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Isolation and characterization of cellulolytic yeasts for bioethanol production

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Abstract: A total of 104 yeast isolates from various natural habitat mainly rotten fruit rinds, forest leaf litter, compost, fruit samples, food stuff and over matured paragrass were isolated on the basis of zone of hydrolysis of cellulose and release of reducing sugars. Among these 31 cellulolytic yeasts were screened. Five efficient strains (CY-52, CY-58, CY-59, CY-62 and CY-81) were selected from total of 104 isolates, isolated from various natural habitats (mainly rotten fruits, vegetables, sugarcane molasses) on the basis of zone of hydrolysis and release of maximum reducing sugars. The efficient yeast strains were subjected on two delignified substrates *i.e.,* paddy straw and sugarcane bagasse. The yeast isolate CY-59 showed the maximum bioethanol production of 48.18 mg g^{-1} and 60.51 mg g^{-1} from paddy straw and sugarcane bagasse, respectively which was on par to reference strain NCIM-3200 and commercial cellulase.

Key words: Alkali, Bioethanol, Carboxy methyl cellulose, Cellulolytic yeast

Introduction

Energy is an important factor for the economic development of a nation. Biomass is a sustainable alternative to fossil energy carriers which are used to produce fuel. Recovery of cellulose is a critical process from the biomass in order to subject them for bioethanol production. The recalcitrant nature of the lignocellulose where the lignin surrounds the cellulose and hemicellulose makes it difficult to convert them into monomeric sugar units.

Ethanol has already been introduced on a large scale in Brazil, USA and some European countries and we expect it to be one of the dominating renewable biofuel in the transport sector within the coming 20 years. Therefore, agriculture and forest residues are mainly used for the production of ethanol. *Saccharomyces cerevisiae* is mainly used for bioethanol production because of its high ethanol productivity, high ethanol tolerance and simplicity of genetic engineering. At present ethanol production from agro residues is by two step process. Therefore, cellulolytic yeasts have employed to make hydrolysis and fermentation process into single step through the cellulase activity.

Material and methods

The samples were selected based on the cellulosic nature of the outer skin of the degraded substrates and other sources which are partially composted. For *e.g.* rotten fruit rinds, forest leaf litter, compost, bagasse, composted plant residues, cactus flower, fruit samples, food stuff, over matured paragrass and FYM etc (total of 17 samples). The isolations were made by following standard serial dilution technique using normal MGYP media and incubated for 24-48 h at 37° C. Based on the colony morphology and microscopic observations, typical yeast colonies were chosen, purified and observed under the microscope. All the 104 isolates were spotted individually on media containing carboxy methyl cellulose as sole source of carbon and incubated at 37°C for 48 hr. The plates were flooded with 0.01 per cent of congo red stain for 15 minutes followed by destaining with 1M sodium chloride solution for 15 minutes (Teather and Wood, 1982). The clear zone around the colonies indicated the hydrolysis of cellulose by yeast.

The isolates were subjected for utilization of various carbon sources *viz. g*lucose, galactose, sucrose, maltose, lactose, starch, D-xylose, arabinose and D-ribose (Ribereau-Gayon *et al.,* 2007). The selected crop residues (bagasse and paddy straw) were delignified to obtain cellulose. Inoculation of efficient strains were carried out to know their performance on substrates. Ten grams of each substrate of 0.5 µ particle sizes were subjected for 2.5 and 3 per cent NaOH (w/v) separately and autoclaved for 6 and 8 hr. The alkali was sufficient enough to moisten entire substrate except in case of bagasse where additional 10 ml was used. The contents were drained and thoroughly washed to remove alkali. The pulp obtained was used for determining neutral detergent fibre (NDF) and acid detergent fibre (ADF) as per method described by Goering and Van Soest (1975).

Enzyme assays such as FPase activity, CMCase activity were performed to know saccharification potential of the strains and commercial enzymes as control was used during the investigation. The amount of reducing sugars was estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959) and the ethanol was estimated colorimetrically as described by Caputi *et al.* (1968).

Results and discussion

A total of 104 yeast isolates were obtained from 17 samples *viz.* sugarcane bagasse, forest litter, rotten fruits and compost residues. Based upon the typical colony morphology and microscopic observations resembling yeast, it was further purified (Table 1) and again subjected for zone of inhibition for screening efficient strain (Table 2).

Thirty one isolates were screened and tested in CMC broth to know the efficiency of the isolates in terms of releasing reducing sugars (Table 3). Among all isolates screened for reducing sugars the CY-59 showed the highest reducing sugars

Table 1. Release of reducing sugars (mg g^{-1}) by the yeast isolates at five days of interval

Isolates	Reducing sugars mg g^{-1} CMC			
	1 DAI	5 DAI	10 DAI	15 DAI
CY-1	0	54.98	87.96.	112.93
CY-2	0	16.33	29.54	65.02
CY-5	0	0	16	39
CY-7	0	24.65	59.09	106.98
CY-13	10.04	28.87	69.85	134.76
CY-14	0	20.64	30.76	64.34
CY-17	1.47	29.67	46.74	98.85
CY-18	0	0	17.33	59.76
CY-20	0	3.07	29.04	56.98
CY-24	0	3.04	39.54	89.03
CY-29	0	0.16	10.33	27.98
CY-30	5.03	29.44	78.09	105.98
CY-31	0	30.03	69.09	124.0
CY-32	0	42.09	88.09	128.08
CY-33	13.03	54.03	75.05	109.03
CY-34	0	3.05	39.04	69.04
CY-39	0	52.03	79.03	119.93
CY-40	2.04	16.94	28.08	65.03
CY-42	0	37.03	67.04	148.03
CY-43	0	34.02	48.02	89.04
CY-45	0	1.98	34.09	29.08
CY-47	8.02	24.06	34.08	67.03
CY-48	0.35	36.03	80.03	120.09
CY-52	13.02	38.02	79.09	167.03
CY-58	10.03	29.03	74.03	159.83
CY-59	16.05	46.03	89.03	174.03
CY-62	14.03	43.03	83.03	170.03
CY-65	0	0	23.02	34.02
CY-68	0	2.03	16.03	36.03
CY-81	13.08	42.03	82.08	169.02
CY-83	2.03	18.03	34.02	78.09
NCIM-3200	17.14	49.20	90.88	186.01
S.Em.±	0.29	0.77	0.87	1.14
C.D. at 1%	0.98	2.68	3.02	3.98

DAI- Days after incubation

Table 2. Release of total sugars by the yeast isolates at five days of interval

Isolates	Total sugars mg g^{-1} CMC			
	1 DAI	5 DAI	10 DAI	15 DAI
CY-1	0	42.08	104.06	139.65
CY-2	0	22.05	34.09	78.05
CY-5	0	0	28	55
CY-7	0	35.07	70.43	116.98
CY-13	14.08	35.05	82.03	145.04
CY-14	0	27.04	39.03	80.87
CY-17	6.08	34.04	64.05	109.05
CY-18	0	0	23.07	70.94
CY- 20	0	7.09	34.06	69.09
CY- 24	0	7.07	45.98	106.09
CY- 29	0	3.97	17.08	40.98
CY- 30	13.98	40.05	89.09	123.06
CY- 31	0	43.08	83.02	139.07
CY- 32	0	52.08	102.08	165.08
CY- 33	19.08	39.05	104.06	132.09
CY- 34	0	7.08	45.06	84.06
CY- 39	0	44.08	93.05	138.05
CY- 40	5.08	29.06	43.08	88.08
CY- 42	0	36.08	84.08	172.09
CY- 43	0	39.05	73.06	112.08
CY- 45	0	5.08	53.08	56.09
CY- 47	14.08	32.08	53.08	81.09
CY- 48	3.08	42.09	96.09	145.09
CY- 52	24.08	47.98	116.08	185.03
CY- 58	21.08	42.09	108.08	180.04
CY- 59	32.08	56.98	132.09	193.05
CY- 62	28.09	53.98	121.09	190.05
CY- 65	0	0	38.09	65.08
CY- 68	0	5.07	34.08	56.08
CY- 81	26.08	50.09	118.07	188.98
CY- 83	9.05	38.08	68.09	104.08
NCIM-3200	34.29	60.90	141.19	206.36
S.Em.±	0.64	1.01	1.29	1.43
C.D. at 1%	2.06	3.09	3.94	4.02

DAI- Days after incubation

Table 3. Effect of alkali on paddy straw and bagasse at different period of incubation

Substrate	Concentration of alkali (NaOH)	Cellulose content (mg g^{-1})		Mean substrate
		6 hr	8 hr	
Paddy straw	2.5%	421	491	489
	3%	483	564	
Sugarcane bagasse	2.5%	471	589	580
	3%	567	695	
Mean concentration of alkali	2.5%	493		
	3%	577		

Source	S.Em.±	C.D. at 1%
Substrate (A)	0.0035	0.013
Incubation period (B)	0.0029	0.011
Concentration of alkali (C)	0.0029	0.011
A x B	0.005	0.019
A x C	0.005	0.019
B x C	0.0041	NS
A x B x C	0.007	NS

Initial cellulose content: Paddy straw - 340 mg g^{-1}, Sugarcane bagasse - 390 mg g^{-1}

(174.03 mg g^{-1}) followed by CY-62 (170.03 mg g^{-1}), CY-8 (169.02 mg g^{-1}), CY 52 (167.03 mg g^{-1}) and CY-58 (159.83 mg g^{-1}) and the viscosity of the CMC broth was reduced.

Among all isolates CY-59 showed the highest total sugars (193.05 mg g^{-1}) followed by CY-62 (190.05 mg g^{-1}), CY-81 (188.98 mg g^{-1}), CY-52 (185.03 mg g^{-1}) and CY-58 (180.04 mg g^{-1})

Table 4. Enzymatic activity of the crude extract

Isolates	Reducing sugars	
	FPase activity (U ml^{-1})	CMCase activity (U ml^{-1})
CY-52	4.47	3.25
CY-58	3.66	2.26
CY-59	16.82	12.99
CY-62	12.80	9.25
CY-81	11.55	7.05
Saccharomyces cerevisiae NCIM-3200 (Reference strain)	18.28	17.24
Commercial Cellulase	24.15	18.28

(Table 4). Five isolates were tested for utilization of nine sugars *viz.* glucose, galactose, sucrose, maltose, lactose, starch, D-xylose, arabinose and D-ribose. All the strains utilized glucose, galactose and maltose and sucrose sugars where as except isolate CY- 58, all isolates were unable to utilize lactose. Out of five isolates, CY-59 and CY-81 utilize pentose sugars. (Table 5).

The two agro-residues selected for bioethanol production studies differed in their composition of cellulose contents. Sugarcane bagasse contained the highest cellulose content of 695 mg g^{-1} followed by paddy straw (564 mg g^{-1}) (Table 6). The difference in chemical composition of the agro-residues is due to variation in the composition of cell wall as it varies with plant species, tissue type and region within the cell wall and development stages of the cell wall (Carpita and McCann, 2000).

Filter paper activity was assayed to know the isolates activity which was expressed in terms of filter paper units (FPU). Among all isolates CY- 59 (16.82 U ml^{-1}) showed the highest activity followed by CY-62 (12.80 U ml^{-1}), CY-81 (11.55 U ml^{-1}), CY-58 (7.66 U ml^{-1}) and CY-52(4.47 U ml^{-1}). Reference strain and commercial cellulase showed 18.28 U ml^{-1} and 24.15 U ml^{-1}, respectively. Similarly, CMCase activity was tested to know the efficiency of the strains *i.e.*, CY-59 showed the highest activity (12.99 U ml^{-1}) followed by CY-62 (9.25 U ml^{-1}), CY-81 (7.05 U ml^{-1}), CY-58 (3.26 U ml^{-1}) and CY-52 (2.25 U ml^{-1}). The CMCase activity of the reference strain and commercial cellulase was 17.24 U ml^{-1} and 18.28 U ml^{-1}, respectively (Table 7).

The delignified substrates were inoculated with the 5 potential cellulolytic yeasts and ethanol was estimated after 5 days of fermentation. Among the all strains CY-59 showed the highest 48.18 mg g^{-1} followed by CY-62 (38.29 mg g^{-1}), CY-81(33.11 mg g^{-1}), CY-52 (28.11 mg g^{-1}) and CY-58 (21.99 mg g^{-1}) from paddy straw. Commercial cellulase and reference strain showed the highest value among all strain *i.e.* 72.29 mg g^{-1} and 64.62 mg g^{-1}, respectively.

The result indicated that, the yeast isolates also efficiently exhibited the cellulase activity and converted the sugars to alcohol, although less than that of commercial enzyme.

The strain CY-59 (60.51 mg g^{-1}) showed the highest ethanol production followed by CY-62 (49.51 mg g^{-1}), CY-81 (43.88 mg g^{-1}), CY-52 (37.14 mg g^{-1}) and CY-58 (28.66 mg g^{-1}) from sugarcane bagasse. Commercial cellulose and reference strain showed the highest value of 76.99 mg g^{-1} and 72.44 mg g^{-1}, respectively as compared to all the isolates.

Thus, it was evident that the yeast can exhibit the cellulase activity when cellulosic substrate is available and can convert to alcohol also, though they yield less than the commercial cellulose enzyme. The strain CY-59 showed maximum saccharification as well as fermentation. It was well established that the strain exhibited all three components of cellulose enzyme. However, extrapolating more sources from nature is essential which may yield better strains to solve the constraints for bioethanol production.

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