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Characterisation of thermotolerant, ethanol tolerant fermentative *Saccharomyces cerevisiae* for ethanol production

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Abstract Of the four thermotolerant, osmotolerant, flocculating yeasts (VS_1, VS_2, VS_3 and VS_4) isolated from the soil samples collected within the hot regions of Kothagudem Thermal Power Plant, located in Khammam Dt., Andhra Pradesh, India, VS_1 and VS_3 were observed as better performers. They were identified as *Saccharomyces cerevisiae*. VS_1 and VS_3 were tested for their growth characteristics and fermentation abilities on various carbon sources including molasses at 30 °C and 40 °C respectively. More biomass and fermentation was observed in sucrose, fructose and glucose. Maximum amount of ethanol produced by VS_3 containing 150 (g/l) of these substrates were 74, 73, and 72 (g/l) at 30 °C and 64, 61 and 63 (g/l) at 40 °C respectively. With molasses containing 14% sugar, the amount of ethanol produced by VS_3 was 53.2 and 45 (g/l) at 30 °C and 40 °C respectively. VS_3 strain showed 12% W/V ethanol tolerance. VS_3 strain was also characterised for its ethanol producing ability using various starchy substrates in solid state and submerged fermentation. More ethanol was produced in submerged than solid state fermentation.

1 Introduction

During conventional yeast alcohol fermentation heat is generated at the rate of 140.2 cal/g glucose fermented. This rise in temperature continues until the temperature of the medium becomes intolerable for growth and activity. This often results in high leftover sugars, low alcohol yields and poor fermentation efficiency which is due to inactivation of yeast cells at higher temperatures [1].

In industries this rise in temperature during fermentation is controlled by cooling the reactor. Thermotolerant yeasts in this situation are more advantageous in that they have faster fermentation rates, avoid the cooling costs, decrease the distillation costs and thereby help in decreasing the over all fermentation costs, so that ethanol can be made available at a cheaper rate [2, 3]. Since the industrially used strain is non amylolytic and non-cellulolytic, starchy and cellulosic substrates should be saccharified into simple sugars for production of ethanol by treating the substrates with amylases and cellulases. The type of sugar produced by the process of hydrolysis varies with the type of substrate used. Mostly starchy substrates produce maltose, glucose, fructose and cellulosic substrates produce xylose, arabinose, glucose, mannose and galactose in various amounts [4]. We have isolated four different strains of thermotolerant *Saccharomyces cerevisiae* from soil samples collected within the hot regions of Kothagudem Thermal Power Plant located in A.P. India.

Of these strains isolated VS_1 & VS_3 are better performers. In view of the potential advantages of solid substrate fermentation over conventional submerged fermentation, VS_3 strain was used for production of ethanol from various starchy substrates in co-culture with local isolate of *Amylolytic Bacillus* and with crude amylase enzyme [5, 6]. Although the ethanol production technology is old, potent strains are required for commercial production in order to economise the process.

Hence in the present study, it is planned to assess the potential of these strains for production of ethanol from various sugars and starchy substrates by submerged fermentation.

2 Materials and methods

2.1 Microorganisms and culture media

VS_1 and VS_3 yeast strains were cultivated in yeast extract peptone dextrose medium (YEPD) as described earlier [6].

2.2 Batch flask experiments

VS_3 strain was tested for ethanol tolerance using 4 to 12% (W/V)of ethanol by adding 5% inoculum. Effect of pH on the growth of VS_3 strain was studied by inoculating culture into different 250 ml flasks containing 100 ml YEPD medium using a pH range of 2.0 to 12.0. Samples were withdrawn after 48 hrs of inoculation for estimation of biomass. 5% of VS_1 and VS_3 yeast inoculum was added to four sets of 250 ml conical flasks with 100 ml yeast fermentation medium (YFM) containing (W/V) yeast extract 0.3% peptone 0.5% and carbon sources each of 15% (Arabinose, sucrose, fructose, glucose, lactose, maltose, xylose, galactose and sorbitol) and molasses with 14% sugar, pH 5.5. Two sets of conical flasks were incu-

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bated at 30 °C and other two at 40 °C for 48 hours. Samples were withdrawn after 48 hours for estimation of biomass, and ethanol. 20% of various starchy substrates (sweet potato, wheat flour, potato starch, rice starch, soluble starch, and sweet sorghum) were added to YFM medium as described above in 250 ml conical flasks with pH 5.0. One set of conical flasks were incubated at 37 and other set at 42 °C.

2.3 Analytical methods

The biomass of VS_1 and VS_3 cultures were estimated both by taking O.D at 660 nm and by taking wet weight in which the cells were centrifuged after 48 hours of incubation and the weight of the pellet was taken. Ethanol produced was estimated by high pressure gas chromatography as described earlier [6].

3 Results and discussion

3.1 Culture identification

VS_1 and VS_3 strains were identified as *Saccharomyces cerevisiae* in our laboratory by studying morphological and biochemical characteristics and our identification was confirmed by Microbial Type Culture Collection (Institute of Microbial Technology Chandigarh). Authority for identification was Meyen-Ex-Hansen.

3.2 Flocculation

Flocculation ability of VS_1 and VS_3 strains was tested by Tullo test based on which these strains were classified as Class IV yeasts [7].

3.3 Effect of pH

It was observed that the optimum pH for growth of VS_3 strain was 5.5, but VS_3 strain could grow from pH 2–10 which is an unusual property of our *Saccharomyces cerevisiae*.

3.4 Ethanol tolerance

50% increase in the biomass after inoculation was taken as tolerance based on which the VS_3 strain is identified as ethanol tolerant yeast which was capable of tolerating an ethanol concentration of 12% (W/V) which may be due to the presence of increased amount of saturated fatty acids as suggested by Judit Krish and Bela Szajani (1997) [8] (Table 1).

Table 1. Biomass of VS_3 strain at various ethanol concentration (% W/V)

Concentration of ethanol	O.D	B.Y
4	1.90	1.20
5	1.85	1.19
6	1.82	1.18
7	1.80	1.17
8	1.78	1.14
9	1.76	1.11
10	1.75	1.08
11	1.65	1.00
12	1.50	0.95

O.D = Optical density of yeast culture measured at 660 nm
B.Y = Biomass of yeast culture in g/l (Wet Weight)

Table 3. Ethanol production from various carbon sources using VS_1 & VS_3 strains at 30 °C and 40 °C

Substrate	Ethanol (g/l) VS_1		Ethanol (g/l) VS_3	
	30 °C	40 °C	30 °C	40 °C
Arabinose	4.0	2.4	5.0	3.0
Fructose	67.0	52.0	73.0	61.0
Galactose	60.0	45.0	63.0	31.0
Maltose	58.0	30.0	60.0	36.0
Glucose	65.0	53.0	72.0	63.0
Sorbitol	3.0	0.8	4.0	1.0
Molasses	52.0	30.0	53.2	45.0
Sucrose	68.0	52.0	74.0	64.0
Xylose	4.0	2.0	5.0	2.5

Table 2. Biomass of VS_1 and VS_3 cultures on various carbon sources at 30 °C & 40 °C

Substrate	VS_1				VS_3			
	30 °C		40 °C		30 °C		40 °C	
	O.D	B.Y	O.D	B.Y	O.D	B.Y	O.D	B.Y
Arabinose	0.90	0.60	0.50	0.30	1.20	0.80	0.60	0.37
Fructose	5.00	3.10	2.80	1.80	5.10	3.15	3.00	1.90
Galactose	3.40	2.20	1.60	1.00	3.70	2.32	2.00	1.20
Maltose	3.20	2.10	1.40	0.90	3.50	2.20	1.60	1.00
Glucose	4.80	3.00	2.20	1.40	5.00	3.10	2.40	1.55
Sorbitol	0.90	0.60	0.53	0.30	1.00	0.70	0.63	0.40
Sucrose	4.90	3.00	2.20	1.40	5.20	3.20	2.60	1.65
Xylose	0.89	0.60	0.60	0.35	1.20	0.80	0.71	0.40

B.Y = Biomass of yeast cultures in g/l (Wet Weight)
O.D = Optical density of the culture measured at 660 nm

Table 4. Production of ethanol by VS_1 & VS_3 strains from various starchy substrates in submerged fermentation

Substrate	VS_1		VS_3	
	Ethanol in g/100g substrate at 37 °C	Ethanol in g/100g substrate at 42 °C	Ethanol in g/100g substrate at 37 °C	Ethanol in g/100g substrate at 42 °C
Potato starch	6.0	4.5	8.0	6.0
Rice starch	5.0	3.0	6.0	3.0
Sweet potato	10.0	5.7	12.0	6.5
Soluble starch	2.0	1.5	3.0	2.0
Sweet sorghum	6.0	4.5	8.0	5.5
Wheat flour	10.0	6.9	14.0	8.5

3.5
Ethanol from various carbon sources

Both VS_1 and VS_3 cultures have grown on all the substrates tested at both 30 °C and 40 °C. Both the cultures produced more biomass on sucrose, fructose and glucose and maltose. VS_1 has produced a maximum biomass of 3.0, 3.10, 3.0 and 2.10 (g/l) at 30 °C, 1.40, 1.80, 1.40 and 0.90 (g/l) at 40 °C from sucrose, fructose, glucose and maltose respectively. VS_3 has produced more biomass than VS_1. The biomass of VS_3 at 30 °C and 40 °C was 3.20, 3.15, 3.10, 2.20 (g/l) and 1.65, 1.90 and 1.55, 1.00 (g/l) respectively. Less biomass was obtained on lactose, xylose, arabinose and sorbitol (Table 2).

More ethanol was produced from sucrose by both VS_1 and VS_3 (68, 52 and 74, 64 (g/l) at 30 °C and 40 °C respectively) which indicates that both the strains have good invertase activity and can be used for production of ethanol from the substrates which contain sucrose. VS_3 has produced relatively more amount of ethanol from fructose, glucose, galactose and maltose as shown in Table 3.

The amount of ethanol produced from these substrates was 73, 72, 63 and 60 (g/l) at 30 °C and 61, 63, 31 and 36 (g/l) at 40 °C respectively. These results indicate that VS_3 strain can be utilised for production of ethanol from starchy substrates, because starchy substrates produce the above sugars upon hydrolysis by amylase enzymes. Banat et al. (1992) reported the isolation of thermotolerant *Kluveromyces marxianus* which has produced a maximum of 68 and 55 (g/l) ethanol at 40 °C and 30 °C respectively. But our VS_3 strain being *Saccharomyces cerevisiae* has produced 74 and 64 (g/l) ethanol at 30 °C and 40 °C respectively. Currently used organism for alcohol production is *Saccharomyces cerevisiae*, in addition our strains are having good flocculating ability which is another desirable advantage for alcohol production because they help in decreasing the separation costs [9].

Both VS_1 and VS_3 have produced less amount of ethanol from arabinose, and sorbitol. The amount of ethanol produced by VS_3 from these substrates was 5.0, and 4.0 (g/l) at 30 °C and 3.0, and 1.0 (g/l) at 40 °C respectively. There are only few reports on *Saccharomyces cerevisiae* strains capable of growing and fermenting xylose. Our *Saccharomyces cerevisiae* strains (VS_1 and VS_3) have grown and fermented xylose. The amount of ethanol produced from xylose by VS_3 was 5.0 (g/l) at 30 °C and 2.5 (g/l) at 40 °C. The amount of ethanol produced by VS_3 on molasses (14% sugar) was 53.2 and 45 (g/l) at 30 °C and 40 °C respectively. VS_1 and VS_3 has produced less ethanol from molasses when compared to glucose medium which might be due to the presence of high concentration of fermentation inhibitors which inhibited the fermentation.

3.6
Ethanol production in submerged fermentation from various starchy substrates

The amount of ethanol produced from various starchy substrates is shown in Table 4. These results indicate that more ethanol was produced by VS_3 from wheat flour and sweet potato than VS_1. The maximum amount of ethanol produced by VS_3 from these two substrates was 14 & 12 g/100 g substrate and 8.5 & 6.5 g/100 g substrate at 37 °C and 42 °C respectively. Less ethanol was produced from rice and soluble starch (6.0 & 3.0, and 3.0 & 2.0 gms of ethanol for 100 g/substrate at 37 °C and 42 °C respectively. On contrary our early experiments in solid substrate fermentation showed that more ethanol is produced from rice starch and sweet sorghum by VS_3 strain 10 g & 3.5 g at 37 & 42 °C from rice starch and 8.5 and 7.5 g/100 g substrate from sweet sorghum at 37 & 42 °C respectively [6].

4
Conclusion

These studies indicate the novelty and suitability of VS_3 strain for production of ethanol from various carbon sources.

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