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Screening and Parameters Optimization of Pentose Fermenting Yeasts for Ethanol Production using Simulated Media

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Saccharomyces cerevisiae **has been widely used for fermentation of refined starch and sugar.** ***S.cerevisiae*** **is unable to utilize pentose sugars such as xylose and arabinose etc. Hence pentose utilizing three standard cultures** ***Candida tropicalis*** **Y-27405,** ***Candida intermedia*** **MTCC-1404 and** ***Candida tropicalis*** **Y-1552 were screened for best ethanol and C. intermedia MTCC -1404 was found to be best ethanol producer. Further the effect of different process parameters like initial pH, temperature, agitation, concentration of xylose, initial glucose concentration, inoculum size and fermentation time on ethanol yield by selected organism** ***C. intermedia*** **MTCC-1404 for ethanol production. The yield of ethanol obtained under optimal conditions was 0.4±0.012 g/g.**

Key words: Pentose fermenting yeats, Ethanol production, Simulated media.

Bioethanol production is an interesting alternative to fossil fuels. In 2010, 86.8 billion liters of ethanol was produced worldwide, mostly in the USA and Brazil[1]. Nowadays most of the bioethanol production is from starch and soluble sugar-based biomass like sugar cane juice and corn starch[2].

The fermentation of sugar mixtures that result from biomass hydrolysis is a significant bottleneck in the overall process. Few fermentative organisms can ferment both hexose and pentose sugars to liquid fuels such as ethanol have been identified[3-6]. Xylose is only second to glucose in natural abundance, and xylose can be fermented by microorganisms like *Pichia stipitis, Candida shehatae* etc., Thus, there has been a great emphasis in the last two decades on developing an efficient organism for pentose fermentation through metabolic engineering[7,8].

S. cerevisiae is robust budding yeast that has been widely used for fermentation of refined starch and sugar into[9] significant titers of fuel ethanol when grown aerobically in batch culture[10] However; *S. cerevisiae* is unable to utilize pentose sugars such as xylose and arabinose that result from the hydrolysis of hemicellulose. The engineering of pentose metabolism into the *S. cerevisiae* genome has been achieved, but problems with co-factor imbalances and gene expression have hindered the efficiency of these mutants[11].

However, the lack of suitable microorganisms ferment pentose and hexose sugars into ethanol has been one of the major problems preventing commercialization of second generation ethanol production[12]. Therefore the researchers are trying to isolate different microorganisms and modify genetically to overcome the low ethanol productivity by traditional organisms .

In the present study, 3 standard cultures *Candida tropicalis* Y-27405, *Candida intermedia*

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MTCC-1404 and *Candida tropicalis* Y-1552 which have ability to ferment D-xylose were screened for best ethanol yield and the effect of different process parameters (pH, temperature, xylose concentration, agitation and aeration) on ethanol yield by selected organism have been studied

MATERIALS AND METHODS

Microorganism and its Maintenance

C. tropicalis Y-27405 and *C. tropicalis* Y-1552 were procured from, The Agricultural Research Service ARS Culture Collection center (NRRL) USA. The stock culture was maintained on (YPX) agar slants containing yeast extract 10 (g/l), peptone (20g/l), xylose (30 g/l), agar (25g/l) and pH to 5 stored at 4^0 C.

C.intermedia MTCC-1404 was obtained from IMTECH, Chandigarh. The stock culture was maintained on (YPX) agar slants.

Inoculum media

The seed culture of three yeasts were prepared by inoculating loopful of each yeast from YPX slants into 25ml sterile media in 100 ml Erlenmeyer flask, consisting of 20 g/l of xylose, 20 g/l of Peptone and 10 g/l of Yeast extract. Inoculum was grown aerobically at 30 °C on a rotary shaker at 150 rpm for 24 h.

Screening of pentose fermenting yeast

For ethanol production, three different pentose utilizing yeasts *C. tropicalis* Y-27405, *C.intermedia* MTCC-1404 and *C. tropicalis* Y-1552) were examined to select the best one based on their ethanol production and their ability to utilize maximum sugar. Each 50 ml of modified MGYP inoculum media containing malt extract 0.5%, glucose + xylose 1.0%, yeast extract 0.5%, peptone 0.5% and pH 5.5 [21] is inoculated with *C. tropicalis* Y-27405, *C. intermedia* MTCC-1404 and *C. tropicalis* Y-1552 separately and incubated at 30°C and 150 rpm for 24 h. This 24 h old inoculum is transferred to fermentation medium. Fermentation medium (20 ml) was taken in 3sets of conical flasks each containing 3% concentration of xylose , inoculated separately with *C. tropicalis* Y-27405, *C. intermedia* MTCC-1404 and *C. tropicalis* Y-1552 , at 30 °C, 150 rpm for 96 h. Samples were collected after every 12 h interval starting from 12h to 96h of fermentation to estimate concentration of ethanol , biomass production and left over sugar present in fermented broth .

Parametric optimization studies of ethanol production by *C.intermedia* MTCC-1404

Effects of various physical factors have been studied to know the best suitable conditions for maximum ethanol production. These parameters include; initial pH (3.5-7.5), temperature (20-40 °C), agitation (static -250 rpm), concentration of xylose (3-7%), initial glucose concentration (0.2-0.5%), inoculum size (5-15%) and fermentation time (24h-96h) which were studied separately in synthetic media. Optimizations of parameters were carried out in a stepwise procedure where specified parameter was varied by keeping all the other parameters constant.

Analytical methods

Total reducing sugars

The total reducing sugars released after acid hydrolysis were estimated by DNS method[13].

Ethanol estimation

The fermented media was centrifuged at 5000g at 40C for 5 min and the supernatant was filtered using 0.22µm cellulose acetate filters and then ethanol produced was analyzed by gas chromatography (GC) (Shimadzu (GC-2011), Japan) using ZB-Wax column (30 mm×0.25 mm) with a flame ionization detector (FID). The analysis was performed according to NREL (National Renewable Energy Laboratory) procedure LAP #001 (David, 1994). The column temperature was 150°C (isothermal), program run time: 5.5 min, ethanol retention time: about 2.3 min and the carrier gas was nitrogen (16 kPa), injector temperature: 175 °C, detector temperature: 250 °C, flow rate: 40 ml/min, spilt ratio: 1/50, velocity of H2 flow: 60 ml/min, sample quantity: 1 µl.

Statistical analysis of data

All the experiments were performed in triplicates and the results were presented as mean ± standard deviation and were also analyzed by ANOVA using statistical software Graphpad-Prism 6 Demo.

RESULTS AND DISCUSSION

Screening of pentose fermentation yeast

Ethanol fermentation of xylose (3%) was performed with *C. tropicalis* (Y-27405) *C.intermedia* (MTCC-1404) and *C. tropicalis* (Y-

1552).

***C. intermedia* (MTCC-1404) was selected for pentose fermentation than *C. tropicalis* (Y-27405)**

The ethanol produced by *C. intermedia* (MTCC-1404) was 9 g L^{-1} with a yield of 0.3± 0.01 g/g at D-xylose concentration of 3% (Figure 1).

Jyothi[15] studied 6 different MTCC strains for ethanol production from synthetic media 30 xylose and reported that *C.intermedia* MTCC-1404 produced 0.35 (g/g) ethanol yield. Table 1 compares the result of present study with previous reports on ethanol production from pentoses sugar.

Rao *et al.*,[16] isolated 35 yeasts from the gut of beetles collected from Hyderabad city, India. They reported 12 strains can convert xylose to xylitol that reduced ethanol production. Jyothi[15] reported that *C. intermedia* MTCC-1404 has produced more amount of ethanol and little amount of xylitol and their results support the present study.

In present study, *C. intermedia* MTCC-1404 has been selected for optimization process parameter ethanol production.

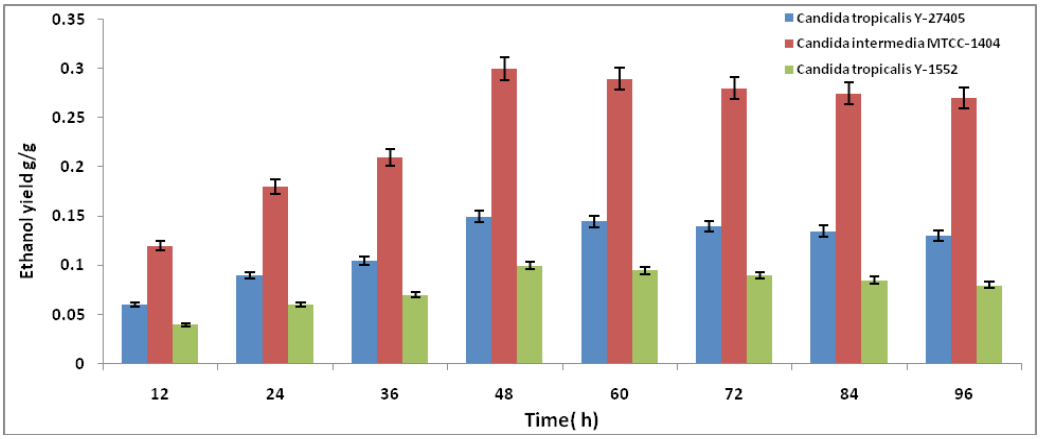

Fig. 1. Ethanol yield using *Candida tropicalis* (Y-27405) *Candida intermedia* (MTCC-1404) and *Candida tropicalis* (Y-1552)

Table 1. Comparison of ethanol production with previous reports of pentose fermenting yeast

S.No	Strain	Ethanol production (g/g)	Reference
1	*Candida boidinii* MTCC288	0.29	[15]
2	*Candida tropicalis* MTCC230	ND	[15]
3	*Candida intermedia* MTCC-1404	0.35	[15]
4	*Candida parapsilosis* MTCC-1744	ND	[15]
5	*Pachysolen tannophiles* MTCC 1077	0.25	[15]
6	*Pichia jadinii* MTCC 185	ND	[15]
7	*Pichia stipitis* NCIM3499	0.44	[17]
8	*Pichia stipitis* NCIM 3498	0.40	[14]
9	*Candida shehatae* NCIM 3501	ND	[14]
10	*Candida tropicalis* Y-27405	0.15	Present study
11	*Candida intermedia* MTCC-1404	0.3	Present study
12	*Candida tropicalis* Y-1552	0.1	Present study

Parametric optimization studies for ethanol production by *C. intermedia* MTCC-1404

To produce maximum ethanol by yeast in synthetic xylose media, optimization of various factors viz. pH, temperature, concentration of xylose, agitation etc has been studied to know the best suitable conditions for maximum ethanol production. The results were presented in the Table 2.

Table 2. Parametric optimization studies for ethanol production by *Candida intermedia* MTCC-1404

S. No	Parameters	Optimum level	Ethanol yield g/g
1	pH	5.5	0.3±0.013
2	Temperature	30	0.3±0.012
3	Concentration of xylose	5%	0.36±0.016
4	Agitation	150rpm	0.36±0.015
5	Initial glucose concentration	0.2%	0.38±0.0162
6	Inoculum size	15%	0.4±0.014

Effect of pH on ethanol production by *C. intermedia* MTCC-1404

The effect of initial pH for ethanol production using xylose by *C. intermedia* MTCC-1404 was investigated within the range of 3.5 -7.5. The pH was adjusted either with 1N NaOH or 1N HCl. *C.intermedia* MTCC-1404 was grown at temperature 30 °C, agitation and 150 rpm. Figure 3 shows that maximum ethanol yield of 0.3±0.013 (g/g) was obtained with initial pH- 5.5 using xylose as carbon source.

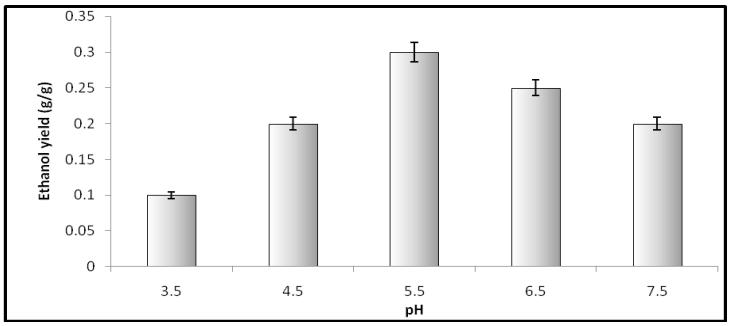

Fig. 3: Effect of pH on ethanol production by *C. intermedia* MTCC-1404

Effect of temperature on ethanol production by *Candida intermedia* MTCC-1404

The effect of temperature on the ethanol production was carried out at the level of 20-40 ^{0}C. The results are shown in Figure 4. The optimum temperature for maximum ethanol yield (0.3±0.012g/g) was found at to be 30 ^{0}C and an increase in the temperature, the ethanol yield was increased upto 30°C and further increase in temperature decreased the ethanol yield.

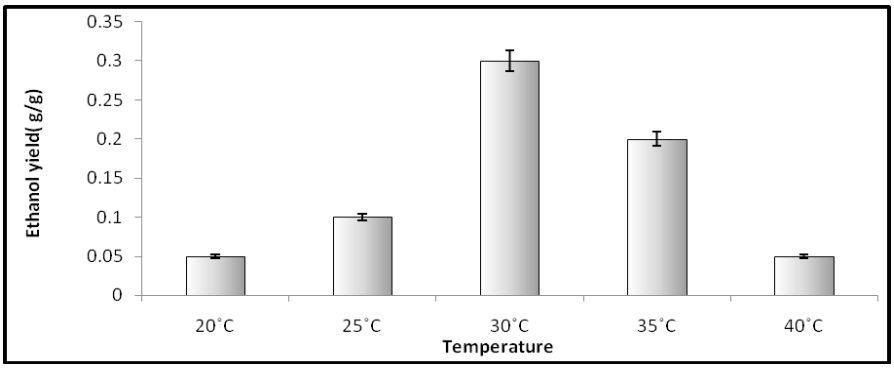

Fig. 4. Effect of temperature on ethanol production by *C. intermedia* MTCC-1404

Effect of different concentration of xylose on ethanol production by *C. intermedia* MTCC-1404

The effect of xylose concentration ranging from 3-7 % was studied for maximum ethanol production. The results were shown in Figure 5. The ethanol yield of 0.36±0.016 g/g was maximum consistent up to 5% xylose concentration thereafter it declined constantly.

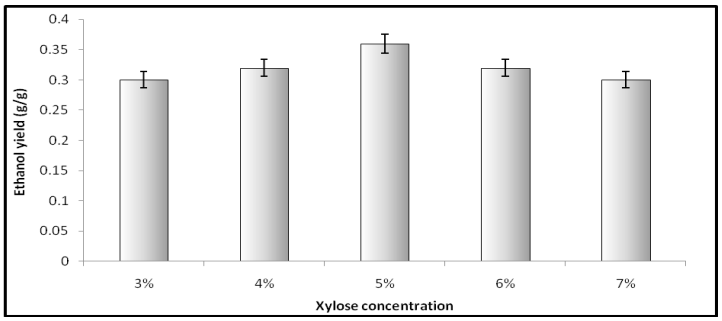

Fig. 5. Effect of different concentration of xylose on ethanol production by *C. intermedia* MTCC-1404

Effect of agitation on ethanol production by *Candida intermedia* MTCC-1404

Ethanol production was carried out in static and shaking condition. Optimization of agitation was performed by varying the agitation speed in the range between 100 to 250 rpm. Agitation at 150 rpm resulted in higher yield of ethanol i.e. 0.36±0.015 g/g when compared with static mode (Figure 6) .

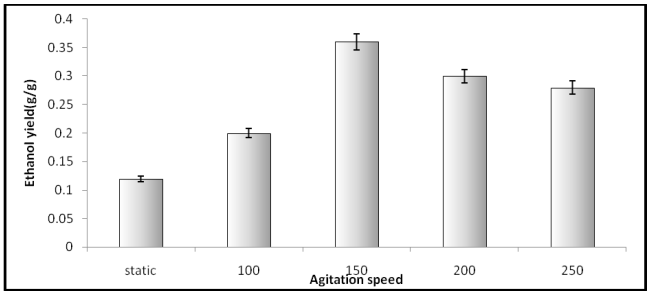

Fig. 6. Effect of agitation speed on ethanol production by *C. intermedia* MTCC-140

Effect initial glucose concentration on ethanol production by *C. intermedia* MTCC-1404

The effect of initial glucose concentration ranging from 0.2-0.5% was studied for maximal ethanol production. A typical profile for consumption of different glucose concentration is shown in (Figure 7). The higher yield of ethanol i.e. 0.38 ± 0.0162 g/g was obtained at 0.2%

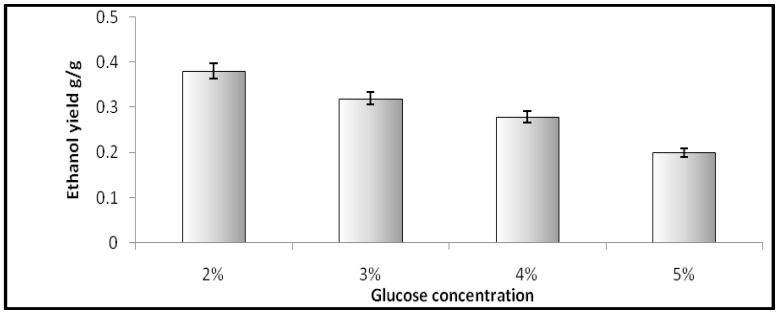

Fig. 7. Effect of different concentration of glucose on ethanol production by *Candida intermedia* MTCC-1404

concentration of glucose.

Effect of different inoculum size on ethanol production by *C. intermedia* MTCC-1404

The effect of different inoculum size on ethanol production was studied to select the best inoculum size for maximum ethanol production. There was a regular increase in ethanol production with the increase in size from 5-15 and thereafter it declined. The maximum ethanol yield 0.4±0.014 g/g was recorded at 15% of inoculum size. (Figure 8)

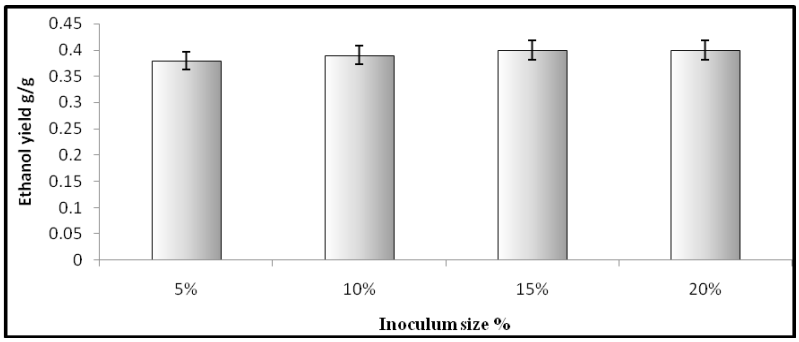

Fig. 8. Effect of different inoculum size on ethanol production by *C. intermedia* MTCC-1404

Effect of fermentation time on ethanol production by *Candida intermedia* MTCC-1404

The effect of fermentation time on ethanol production was studied to know the optimum time for maximum ethanol production. There was a regular increase in ethanol production up to 48h; further increase in the fermentation time decreased the ethanol production. Maximum ethanol of 0.4±0.012 g/g was produced at 48h (Fig. 9).

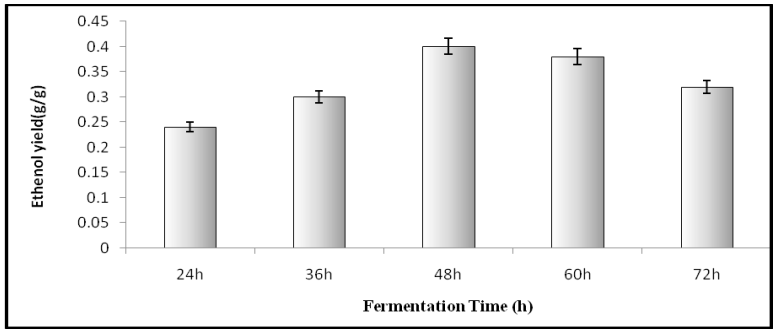

Fig. 9. Effect of fermentation time on ethanol production by *Candida intermedia* MTCC-1404

In the present work, optimum pH and temperature were found to be 5.5 and 30°C. With more than these levels ethanol productivity decreased. Membre *et al*.,[18] reported that generally low pH and high sugar concentration is adverse for the bacterial growth hence bacterial contamination could be reduced**.** Maximum ethanol yield of 0.3±0.013 g/g was obtained with initial pH- 5.5. Abbi *et al*., [19] mentioned that at low temperature, the decline in ethanol production was probably due to inactivation of cellular activities and at higher temperatures the enzymatic reactions in the cell are destroyed these results are supporting the present work result. In the present study, maximum ethanol yield 0.36±0.016 g/g obtained was consistent up to 5% xylose concentration thereafter it declined constantly. According to Nigam[20], this is probably because of higher sugar concentration more ethanol was produced which limits ethanol tolerance of the yeast. Kumar[17] reported 6% xylose concentration for maximum ethanol productivity of *P. stipitis*, which is comparable with the current study.

Statistical evaluation of parametric optimization data

To assess whether fermentation parameters have any significant effect on ethanol production, ANOVA analysis was carried out using Graphpad software. The results infer that all the parameters have significant effect on ethanol production (Table 3), all the F-values yielded were positive ($p<0.05$), among all parameters temperature with F-value of 576.98 and R square of 0.99569 showed more significant effect and initial glucose with F-value of 10.279 and R square of 0.80436 showed least significant effect.

Table 3. Statistical evaluation of parametric optimization data -ANOVA

Parameters	Lower	Upper	Difference	R square	F-value	P-value	Sig ?Y/N
pH	0.1	0.3	0.2	0.98534	168	P < 0.0001	Y****
Temperature	0.05	0.3	0.25	0.99585	600.26	P < 0.0001	Y****
Concentration of xylose	0.3	0.36	0.06	0.77558	8.6401	P = 0.0028	Y**
Agitation	0.12	0.36	0.24	0.98853	215.41	P < 0.0001	Y****
Concentration of glucose	0.2	0.38	0.18	0.97198	92.491	P < 0.0001	Y****
Inoculum size	0.38	0.4	0.06	0.24836	0.88112	P = 0.4906	N
Time	0.24	0.4	0.16	0.97161	85.555	P < 0.0001	Y****

CONCLUSION

Three different pentose utilizing yeasts *C. tropicalis* Y-27405, *C.intermedia* MTCC-1404 and *C. tropicalis* Y-1552) were examined to select the best one based on their ethanol production and their ability to utilize maximum sugar *C. intermedia* MTCC-1404 was found to be best organism for ethanol production. Parametric optimization studies were carried out to know the most influential range of parameters like pH, temperature, agitation, concentration of xylose, initial glucose concentration, inoculum size, fermentation time and ethanol production by for *C. intermedia* MTCC-1404. The yield of ethanol obtained under optimal condition at pH 5.5, 30°C, 150 rpm, 5 percent xylose, 0.2 percent initial glucose, 15percent inoculum size and 48h was 0.4±0.012 g/g.

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