Indian J. Microbiol. (December 2007) 47:353–357

ORIGINAL ARTICLE

Cauliflower waste incorporation into cane molasses improves ethanol production using *Saccharomyces cerevisiae* MTCC 178

Gurpreet Singh Dhillon · Sunil Bansal · Harinder Singh Oberoi

Received: 21 May 2007 / Final revision: 19 September 2007 / Accepted: 6 November 2007

Abstract Diluted cane molasses having total sugar and reducing sugar content of 9.60 and 3.80% (w/v) respectively was subjected to ethanol production by *Saccharomyces cerevisiae* MTCC 178. Incorporation of dried Cauliflower Waste (CW) in molasses at the level of 15 % increased ethanol production by nearly 36 % compared to molasses alone. Addition of 0.2 % yeast extract improved ethanol production by nearly 49 % as compared to molasses alone. When the medium containing diluted molasses and 0.2 % yeast extract was supplemented with 15 % CW, 29 % more ethanol was produced compared to molasses with 0.2 % yeast extract. Cell biomass, ethanol production, final ethanol concentration and fermentation efficiency of 2.65 mg mL^{-1}, 41.2 gL^{-1}, 0.358 gg^{-1} and 70.11 % respectively were found to be best at 15% CW supplementation level besides reduction in fermentation time but further increase in CW level resulted in decline on account of all the above parameters. This is probably the first report to our knowledge, in which CW was used in enhancing ethanol production significantly using a small quantity of yeast extract.

Keywords Molasses · Cauliflower waste · Yeast extract · Ethanol production · Total sugars · Fermentation efficiency

G. S. Dhillon · S. Bansal · H. S. Oberoi (✉)
Central Institute of Post Harvest Engineering and Technology,
PAU Campus,
Ludhiana – 141 004, India

Tel: +91 / 161 / 2808825,2808669,
Fax: +91 / 161 / 2808670
Email: hari_manu@yahoo.com;
harinderoberoi@hotmail.com

Introduction

Cauliflower (*Brassica oleracae* L) is an important vegetable grown all over the world. The total cauliflower production in India in 2005 was 4.5 million tones which is about 28% of the total world production[1]. Cauliflower has the highest waste index i.e. ratio of edible portion to non edible portion[2] and thus enormous amount of organic solid waste is generated. Cauliflower waste (CW) constitutes about 48–58% of the total weight of cauliflower and on dry matter basis consists of 85.5% organic matter, 16.6% cellulose, 14.9% crude protein, 8.4% hemicellulose, 17% total sugars, 3.9% reducing sugars, 6.25% phenolics, 14% ash; the main minerals present are 9.83% Ca, 6.12% Mg, 4.26% Na, 28.74% K and 0.62% S (Oberoi *et al*)[3]. Disposal of this nutritionally rich CW in municipal bins results in rotting, which creates foul smell thereby adding to the environmental problems and jeopardizes public health. Due to its nutritional value, this waste can be utilized as an important substrate for production of industrially important products such as bio-ethanol or enzymes.

The rapidly depleting fossil fuels and constant rising crude oil prices all over the world has increased interest in alternative sources of energy[4]. It is well established that harmful gases are produced due to incomplete combustion of fossil fuels during their burning. Ethanol contains 35% oxygen, which results in a complete combustion of fuel & thus lowers the emission of harmful gases. Moreover ethanol production process uses energy from renewable sources only hence no net carbon dioxide is added to the environment, thus reducing green house gas emissions[5]. It has also been well established now that ethanol increases the octane number, decreases the reid vapour pressure and produces fuel with clean burning characteristics. In devel-

oping countries like India where the technology of ethanol production from lignocellulosics is not established at even pilot scale and starchy material available in form of corn, sorghum grains or wheat and rice is not even sufficient to meet the food needs of growing population, the only option left is sugar from sugarcane or sugar beet. Molasses, which is a by-product of sugar industry, is extensively used for ethanol production in India as it is rich in total sugars and contains some amount of proteins and minerals and thus serves as an ideal substrate for the growth of yeast cells and bioconversion of fermentable sugars into ethanol. The present study was thus planned with an objective of producing ethanol with higher efficiency and less time by supplementing cane molasses with CW which otherwise does not have any use in the Industry but contains substantial amount of minerals and crude protein as mentioned above.

Material and Methods

Materials : Cauliflower for experimental purpose was procured from the local vegetable market in Ludhiana, India and the edible portion was removed and non-edible portion (CW) was oven dried, powdered and retained for future trials. CW was cleaned with sterile water, cut into small fractions of nearly 0.5 cm manually and was completely dried at 50^0C in a hot air oven for 24 h, ground using cyclotec sample mill (Tecator, Sweden). The powdered material was added into the medium in different concentrations after moisture equilibration. Cane molasses was procured from Jagatjit Industries Ltd, Hamira, Punjab, India.

Organisms: *Saccharomyces cerevisiae* MTCC 178 was procured from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India .The strain was maintained on YEPD medium containing (gL^{-1}): yeast extract, 3; peptone, 10; dextrose, 20; agar,15 and stored in the refrigerator at 4–5^0C and was sub cultured regularly at an interval of 15 days.

Fermentation medium : For the preparation of inoculum, *S. cerevisiae* MTCC 178 was transferred from agar slants into 250 mL Erlenmeyer flasks each containing 100 mL of sterile YEPD broth and incubated in an incubator shaker at 30^0C for 24 h or more till a required cell count was attained. The media used for fermentation are:

(a) Molasses diluted to 9.6% total sugars
(b) Molasses containing 9.6% total sugars + 0.2% yeast extract
(c) Molasses containing 9.6% total sugars + 0.2% yeast extract + 5-20%, w/v CW
(d) Molasses containing 9.6% total sugars + 5-20% CW.

200 mL of the fermentation medium in 500 mL Erlenmeyer flasks was used for experimentation purpose and the flasks were sterilized at 121^0C at 15 psi for 20 min. The initial pH of 5.0 was set for all the experiments by using 1 N HCl / 1N NaOH. The flasks were inoculated with 5% (w/v) inoculum of yeast culture containing 1 x 10^8 cells mL^{-1} and were incubated at 28 $\pm$ 1^0C on an incubator shaker at 120 rpm for 48 h.

Analytical: The samples were drawn regularly at 6 h interval after incubation and were analyzed for ethanol production, residual sugars and cell biomass. The samples were centrifuged at 5,000 rpm for 10 min at 4^0C and the supernatant was analyzed for ethanol production and residual sugars. Ethanol was analysed using Gas Chromatograph, GC 2010 (CIC, Baroda, India). The injector and detector were maintained at 120^0C and the oven was maintained at 100^0C. Nitrogen was used as a carrier gas with linear velocity of 30 mL min^{-1}. Total sugars, reducing sugars and xylose were determined by the method of Dubois *et al*.[6] , Miller[7] and Standing *et al*.[8] respectively. Reducing sugars and xylose were analysed to see the effect of steam treatment on the CW component in the medium in different treatments at 0 h just prior to inoculation. All the colorimetric observations were done using microprocessor based double beam UV–VIS spectrophotometer (Spectroscan DV 80). The pellet was used for yeast cell biomass calculations and the biomass was calculated by subtracting the weight of Whatman no.1 filter paper with yeast cell pellet after drying in hot air oven at 105^0C from the weight of pre weighed filter paper. Fermentation Efficiency was calculated as total ethanol produced gL^{-1}/ expected yield of ethanol on theoretical basis multiplied with 0.51. The total residual sugars were subtracted from the total fermentable sugars prior to fermentation for calculating fermentation efficiency. The ethanol yield was calculated as the ratio of ethanol produced to the total sugars consumed and was expressed as gg^{-1}.

Statistical Analysis: All the experiments related to sugar, ethanol and cell biomass were conducted in triplicate using simple CRD and the statistical analysis was done using simple ANOVA.

Results and Discussion

Cane molasses on an average contained 48% total sugars which included 10% non fermentable sugars on total sugar basis as clarified from the source of procurement and the same was diluted five times such that it contained 9.6% total sugars and 3.8 % total reducing sugars respectively.

Table 1 Analysis of sugars for different treatments prior to inoculation.

Treatment	Total sugars (%)	Total Reducing Sugars (%)	Xylose concentration (%)
M + 0.2% ye	9.60 ± 0.04	3.80 ± 0.04	0.036 ± 0.002
M + 0.2% ye + 5% CW	10.35 ± 0.06	3.95 ± 0.06	0.044 ± 0.004
M + 0.2% ye + 10% CW	11.15 ± 0.06	4.13 ± 0.07	0.053 ± 0.005
M + 0.2% ye + 15% CW	12.05 ± 0.09	4.32 ± 0.05	0.071 ± 0.003
M + 0.2% ye + 20% CW	12.57 ± 0.06	4.50 ± 0.06	0.91 ± 0.007

Values are Mean ± SD.

Molasses with this dilution was used for all future experiments.

Effect of CW supplementation alone on ethanol production parameters: Table 1 clearly depicts an increase in reducing sugars, total sugars and xylose concentrations with increase in cauliflower waste in the fermentation medium prior to inoculation but after sterilization which is mainly due to the breakdown of hemicellulosic fraction of cauliflower as the medium was maintained at acidic pH of 5.0. The available literature suggests that mild acid hydrolysis coupled with steam explosion results in the release of sugars like xylose, arabinose, galactose and rhammose from lignocellulosics of which xylose is a major constituent[9,10], however these are not fermented by the *S. cerevisiae* strain used in this study and thus resulted in higher concentration of total residual sugars once concentration of CW in the medium was increased (Table 1).

An initiation in ethanol production at the end of 6 h for all the treatments is observed (Fig.1), as at this stage the available sugars are consumed by the yeast cells for their growth resulting in a lag phase. It is clear from the Figure that maximum ethanol production was observed after 42 h for all the treatments except control involving only molasses where maximum ethanol production was observed after 48 h. The decrease in lag phase due to incorporation of CW in molasses could mainly be due to the presence of proteins and minerals in CW. In molasses, sodium and phosphorous have been found to be limiting nutrients [11] whereas sodium is present in reasonable quantity in CW. The decline in ethanol production after reaching maximum value at the end of 42 h (Fig. 1) could be due to the oxidation of ethanol and utilization of ethanol as substrate due to limiting available fermentable sugars in the medium. The highest value for ethanol production was found at 15% CW supplementation level (Table 2) which was nearly 36% more than control but a decrease in ethanol production and productivity parameters were observed when CW level was increased beyond 15% which may probably be due to the presence of phenolics and sulphur which increase with the increase in CW incorporation having an adverse

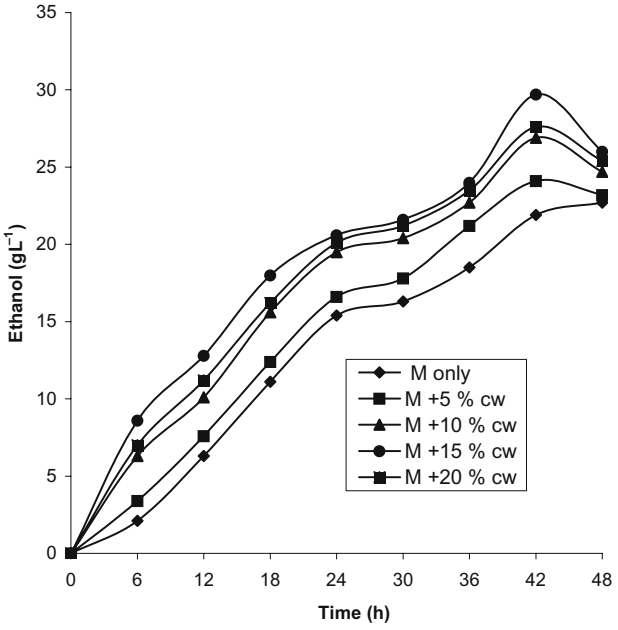

Fig. 1 Effect of supplementation of molasses with cauliflower waste on ethanol production.

effect on the growth of the yeast cells. Zanichelli *et al*.[12] have reported a ethanol yield of 8–12 % (v/v) from olive oil milling waste water after removing phenolic fraction which is known to inhibit yeast growth . Our results are in line with the results of Rozes *et al.*[13] who have reported that sugar uptake by *Oenococcus oeni* decreased due to the presence of phenolic compounds. The improvement in ethanol production and fermentation efficiency by supplementation of CW could be the cumulative effect of protein and minerals like Ca, Mg, Na and K which help in the respiratory and fermentative ability of yeast cells. Stehlik –Tomas *et al.*[14] have reported that zinc, copper and manganese incorporation into medium containing molasses enhanced the kinetics of ethanol fermentation by *Saccharomyces cerevisiae* in aerobic and semi aerobic conditions. The CD value of 1.183 clearly indicates a significant improvement in ethanol production by supplementation of molasses with CW (Table 2).

Table 2 Effect of CW supplementation on ethanol production and related fermentation parameters after 42 h of incubation.

Treatment	Cell biomass (mg/ml)	Total residual sugars (%)	Ethanol produced (gL^{-1})	Final Ethanol concentration (gg^{-1})	Fermentation Efficiency (%)
M only	1.60	2.35	21.9	0.267	52.30
M + 5% CW	1.62	2.48	24.1	0.269	52.74
M + 10% CW	1.76	2.64	26.9	0.276	54.21
M + 15% CW	1.82	2.89	29.7	0.283	55.41
M + 20% CW	1.79	3.05	27.6	0.251	49.20
CD (0.05)	0.095	0.111	1.183		

Table 3 Effect of CW supplementation with 0.2%, w/v yeast extract on ethanol production and fermentation parameters after 36 h of incubation.

Treatment	Cell biomass (mg/ml)	Residual sugars (%)	Ethanol produced (gL^{-1})	Specific growth rate (μh^{-1})	Final Ethanol concentration (gg^{-1})	Fermentation Efficiency (%)
Control	1.90	1.22	32.7	0.13	0.350	68.85
5% CW	1.98	1.37	35.8	0.14	0.356	69.71
10% CW	2.37	1.50	38.7	0.14	0.356	69.81
15% CW	2.65	1.99	40.8	0.14	0.358	70.11
20% CW	2.57	2.45	39.7	0.13	0.342	67.11
CD (0.05)	0.092	0.095	0.849			

Control: Molasses + 0.2% yeast extract M: Molasses CW: cauliflower waste.

Effect of CW supplementation with yeast extract on ethanol production and fermentation parameters : Addition of yeast extract (0.2 %, w/v) into the medium containing molasses and different levels of CW stimulated ethanol production both in terms of reduction in time and increased efficiency for all the treatments. While analysing the results of Table 2 and Table 3 and Figs. 1 and 2, it is observed that the treatment involving supplementation of molasses with 0.2% yeast extract and 15% CW when compared to molasses with 0.2% yeast extract resulted in nearly 25% higher ethanol (Table 3). Addition of yeast extract to molasses increased the ethanol production by about 49% as compared to control and substantial increase could be observed when 15% CW was added along with yeast extract in fermentation medium (Table 3). Besides, fermentation time for maximum ethanol production also reduced by 6–12 h depending upon treatment by addition of yeast extract into the medium (Fig. 2). The effect of yeast extract addition in the fermentation medium improves the fermentation efficiency[15]. Our results both in terms of cell biomass, ethanol production and fermentation time are in consonance with the results of Reddy and Reddy[16] who have reported a 50% increase in ethanol production and reduction in fermentation time while producing ethanol from dried mango peel by the use of yeast extract in fermentation medium. Although, molasses alone contains some amount of vitamin B6[17], there is no available report suggesting the presence of other B vitamins in it. Nancib *et al.*[18] reported an increase

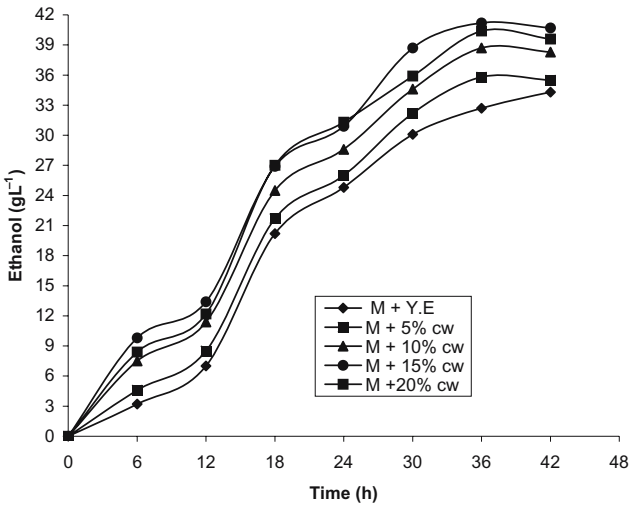

Fig. 2 Effect of supplementation of molasses with cauliflower waste and yeast extract on ethanol production.

in lactic acid production from date juice by the use of yeast extract in the medium. Stehlik –Tomas *et al.*[14] have reported that molasses alone does not contain elements like manganese, zinc and copper which are required for adequate growth of yeast cells. Supplementation of molasses with yeast extract and CW could have resulted in higher concentrations of minerals, essential vitamins and amino acids which in turn might have stimulated the yeast cell growth and subsequent ethanol production capability. Patil and Pa-

til[19] have reported an increase in rate of fermentation by the use of 0.2% chitin, xylan and acacia gum and a reduction in fermentation time by about 42 h as compared to molasses without the above supplements. It is noteworthy to mention here that the hemicellulosic fraction upon hydrolysis through steam sterilization makes the structure of hemicellulosic fraction more amenable to saccharification and results in higher proportion of xylan which could have been true for CW which contains hemicellulose in appreciable amount. There is a scanty literature available on use of vegetable wastes in improving the ethanol productivity and to the best of our knowledge, this is the first ever study reporting the use of CW as supplement in improving ethanol yields. The CD values in Table 3 indicate significant difference in treatments for all the parameters.

Conclusion

It could be concluded from the present study that supplementing molasses with 15% dried CW improved ethanol production by about 36% as compared to control. However when 0.2% yeast extract was used along with 15% CW, ethanol production increased substantially as compared to control besides lowering the overall fermentation time. Since CW does not have any commercial value it can be used as a good supplement with molasses for improving ethanol productivity parameters. This probably is the first study of its kind reporting the use of CW as supplement for improving ethanol productivity

References

1. FAO (2006) Statistical data for agricultural crops. www.fao.org
2. Kulkarni M, Motey R & Lele SS (2001) Biotechnology in Agriculture, Industry and environment. Proc. of the International conference of SAARC countries organized by Microbiologists society at Karad, India pp. 24–31
3. Oberoi HS, Kalra KL, Gupta AK & Uppal DS (2007) Effect of addition of cauliflower waste on glucoamylase production by *Aspergillus niger* NCIM 1054. J Fd Sci Technol 44:509–512
4. Lin Y & Tanaka S (2006) Ethanol fermentation from biomass resources: Current state and prospects. Appl Microbiol Biotech 69:627–642
5. Kheshgi HS, Prince RC & Marland G (2000) The potential of biomass fuels in the context of global climate change; Focus on transportation of fuels. Ann Rev Energy Environ 5:199–244
6. Dubois M, Giles KA, Hamilton JK, Robert PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 25:350–356
7. Miller G (1959) Dinitro salicylic acid reagent for determination of reducing sugars. Anal Chem 31:426–428
8. Standing CN, Fredrickson AG & Tochina HN (1972) Batch and continuous culture for two substrate systems. Appl Microbiol 23:354–364
9. Rao JS, Singhal, RS & Kulkarni PRJ (1998) Xylose syrup from cottonseed hulls: optimization of hydrolysis conditions and purification by ion exchange resins. J Sci Ind Res 57: 196–200
10. Devi SP & Singh HD (1995) Bioconversion of water hyacinth hemicellulose and xylose rich sugar mixtures to ethanol by *Neurospora crassa*. Enz Microb Technol 8: 149–152
11. Gil-Hwan An, Byung-Gui Jang & Myung-Haing Cho (2001) Cultivation of the carotenoid-hyperproducing mutant 2A2N of the red yeast *Xanthophyllomyces dendrorhous* (Phaffia rhodozyma) with molasses. J Biosci Bioengg 92: 121–125
12. Zanichelli D, Carloni F, Hasanaj E, Andrea ND, Filippini A & Setti C (2007) Production of ethanol by an integrated valorization of olive oil by products. The role of phenolics in inhibition. Env Sci Poll Res 14:5–6
13. Rozes, N, Arola, NL & Bordans A (2003) Effect of phenolic compounds on the co- metabolism of citric acid and sugars by *Oenococcus oeni* from wine. Lett Appl Microbiol. 36: 337–341
14. Stehlik- Tomas V, Zetic VG, Stanzer D, Grba S & Vahcic N (2004) Copper and managnese enrichment in yeast *Saccharomyces cerevisiae*. Food Technol Biotechnol 42: 115–120
15. Grohmann K, Cameron RG & Buslig BS (1996) Fermentation of orange peel hydrolysates by ethanologenic *Escherichia coli*. Effects of nutritional supplements. Appl Biochem Biotechnol 57–58:383–388
16. Reddy LV & Reddy OVS (2006) Ethanol production from dried mango peel and effect of nutrient supplementation. Poster no. [P9.08]. Sustainability of the Agri Food Chain, The Hague, Netherlands, Nov 7–9
17. Ensminger AH & Esminger MKJ (1986) Food for Health: A Nutrition Encyclopedia. Clovis, California: Pegus Press; PMID: 15210
18. Nancib A, Nancib N, Meziane-Cherif D, Boubendir A., Fick M & Boudran J (2005) Joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by *Lactobacillus casei* subsp. *rhamnosus*. Bioresour Technol 96:63–67
19. Patil SG & Patil BG (1989) Chitin supplement speeds up the ethanol production in cane molasses fermentation. Enz Microb Technol 11:1138–1143