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Isolation and Evaluation of Cellulolytic Yeasts for Production of Ethanol from Wheat Straw

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A B S T R A C T

The cellulolytic efficiency of yeast isolates obtained from different fruit and fruit wastes was evaluated in terms of degradation of cellulose, release of reducing sugars and bioethanol production. The isolates CY-3, CY-4, CY-8 and CY-15 together with a reference cellulolytic strain *Saccharomyces cerevisiae* (NCIM 3220) displayed maximum hydrolysis of carboxy methyl cellulose (CMC) to yield reducing sugars when determined in 2 days interval for up to 22 days, Based on this, the isolates were further evaluated for combined saccharification and fermentation of a pretreated lignocellulosic substrate such as wheat straw in flask culture studies. The concentration of ethanol produced by the cellulolytic yeasts at the end of fermentation was determined by Gas Chromatography. Strain CY-15 produced 0.573g/L of ethanol which was on par with the reference strain which produced 0.68 g/L. Thus the study attempts to develop a sustainable bioethanol production technology by simultaneous saccharification and fermentation of wheat straw using native yeasts.

Introduction

Production of ethanol from lignocellulosic biomass seems very attractive and sustainable due to several reasons, among which the renewable and ubiquitous nature of biomass and its non competitiveness with food crops are the major ones. The utilization of lignocellulosic biomass for ethanol production necessitates the large-scale production technology to be cost effective and environmentally sustainable. Extensive research has been targeted in the last decade to come out with a self sufficient process to convert the lignocellulosic biomass to bioethanol so as to meet the increasing bioenergy demands. However the development of a single bioprocess combining cellulase production, saccharification, and fermentation into one step has still remained a challenge (Ask *et al.*, 2012). This consolidated bioprocessing (CBP) has been proposed as the most efficient way to reduce the production cost of cellulosic bioethanol (Kang *et al.*, 2015).

Several raw materials are being used for the bioethanol production *viz*. easily fermentable sugary feed stocks (molasses), starchy feed stocks (grains) and feed stocks containing complex sugars in the form of cellulose and hemicelluloses (Li *et al.,* 2009; Choi *et al.,* 2010; Zhao and Xia, 2010). The hydrolysis of cellulose and hemicelluloses polysaccharides into their respective monomers called as saccharification involves cellulolytic microorganisms or their enzymes namely cellulase and hemicellulase (Erdei *et al.,* 2012 and Lu *et al.,* 2013). Yeast strains are known for fermentation of sugars to yield ethanol. Since lignocellulosic substrates contain pentoses as well as hexoses, only few types of yeast are known to utilize it. If the yeast can do the process of hydrolysis as well as fermentation, then the cost of hydrolytic enzymes can be avoided.

Thus the current work deals with isolation of native yeasts with saccharolytic and fermentative abilities for application in bioethanol production from lignocellulosic waste wheat straw.

Materials and Methods

Pure cultures and growth medium

Yeasts were isolated from different sources such as fruits and fruit wastes (Table 1). The isolates were purified, subcultured and maintained on MGYP Agar (Malt Extract Glucose Yeast Extract Peptone) medium. A reference strain of yeast *Saccharomyces cerevisiae* (NCIM 3220) with cellulolytic ability was procured from National Collection of Industrial Microorganisms (NCIM), Pune.

Screening of the yeasts for cellulolytic ability

All the yeast isolates were subjected to test for saccharification of carboxy methyl cellulose (CMC). The amount of reducing sugars liberated was estimated by dinitrosalicylic acid (DNSA) method (Miller *et al.,* 1960). Carboxymethyl cellulose (CMC) of 0.50 g was dissolved in 49.50 ml of sodium citrate buffer (0.50 M, pH 4.8) by vigorous stirring until the CMC was completely dissolved. This solution had the cellulose concentration of one mg per ml. To 0.80 ml of CMC solution (1mg ml^{-1}) 0.80 ml of appropriately diluted culture supernatant was added. The reaction system was incubated for 10 min at 50 ^{0}C. After the incubation, the volume was made up to 3 ml using distilled water. 3 ml of DNSA reagent was added to each sample, mixed well. The reagent blank containing 3 ml of distilled water and 3 ml of DNSA reagent was also prepared. Similarly, standards were also included whose glucose concentration ranged from 10 µg to 100 µg. All tubes *viz.*, samples, standards and blank were kept on boiling water bath for 5 minutes. After this one ml of 40 per cent Rochelle salt solution was added when the reaction mixture was still warm. Then the tubes were cooled. The absorbance in terms of optical density of the standards and sample were read at 510 nm using Systronics UV Spectrophotometer-117.

Saccharification and fermentation of wheat straw by the yeasts

To determine the period for maximum saccharification, the following experiment was undertaken. Wheat straw was pretreated by the method standardised earlier in our laboratory using 3% NaOH for 8 h. About 5g each of oven dried substrates was sterilized in an autoclave in 250ml Earlen Mayer flask. To this 100 ml of sterile distilled water was added to get 5% substrate concentration. The pH was adjusted to 5.5. Fresh cultures of cellulolytic yeast isolates were inoculated at the rate of 2%. To this nutrients consisting of Urea 0.64%, KH_2PO_4 at 0.2% and $MgSO_4$ at 0.1% concentration was added. The flasks were kept

under aerobic condition and reducing sugar was analyzed at 24h intervals. The flasks were incubated for 5 days under aerobic condition followed by creating an anaerobic condition for about 7 days or till the carbon dioxide evolution ceased. Later the residual reducing sugar and ethanol produced was analysed.

Analysis of ethanol using gas chromatography

The ethanol obtained after fermentation of Wheat straw using cellulolytic yeast isolates was analysed using GC (Table 2). For sample preparation, 2 ml of the supernatant from the individual treatments at end of fermentation was centrifuged at 10,000 rpm for 10 min. Ethanol standard as well as the samples were filtered through syringe driven filter nylon 66 of 0.22µ pore size and stored frozen until use.

The GC used in this study was an Agilant 7260, USA with a flame ionization detector. Separation took place in 2m DB wax column using nitrogen as the carrier gas. The GC oven temperature was initially controlled at 65 ^{0}C for 5 min, and then it was increased at a rate of 40 ^{0}C per min to a final temperature of 140 ^{0}C, which was held for 3 min. The temperature of the injector was set at 240 ^{0}C and the detector was set at 250 ^{0}C with H_2 flow at 25ml/min, air 100ml/min and N_2 at 25 ml/min. The injection volume was 0.1µl with split mode of ratio 65:1. Data acquisition software was used to integrate the data.

Results and Discussion

Our study emphasizes on the dire need to isolate and explore potential native yeasts with natural cellulolytic and fermentative abilities. In the present study, fruit and fruit wastes were used as source of cellulolytic yeasts. Fruit and fruit wastes are a natural source of mono and disachharides for yeast growth. A total of twenty yeasts were isolated from different fruit and fruit wastes as shown in table 1. The ability of the isolates to convert carboxy methyl cellulose to yield reducing sugars was tested in order to determine their saccharification potential. Several yeast species have been isolated from different fruits, fruit juices and wastes with ability to produce extracellular enzymes and ethanol production (Rao *et al.,* 2008, Chatterjee *et al.,* 2011 and Zahida *et al.,* 2014). Bioconversion of lignocellulosic sugar into ethanol by genetically engineered cellulolytic yeasts has been investigated by a number of researchers (Kuhad *et al.,* 2011). Fujita *et al.,* (2003) studied the synergistic saccharification and direct fermentation to ethanol of amorphous cellulose by engineered yeast strain displaying all three types of cellulolytic enzymes. However, wild yeasts from different natural substrates possess innate saccharification and ethanol producing capabilities.

In our study, isolates CY-3, CY-4, CY-8 and CY-15 displayed maximum hydrolysis of CMC to yield reducing sugars when determined in a 2 day interval for up to 22 days**.** Isolate CY-4 from the pulp of sweet lemon yielded a maximum of 550.66 mg of reducing sugars/gm of CMC on the 22nd day. Based on this, the isolates were further evaluated for combined saccharification and fermentation of a lignocellulosic waste such as wheat straw in flask culture studies. A reference strain of *Saccharomyces cerevisiae* with cellulolytic ability was included in the study.

When using lignocellulosic waste as substrate for bioethanol production, pretreatment becomes necessary by either physical or chemical methods to make the substrate accessible for further enzymatic saccharification (Srichuwong *et al.,* 2009 and Koo *et al.,* 2011). It can have significant effect on both saccharification and production of ethanol. It also influences the amount of ethanol yield and production cost (Koo *et al.,* 2011).

Table.1 Cellulolytic activity of the yeast isolates in terms of release of reducing sugars (mg g-1 of CMC)

Sl. No	Isolate no. and Source of isolation	Reducing sugars mg g^{-1} Carboxy Methyl Cellulose										
		Period of Incubation (Days)										
		2	4	6	8	10	12	14	16	18	20	**22**
1	CY-1 Straw berry	0	0	0	66	107.33	125.33	161.0	180.33	234.0	261.66	**278.33**
2	CY-2 Straw berry	2.0	3.66	16.33	25.33	28.33	52.10	110.66	194.0	304.66	326.66	**460.33**
3	CY-3 Sweet lemon skin	0	0	4.0	10	64.0	208.66	271.0	275.66	313.66	428.66	**463.33**
4	CY-4 Pulp of sweet lemon	16.33	24.66	80.66	201.33	285.0	322.33	398.0	443.33	475.66	509.0	**550.66**
5	CY-5 Papaya	0	0	20.66	22.66	24.0	124.0	172.66	177.33	164.33	220.73	**220.70**
6	CY-6 fermented strawberry	0	4.16	8.33	19.06	20.33	25.00	27.53	39.93	46.66	48.53	**50.00**
7	CY-7 Apple skin	0	0	29.33	40.66	49.33	52.33	49.16	133.0	201.33	211.66	**224.33**
8	CY-8 Pulp of apple	0	0	15.33	17.66	79.0	122.0	150.33	176.66	223.33	262.0	**271.66**
9	CY-9 Fermented silage	0	0	4.093	19.66	21.93	22.86	28.30	32.26	38.66	41.90	**55.80**
10	CY-10 Fermented Laquot fruit	0	3.00	6.90	17.33	23.76	24.60	25.36	29.13	49.20	68.66	**76.76**
11	CY-11 Skin of pomegranate	0	0.166	0.833	3.833	8.20	15.20	15.60	18.06	23.06	39.03	**48.46**
12	CY-12 Pomegranate	0	7.0	8.0	34.0	41.0	47.0	48.0	127.3	141.0	434.0	**92.0**
13	CY-13 Inner skin of pomegranate	0	13.33	19.40	22.00	22.26	28.13	37.46	40.93	51.40	57.33	**64.26**
14	CY-14	0	22.10	22.60	38.80	41.20	41.76	49.43	57.16	61.36	64.16	**66.46**

	Inner skin of pomegranate											
15	CY-15 Fermented juice of pomegranate	8.166	16.033	25.10	29.06	35.46	48.96	56.46	68.00	76.43	80.03	**100.36**
16	CY-16 Pomegranate fermented juice	73.33	203.33	230.33	250.0	198.66	118.66	92.66	66.66	13.0	12.66	**10.0**
17	CY-17 Pulp of Apple	0	13.06	18.83	26.20	35.26	46.43	52.13	68.10	71.33	88.36	**89.00**
18	CY-18 Skin of pomegranate	6.00	11.16	19.36	22.83	49.00	55.03	61.43	78.26	80.03	90.03	**126.20**
19	CY-19 Inner skin of Baer fruit	0	2.33	4.03	13.86	16.50	29.50	31.06	36.56	40.50	60.33	**63.36**
20	**CY-20 Pulp of Baer fruit**	**1.33**	**2.56**	**8.63**	**11.43**	**26.56**	**37.10**	**41.86**	**49.80**	**55.06**	**63.30**	**78.03**

Table.2 GC analysis of yeast isolates for ethanol production from wheat straw

Sl. No.	Strain Code	Retention time	Concentration of Ethanol (g/L)
1	CY-3	3.9	0.236
2	CY-4	3.9	0.316
3	CY-8	4.0	0.350
4	CY-15	3.8	0.573
5	*Saccharomyces cerevisiae* (NCIM 3220)	3.9	0.68 g/L

Using yeasts to generate fuel ethanol has advantage of low distillation cost as it gives a high ethanol yield, a high productivity and can withstand high ethanol concentration (Kasavi *et al.,* 2012). Particularly, *S. cerevisiae* is the most attractive yeast in industrial ethanol production as it tolerates a wide range of pH (Lin *et al.,* 2012), thus reducing the contamination problem in the process. Yeast strains such as *Pichia stipitis* (NRRL-Y-7124), *S. cerevisiae* (RL-11) and *Kluyveromyces fagilis* (Kf1) were reported to produce good amount of ethanol from different types of sugars (Mussato *et al.,* 2012).

In the present study, yeast CY-15 isolated from fermented juice of pomegranate showed maximum ethanol production amounting to 0.573g/L in flask culture studies using pretreated wheat straw. Mellitzer *et al.,*(2012) opined that, lignocellulolytic yeast such as of *Pichia* have simple media requirements and their relative easy handling in bioreactors enable inexpensive large-scale cultivations. Bioethanol production from biomass through enzymatic route has been investigated by several researchers with several amendments in the commercial preparation of cellulase for biomass hydrolysis, which contains higher and improved beta-glucosidase for efficient biomass conversion (Singhania *et al.,* 2013).

From the results obtained and the discussion followed, it can be concluded that the characteristic features of yeasts in general and their possible cellulolytic enzymatic capabilities contribute to their high potential for cost reduction during the production of ethanol, thus obviating the need for expensive pure active lignocellulolytic enzymes. Optimization of factors which influence the production of bioethanol including temperature, sugar concentration, pH, fermentation time, agitation rate, and inoculum size is warranted for the development of a successful and sustainable bioethanol production process.

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References

Ask, M., Olofsson, K., Di Felice, T. *et al.,* 2012. Challenges in enzymatic hydrolysis and fermentation of pretreated *Arundo donax* revealed by a comparison between SHF and SSF. Process Biochem. 47:1452–1459.

Chatterjee, S., Barnita Ghosh and Rina Rani Ray. 2011. Isolation and characterization of local yeast strains from waste fruit juices, jaggery and dahi samples. Int. J. Chem. Sci. 9(2):647-656.

Choi, G.W., Um, H.J., Kim, Y. *et al.,* 2010. Isolation and characterization of two soil derived yeasts for bioethanol production on Cassava starch, Biomass- Bioenergy. 34: 1223–1231.

Erdei, B., B. Franko, M. Galbe and G. Zacchi. 2012. Separate hydrolysis and co-fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw, Biotechnol. Biofuels. 5: 1–12.

Fujita, Y., Junji Ito, Mitsuyoshi Ueda, Hideki Fukuda, Akihiko Kondo. 2004. Synergistic Saccharification, and Direct Fermentation to Ethanol, of Amorphous Cellulose by Use of an Engineered Yeast Strain Codisplaying Three Types of Cellulolytic Enzyme. Applied and Environmental Microbiology. 70(2):1207–1212.

Kang, K.E., Chung, D.-P., Kim, Y. *et al.,* 2015. High-titer ethanol production from simultaneous saccharification and fermentation using a continuous feeding system. Fuel. 145: 18–24.

Kasavi, C., I. Finore, L. Lama, *et al.*, 2012. Evaluation of industrial *Saccharomyces cerevisiae* strains for ethanol production from biomass. Biomass- Bioenergy. 45:230–238.

Koo, B., H. Kim, N. Park, *et al.*, 2011.Organosolv pretreatment of *Liriodendron tulipifera* and simultaneous saccharification and fermentation for bioethanol production. Biomass-Bioenergy. 35: 1833–1840.

Kuhad, R C., Rishi Gupta, Yogender PalKhasa, Ajay Singh Y.-H. Percival Zhang. 2011. Bioethanol production from pentose sugars: Current status and future prospects. Renewable and Sustainable Energy Reviews. 15(9): 4950-4962.

Li, H., N.J. Kim, M. Jiang, *et al.*, 2009. Simultaneous saccharification and fermentation of lignocellulosic residues pretreated with phosphoric acid-acetone for bioethanol production, Bioresour. Technol. 100: 3245–3251

Lin, Y., W. Zhang, C. Li, *et al.*, 2012.Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. Biomass- Bioenergy. 47: 395–401.

Lu, J., X. Li, R. Yang, *et al.*, 2013. Fed-batch semi-simultaneous saccharification and fermentation of reed pretreated with liquid hot water for bio-ethanol production using *Saccharomyces cerevisiae*, Bioresour. Technol. 144: 539–547.

Mellitzer, A., Roland Weis, Anton Glieder and Karlheinz Flicker.2012. Expression of lignocellulolytic enzymes in *Pichia pastoris*. Microbial Cell Factories. 11:61.

Miller, G.L., Blum R, Glennon WE, Burton AL. 1960. Measurement of carboxymethylcellulase activity. Anal Biochem. 2:127–132.

Mussato, S.I., E.M.S. Machado, L.M. Carneiro, J.A. Teixeira. 2012. Sugar metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. Appl. Energy 92: 763–768.

Rao, R.S., B. Bhadra and S. Shivaji. 2008. Isolation and characterization of ethanol-producing yeasts from fruits and tree barks. Letters in Applied Microbiology. 47:19–24.

Singhania, R., Anil Kumar Patel, Rajeev K.Sukumaran, Christian Larroche, Ashok Pandey. 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresource Technology. 127: 500-507.

Srichuwong, S., M. Fujiwara, X. Wang, *et al.*, 2009. Simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol. Biomass- Bioenergy. 33: 890–898.

Zahida Nasreen, Shaista Jabeen, Muafia Shafique, Shumaila Usman, Tehseen Yaseen, Ammara Yasmeen and Saima Nazir. 2014. Production of alcohol by yeast isolated from apple, orange and banana. International Journal of Food and Nutrition Sciences.1 (2): 016-019.

Zhao, J. and L. Xia. 2010. Bioconversion of corn stover hydrolysate to ethanol by a recombinant yeast strain. Fuel Process Technol. 91:1807–1811.

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