicator. The conical flask solution was titrated with burette solution in boiling conditions until disappearance of blue

Volume : 6 | Issue : 8 | August 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

color. The sugar concentration was calculated by using the formula given below:

$$US\ (\%gm) = \frac{5.128}{\text{Dilution factor} \times \text{Fehling factor} \times \text{Titratevalue}}$$

Dilution factor: 20/100 = 0.2

Ethanol content:
Ethanol content of the fermented samples was measured by ebulliometer approved in distillation factories (Fadel et al.,2014).

Increase in ethanol %
Increase in ethanol % was calculated as follow:
Ethanol in fermented mash treated by enzyme minas ethanol in fermented mash without treated by enzyme x100divided by ethanol in fermented mash without treated by enzyme.

Results and Discussion
Effect of alpha amylase
Data presented in Table (2) show that little increase in ethanol yield was achieved by addition of alpha-amylase enzyme to fermentation molasses medium.The decrease in US and increase in ethanol yield were more when the enzyme was supplemented at 4mg /100 g US. The obtained results can discussed on the light ofβ-amylase performs the first step of breaking starch into small pieces acting at random locations along the starch chain breaks down, yielding maltotriose and maltose from amylose, or maltose,glucose and "limit dextrin" from amylopectin (Ghalanbor, et al., 2008 andKadziola et al .,1998). Glucose only instead of other product can be ingest by ***S.cerevisiae*** and excrete alcohol in the fermentation medium. Alpha amylase was used previously to enhance ethanol yield from sugar cane molasses(Viviani et al.,2014).

Table(2):Effect of α- amylase on the ethanol yield from cane molasses fermentation *by S.cerevisiae* F-514

α-amylase mg/100g UN	US* %	Ethanol yield %	% Ethanol increase Related to control
0	1.28	8.17	0.0
1	1.25	8.18	0.12
2	1.22	8.19	0.25
3	1.19	8.21	0.45
4	1.18	8.22	0.61
5	1.20	8.20	0.37

*Unfermentable sugars

Effect of glucoamylase
Remarkable decrease in US followed by increase in ethanol yield was achieved by addition glucoamylase to fermentation medium along withalpha-amylase Table (3). The most suitable enzyme activity was achieved when 6mg/100 g US was added.Alpha-amylase begins the process of starch digestion as a liquefaction enzyme since it a takes starch chains and breaks them into smaller pieces with two or three glucose units. Glucoamylase is an enzyme which decomposes the above smaller fragments into glucose by tearing-off glucose units from the non-reduced end (Juge et al.,2002and Hiromi et al.,1966). The liberated glucose resulted in availability of surplus fermentable sugars which fermented by ***S.cerevisiae***to ethanol yield,and thus added value to ethanol production from molasses.

Table(3):Effect glucoamylase on the ethanol yield from sugar cane molasses fermentation by S.cerevisiae F-514

A A* mg/100g US	GA** mg/100g US	US*** %	Eth-anol yield %	% Ethanol increase Related to control
0	0	1.28	8.17	0.0
4	0	1.18	8.22	0.61
4	2	1.06	8.52	4.28
4	4	0.98	8.59	5.14
4	6	0.89	8.68	6.24
4	8	0.86	8.65	5.88

**Alpha amylase ** Glucoamylase
***Unfermentable sugars

Effect of dexranase
Molasses contains biopolymer dextran which produced from sucrose by microorganisms (specially***Leuconostocrnesenteroides***). Dextran cannot crystallize during sugar manufacture, so go to lossinmolasses. Dextranasehydrolyzes the biopolymer dextran to glucose units (Fischer and Stein,1960).The glucose units increased the fermentable sugars concentration in the fermentation medium led to more ethanol yield by yeast. Table (4) reveals that addition of dextranase at level 3 mg/100g US was more suitable than other used levels, where unfrmantable sugars was reduced from 0.89 % t0 0.68% resulting in other increase in ethanol yield in fermentation mash from 8.68% v/v to 8.76 v/v resample 7.22v/v % in ethanol yield related to control without enzymes treatment.

Table(4): Effect of dextranase on the ethanol yield from sugar cane molasses fermentation by S.cerevisiae F-514

AA*mg/100g UN	GA** mg/100g UN	DX*** mg/100g UN	US **** %	Ethanol yield %	% Ethanol increase Related to control
0	0	0	1.28	8.17	0.0
4	0	0	1.18	8.22	0.61
4	6	0	0.89	8.68	6.24
4	6	1	0.78	8.72	6.73
4	6	2	0.72	8.74	6.98
4	6	3	0.68	8.76	7.22
4	6	4	0.68	8.76	7.22
4	6	5	0.62	8.72	6.73

*Alpha aamylase ** Glucoamylase ****Dextranase

***Unfermentable sugars

4-Effect of cellulase
Data tabulated in Table (5) show an increase in ethanol yield in the fermentation mash by increase the amount of cellulase till 2mg/100g US. Cellulase actions achieved hydrolyzing for US to glucose and decreasingit from 0.62% to 0.42%. So, the fermentable glucose was increased in the fermentation medium and lead to an increase in ethanol yield from 8.76 % to 8.89 % v/v. Another enzyme addition was no efficient. Cellulase contains there types of enzymes responsible for saccharification of cellulose polymer to glucose (Worthington Biochemical Corporation, 2014). 1-Endocellulase which breaks dawn the noncovalent interactions present in the amorphous structure of cellulose; 2- Exocellulase which hydrolysis of cellulose chain from re-

Volume : 6 | Issue : 8 | August 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

ducing ends to break the polymer into smaller sugars. The results of cellulose hydrolysis by both endocellulase and exocellulasewere disaccharides(cellibioses) or tetra-saccharides and3- Beta-glucosidase responsible for hydrolysis of disaccharides and tetra-saccharides into glucose (Zverlov et al.,2005 and Telke, et al.,2013).

Table(5): Effect of cellulase on the ethanol yield from sugar cane molasses fermentation *by S.cerevisiae* F-514

AA* mg/100g UN	GA** mg/100g UN	DX*** mg/100g UN	CEL**** mg/100g UN	US ***** %	Ethanol yield %	% Ethanol increase Related to control
0	0	0	0	1.28	8.17	0.0
4	0	0	0	1.18	8.22	0.61
4	6	0	0	0.89	8.68	6.24
4	6	3	0	0.62	8.76	7.22
4	6	3	1	0.56	8.79	7.59
4	6	3	1.5	0.46	8.82	7.96
4	6	3	2.0	0.42	8.89	8.81
4	6	3	2.5	0.42	8.80	7.71

*Alpha amylase ** Glucoamylase ***Dextranase **** Cellulase *****Unfermentable sugars

Conclusion:

The addition of commercial alpha amylase, glucoamylase, dextranase and cellulase to sugar cane molasses medium contained total sugars 16.6% involved 1.8 unfermentable sugars was examined in this study for ethanol production. Fermentation was carried out for 36 hrs at 35°C by ***Saccharomyces cerevisiae*** F-514 giving ethanol yield 8.89% v/v compared to 8.17% v/v in untreated enzyme molasses medium.The obtained ethanol yield in enzyme treated molasses increase by 8.81 % v/v relative to molasses without enzymes treatment.

Acknowledgements

The authors gratefully acknowledge the financial support of Egyptian Sugar and Integrated Industries Company (ESIIC), Egypt provided by donating the raw-material and SternEnzym GmbH and Co.KGfor supplying the enzymes. The authors deeply thank Engineer M. Abd-Alraheem, Chairman and Managing Director of Egyptian Sugar and Integrated Industry Company. Also,deeplythank to the M. El – Heeny Chairman of factories sectors and members of Chemicals Factories, Egyptian Sugar and Integrated industries Company,Egypt.

References

1. Fadel M., Abdel Naser A. Zohri. MakawyM,Hsona. M.S. and Abdel-Aziz A M (2014): Recycling of vinasse in ethanol fermentation and application in EgyptainDistillery Factory.African Journal of Biotechnology13 (47):4390 – 4398.
2. Fadel M, Abeer A. Keera, Foukia E.Moufi and KahilT. (2013): High level ethanol from sugar cane molasses by a new thermotolerant***Saccharomyces cerevisiae***strain in industrial Scale. Biotechnology Research International,ID253286 6 pages.
3. Fischer E. and Stein, E.A. (1960): Cleavage of O- and S-glycosidic bonds (survey). InBoyer, P.D., Lardy, H. and Myrbäck, K. The Enzymes 4 (2nd ed.). New York: Academic Press. pp. 301–312.
4. Ghalanbor Z (2008): Binding of Tris to ***Bacillus licheniformis***alpha-amylase can affect its starch hydrolysis activity.Protein PeptideLett., 15 (2): 212–214.
5. Hiromi K, HamauzuZ.I. and TakahashiK. (1966): Kinetic studies on glucamylase. II. Competition between two types of substrate having K-1,4 and K-1,6 glucosidic linkages, J. Biochem. (Tokyo), 59: 411-418.
6. Juge N., Le Gal-Coëffet M-F., FurnissC.S.M., Gunning A.P., GiardinaT., Kramhøft, B., Morris V.J., Svensson B.and Williamson G. (2002): The starch binding domain of glucoamylase from ***Aspergillusniger***: overview of its structure, function, and role in raw starch hydrolysis. Biologia, 57: 230 – 245.
7. Kadziola A., Søgaard M., Svensson B. andHaser R. (1998): Molecular structure of a barley alpha-amylase-inhibitor complex: implications for starch binding and catalysis. J. Mol. Biol. 278 (1): 205–217.
8. KubikC.P. (1981): Release of carboximethylcellulaseand B-glucosidase from cell wall of ***Tricodermareesei***. Eur.J. Appl.Microbial.Biotecnol.,13: 226-231.
9. Lane J.H. and EynonL. (1923): Determination of reducing sugars by means of Fehling's solution with methylene blue as internal indicator. J. Soc. Chem. Ind. 42,32.
10. Mandels M., Hontz L. and Nystrom J. (1974): Enzymatic hydrolysis of waste cellulose. Biotechnol.Bioeng., 16(11): 1471-1493.
11. Manning B.G. and Campbell L.L. (1961): Thermostable β–amylase of ***Bacillus phillus***.Journal of Biological Chemistry 236: 2952–2955.
12. MillerL. (1959): Use of denitrocellulosic acid reagent for determination of reducing sugar. Analytic Chem 31: 426-29.
13. Telke A.A.,Zhuang N., GhatgeS.S., Lee S.H., and Ali Shah A. (2013): Engineering of family-5 glycoside hydrolase (Cel5A) from an uncultured bacterium for efficient hydrolysis of cellulosic substrates.PLoS ONE 8 (6): e65727.
14. Viviani C.,OnishiJosé E.,Olivo G., ZaninM., Flavio F. and Moraesal(2014): Enzymatic hydrolysis of sugarcane molasses aspre-treatment for bioethanol production International Sugar Journal. 907-910.
15. Worthington Biochemical Corporation (2014):Cellulase. accessed on 2014-07-03.
16. Zverlov V.V., Schantz N. and Schwarz W.H. (2005): A major new component in the cellulosome of ***Clostridium thermocellum*** is a processive endo-beta-1,4-glucanase producing cellotetraose". F.E.M.S.Microbiol. Lett. 249 (2): 353– 358.