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Research Article

Process optimization, enhancement and production of Pyruvate decarboxylase by *Hansenula polymorpha*

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Abstract

The value of enzymes as analytical tools and as industrial catalysts is often limited by their requirements for mild storage and reaction conditions as well as their poor operational or long-term stability in some cases. Time course studies and kinetic characterizations using shake flasks marked the PDC producing potential of the strain. The enhancement in PDC production fell in the recommended range (2-3 folds) for industrial use of the yeast culture. Growth curves of *Hansenula polymorpha-CNC 1* were studied using different concentration changes in media (pH 5.0) at 34^0C. Comparatively higher values of Pyruvate decarboxylase were found in whole cell PDC for (Shake flask 2.36±0.16 U/mg protein).Maximum Pyruvate decarboxylase production the results of glucose (7.25 g/100 ml), the results of (0.90g/100 ml) the results of Malt extract (7.25 g/100 ml) the results of $(NH_4)_2SO_4$ (1.20 g/100 ml) the results of Na_2PO_4 (0.60 g/100 ml) the results of $CaCl_2$ (0.060 g/100 ml) the results of Agitation (250 rpm) . An increase or decrease in the values of process parameters resulted in the decline of maximum production.

Keywords: Biotransformation, Pyruvate decarboxylase, *Hansenula polymorpha*.

Introduction

The use of biocatalysts in biotransformations minimizes many problems encountered in chemical processes. Biocatalysts can be used in simple and complex transformations without the need for tedious blocking and deblocking steps that are common in organic synthesis (**Dordick *et al.*, 1998**). In addition, close to quantitative substrate conversion can be achieved under milder condition than those of chemical processes (**Schmid *et al.*, 2001**). Biocatalysts are considered as environmentally friendly alternatives to chemical catalysts as they are biodegradable and meet the requirements of efficient reactions with fewer by-products.

PDC is currently industrially produced using yeast culture fermentation by the biotransformation of benzaldehyde, giving industrial relevance to research into whole cell biotransformation for PDC production. Both active microbes as well as purified enzymes can be used as biocatalysts, with whole cell biotransformation remaining more industrially appealing. Whole microbes contain the cellular machinery and enzymes required to perform complex biotransformations. Glycolysis in yeast generates pyruvate from glucose. Under aerobic conditions, pyruvate can be further utilized in the Citric Acid Cycle, but anaerobic conditions cause the accumulation of pyruvate and PDC. When benzaldehyde is added to the system, PDC catalyzes the reaction of benzaldehyde and pyruvate to PAC.

The primary goal of the present work was to evaluate different Pyruvate Decarboxylase preparations in *Hansenula polymorpha*.The criteria used for these evaluations are cultivation conditions for optimum production of Pyruvate dercarboxylase and study of process parameters for biotransformation.

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Materials and Methods

Isolation of yeast

Wild yeast strains were isolate from sugar mill process streams. Collected Sugar cane juice (10ml) was added into 250 m L conical flasks containing 90 ml sterile distilled mixed to prepare homogenous suspension.The suspension was further diluted upto $10^{-7,}$ 0.5 ml of the dilution was transformed to a separate petriplates containing YEMA medium (Yeast Extract 0.4g, Dextrose 0.4g, Agar 1.75 g, Malt Extract 1 g, to make up to 100 ml pH 5.5. The obtained yeast colonies were further purified by streaking on pertriplates containing the YEMA medium.

Screening of yeast isolate

Primary screening

The yeast strains were primarily screened for acetaldehyde tolerance after (**Agustin** and **Marcel, 2004)**.

Secondary screening

The cultures obtained after acetaldehyde-based screening was further tested by secondary screening in terms of Pyruvate decarboxylase production using submerged fermentation in 1L Erlenmeyer flask (**Shukla *et al.*, 2001**). The cells were allowed to adapt the medium for one hour in a rotary shaker incubator at 300 rpm. Five sequential doses (each 1.20 ml/l acetaldehyde + 1.20 ml/l benzaldehyde) were added after 1.0 h intervals. The process was terminated after 1.0 h of last dose and broth was used for further analysis.

Identification of the selected yeast isolate

Morphological characterization

According to the method of (**Kreger-van Rij, 1984)** and (**Kurtzman** and **Fell, 1997)**, the morphology of the vegetative cells was grown in liquid and on solid media.

Biochemical characterization

API biochemical strip was used to investigate the biochemical reaction. This strip consists of 10 tubes containing dehydrated substrates, which enable the performance of 12 identification tests (sugar acidification of enzymatic reactions). The reactions produced during incubation were revealed by spontaneous color changes. After 24 h of incubation at 37 °C, the reactions were read visually according to the reading table and identification was obtained by using the identification software.

Molecular characterization

The genotypic identification of yeast (CNC-1) was conducted by reading of the nucleotide sequence of DNA encoding 18S rRNA (rDNA). The rDNA was amplified using universal fungal primers , forward primer, NS1 (5'-GTAGTCATATGCTTGTCTC-3') and reverse primer,NS2 (5'-GGCTGCTGGCAC CAGACTTGC-3') **(White *et al.*, 1990)**. Nucleotide sequences were analyzed. All the sequences were compared for their similarity with reference yeast strains by a BLAST search.

Sequence analysis

The sequence data was compared to gene libraries (GenBank) with BLAST and FASTA programs. Multiple sequence alignments of translated gene sequence were carried out with the program CLUSTALW (version 1.83). Genetic distances were estimated by using the Maximum parsimony with bootstrap method employed by PHYLIP (**Felstein, 1993**). The tree was displayed with the TREEVIEW program.

Selection and maintenance of isolate

Glycerol stocks were prepared and stored at -80^{0}C for long term preservation. Pure culture (CNC-1) was incubated at 38^{0}C for 72 h in isolation broth. Then 0.5 ml of each of the culture (CNC-1) was transferred into cryotubes and 0.5 ml broth containing 40% glycerol was added. The samples were mixed gently and stored at 80°C for further uses.

Optimization process for the Pyruvate Decarboxylase synthesis

Different concentration of Growth medium were prepared and inoculated the yeast (CNC-1) strain in conical flask separately, incubated in the room temperature for 48-72 hrs and measure the optical density at 660 nm. The organism was grown in selective

growth medium by varying Glucose, Yeast extract, Malt extract, pH, Temperature, Agitation, $(NH_4)_2SO_4$, KH_2PO_4, Na_3PO_4, $CaCl_2$ by changing one factor at a time keeping the other factors constant. (**Rosche *et al.*, 2002**)

Fermentation Process Conditions

Pyruvate Decarboxylase enzyme was produced via a yeast fermentation of glucose-based optimized media. Various scales and methods of fermentation were employed: (I) 0.5 L shake flask fermentations to culture of *Hansenula polymorpha*.

Enzyme assays

Extraction of enzymes from cells

Cells were recovered from 5 ml of the culture broth, washed with 30 mM tris (hydroxymethyl) aminomethane buffer (pH 6.5) and weighed. The cells were suspended in the chilled buffer and added glass beads (0.5 mm diameter) in the ratio of 3 g/g biomass. The mixture was vortexed at maximum speed for 5 min. For every 30 seconds of vortexing, the sample was cooled for 1 min in an ice bath. Cell debris was removed by centrifugation and the supernatant was collected for enzyme assays and proteins estimation.

Pyruvate decarboxylase

Sodium citrate buffer (0.02M, pH 6.0) 950 µl, NADH+, Na salt (0.03M, 10 mg/ml) 10 µl, sodium pyruvate (0.03M, 100 mg/ml) 32 µl and alcohol dehydrogenase (10 KU/l, 10 mg/ml of ammonium sulphate) 03 µl were pipetted out in a glass cuvette (light path length, λ = 1.0 cm). The cuvette was incubated in the spectrophotometer at 25°C for 5 min to achieve temperature equilibration and then recorded absorbance at 340 nm (blank). The reaction was initiated by adding 5 µl of enzyme sample to the cuvette. The decrease in absorbance was recorded for 5 min and used to calculate the ΔE340nm /min. Specific PDC activity was determined using the same formula as previously applied for ADH assay. One unit of enzyme activity is defined as that activity which converts 1.0 µ mole of pyruvate to acetaldehyde/min at 25ºC and pH 6.0.

Results

Screening of yeast strains

Wild yeast strains were isolated from sugar mill process streams. A total of twenty eight dominative isolated from the samples of sugarcane juices were collected in different area. Amongst the isolates sixteen was absence of grow on acetaldehyde (1.5 g/l) added YEMA plates during primary screening in Table 4.1. Out of remaining 13 isolates (acetaldehyde tolerant), 4 gave almost negligible amounts of Pyruvate decarboxylase production (≤0.6 g/l) in glucose/peptone medium using shake flasks fermentation, respectively. Four dominative isolates were producing Pyruvate decarboxylase, the product in the range of 0.02- 0.80g/l in Table 4.2. However, sugar consumption and dry biomass ranged from 1.64-2.74 and 0.24-0.82 g/l, respectively. The Isolate of CNC-1 produced higher Pyruvate decarboxylase (0.80g/l).

Table 4.1. Isolation sample and primary screening of Acetaldehyde tolerant

S.No	Isolation Samples	Absence of Acetaldehyde tolerant	Presence of Acetaldehyde tolerant
		Yeast isolate - CNC	
1	**SCJ-** 1	CNC-2, CNC-4, CNC-5	CNC-1, CNC-3
2	**SCJ** -2	CNC-9, CNC-12, CNC-13, CNC-14	CNC-6,CNC-7, CNC-8, CNC-10, CNC-11
3	**SCJ** -3	CNC-17, CNC-18, CNC-20	CNC-15, CNC-16, CNC-19
4	**SCJ** -4	CNC-21, CNC-22, CNC-23	CNC-24
5	**SCJ** -5	CNC-25, CNC-26, CNC-27	CNC-28

Table 4.2. Screening of dominative Pyruvate decarboxylase producing Yeast strain in glucose medium using SSF

S.No	Isolation sample	Yeast isolate	Glucose consumed (g/l)	Dry biomass formed (g/l)	Pyruvate decarboxylase (g/l)
1.	**SCJ-** 1	CNC-1	1.81±0.09	0.82±0.08	0.80±0.08
2.		CNC-3	1.95±0.15	0.34±0.03	0.20±0.10
3.	**SCJ-** 2	CNC-6	1.78±0.2	0.38±0.12	0.16±0.08
4.		CNC-7	1.99±0.18	0.32±0.03	0.16±0.08
5.		CNC-8	1.94±0.15	0.24±0.07	0.16±0.11
6.		CNC-10	1.72±0.07	0.60±0.09	0.58±0.15
7.		CNC-11	1.92±0.18	0.28±0.05	0.65±0.06
8.		CNC-12	2.71±0.18	0.34±0.04	0.16±0.12
9.	**SCJ-** 3	CNC-15	1.87±0.23	0.29±0.03	0.09±0.08
10.		CNC-16	2.27±0.20	0.46±0.06	0.02±0.10
11.		CNC-19	1.79±0.15	0.45±0.05	0.12±0.13
12.	**SCJ-** 4	CNC-24	1.64±0.09	0.37±0.08	0.16±0.12
13.	**SCJ-** 5	CNC-28	2.27±0.27	0.37±0.05	0.42±0.12

Table 4.3. Comparison of morphological and cultural characteristics of yeast strain CNC-1

Characteristic	Character	Yeast strain
A. Morphological on		
YPD **broth**	Cell size	5×9 mμ
	Cell shape	Oval
	Cell Arrangement	Single, pair or group
YPD **agar**	Cell size	5×9 mμ
	Cell shape	Oval
	Cell	white
B. Cultural Streak culture	Arrangement	
	Colony color	white
	Appearance	Shiny
	Texture	Soft, smooth
	Margins	Wrinkled
Slide culture	Pseudomycelium	present
Sporulation	Ascospores per ascus	4

Biochemical characterization of yeast strain of CNC-1

The yeast strain **CNC-1** was identified based on biochemical characterization by using API Candida strip. The result which was compared the similarity percentages of sugar acidification and/or enzymatic reactions patterns with reference strain of the APILAB Plus software (version 2.0) was demonstrated in Table 4.4.

Table 4.4. Biochemical characterization

Characteristic	Reference strain (*Pichia sp*)	Yeast strain CNC-1
Sugar acidification		
1. Glucose	+	+
2. Galactose	-	-
3. Saccharose	-	-
4. Trehalose	-	-
5. Raffinose	-	-
Enzymatic reactions		
6. beta-Maltosidase	-	-
7. alpha-Amylase	-	-
8. beta-Xylosidase	-	-
9. beta-Glucuronidase	-	-
10. Urease	-	-
11. N-Acetyl-beta-glucosaminidase	-	-
12. beta-Galactosidase	-	-

Symbol: +, positive; -, negative

18S rDNA sequence analysis

The partial sequence of 18S rDNA of the yeast strain CNC-1 as shown in Figure 4.1 was obtained from the sequence analysis, and compared with other 18S rDNA sequences of yeasts from GenBank database. When examined by BLAST similarity analysis, the 18S rDNA sequence from S1 was produced closely related with *Hansenula polymorpha*. The result showed that the CNC-1 strain has 99.5% similarity of 18S ribosomal RNA gene sequence with *Hansenula polymorpha.*

1 TTTCCTCAGT CCGTTTAGTA TTTACATTGT ACTCATTCCA ATTACAAGAC
51 CAAAGGCCCT GTATCGTTAT ATATTGTCAC TACCTCCCTG TGTCAGGATT
101 GGGTAATTTG CGCGCCTGCT GCCTTCCTTG GATGTGGTAG CCGTTTCTCA
151 GGCTCCCTCT CCGGAATCGA ACCCTTATTC CCCGTTACCC GCGAAAACCA
201 TGGTGGGACA CTATTATATA TCACTAGCTG ATGATGACAA ATTATATCTT
251 TTTATGCACC ATCTGCCGTC CCCCGGTCCT GTTATTTCAC AAATTATCAT
301 TAATTACCTT TATACCGGTC CACCCCATAA TCATTCTTTC ACTCCATAAA
351 TGCCCCCCTT CTAGAGGTCA GATAACGCGC CACTCCTCTA TAATTTACAC
401 TCTCTATTGC CCATCTATAC TAGCTAGCGT ACCACTTACT GAAACCCCCC
451 CCCAATTTCA CTCTTCCATC CCTCTTTTTC CTCCCCATAC ATTCAACACA
501 TACCTTATAC ATTGTTATAG TTATACCTCT ATTCCCCATC TTTTCTCTTT
551 CAACCAACAA CCTCCAAAAC ATCTTTTTAT CCCCTTTTAT AATTCATCAT
601 ACTCCCCCCC TTCACATCCC CACACTACAC AAAAGCCTAA TTCTTTAATT
651 TTACCCCTTC CACATTTATC ACCACCACCA ACCTCCCCTT ATAAAAACCA
701 ATAAAATAAT ACCCCCACTT ATTATATCCT CACCACACCC TAATTTCCAC
751 CCCCCCTTTC ACCTTAATAC TATATAACCT CTACTACGCC CTTTATCTC

Figure 4.1. Partial 18S rDNA sequence of the yeast strain CNC-1

Query 18 GTATTTACATTGTACTCATTCCAATTACAAGACCAAAGGCCCTGTATCGTTATATATTGT 77
Sbjct 365 GTATTTACATTGTACTCATTCCAATTACAAGACCAAAGGCCCTGTATCGTTATATATTGT 306
Query78 CACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGCCGCTGCTGCCTTCCTTGGATGT 137
Sbjct305 CACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGCCGCTGCTGCCTTCCTTGGATGT 246
Query128TAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTTATTCCCCGTTACCCGCGAAAA 1 97
sbjct 245 TAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTTATTCCCCGTTACCCGCGAAAA 186
Query 198 CCATGGT 204
Sbjct 185 CCATGGT 179

Figure 4.2. 18S rDNA of CNC-1 which was performed in the GenBank data library by using Basic Local Alignment Search Tool program.

Effect of composition of growth-cum-fermentation medium

Effect of initial pH

Comparison in product formation and initial pH (3.0-13.0) of the fermentation medium was studied. The data of the results is highlighted in Table 4.5. Significantly ($p \leq 0.05$) higher value of Pyruvate decarboxylase (0.68 U/mg proteins) was found at pH 5.0. The concentrations decreased gradually at lower or higher pH values. Sp. Growth rate increased with increase in pH, becoming maximal (0.25 μ (h-1)) at pH 5.0. The maximal concentration of Dry biomass (13.0g/l) was observed at pH 5.0.

Table 4.5. Effect of pH

Component P^H	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. Growth rate, μ (h-1)	Product Specific enzyme activities (U/mg proteins)
			Pyruvate decarboxylase
3	5.25±0.15	0.11	0.20±0.05
5	13.0±0.18	0.25	0.68±0.02
7	10.0±0.10	0.21	0.10±0.07
9	12.20±0.25	0.23	0.13±0.05
11	8.10±0.20	0.18	0.30±0.05
13	6.0±0.22	0.13	0.18±0.02

Effect of incubation temperature

The effect of incubation temperature on product formation was investigated. The results are given in Table 4.6. A significantly higher ($p \leq 0.05$) Pyruvate decarboxylase production (0.92 U/mg proteins) was achieved at 34°C. The product however, steadily decreased at a higher or lower temperature other than the optimal. Similarly higher concentration of Dry biomass (18.0 g/l) was found. However, the concentration of Sp. Growth rate increased to a maximal amount of 0.36 μ (h-1).

Table 4.6. Effect of incubation temperature

Component Temperature	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. Growth rate, μ (h-1)	Product Specific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
4^0C	7.14±0.26	0.15	0.12±0.07
14^0C	10.10±0.25	0.20	0.16±0.02
24^0C	13.20±0.20	0.24	0.30±0.07
34^0C	18.0±0.25	0.36	0.92±0.15
44^0C	6.11±0.20	0.13	0.80±0.05

Effect of agitation

The effect of agitation rate (50-250 rpm) on Pyruvate decarboxylase production was observed in a Shake flask broth. The results are shown in Table 4.7. Maximal Pyruvate decarboxylase (0.94 U/mg proteins) accompanied by Dry biomass (14.16 g/l) and Sp. Growth rate (0.34 μ (h-1)) was noticed at 250 rpm. Whereas, the second highest Pyruvate decarboxylase production (0.49 U/mg proteins) was found at 200 rpm. However, Dry biomass and Sp. Growth rate were found to be 0.28 μ (h-1) and 13.0g/l, respectively. The optimized conditions i.e. 250 rpm were applied in the subsequent studies.

Table 4.7 Effect of agitation

Component Agitation rpm	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. growth rate, μ (h-1)	Product Specific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
50	7.14±0.26	0.12	0.14±0.37
100	10.10±0.83	0.19	0.19±0.72
150	9.19±0.75	0.15	0.32±0.12
200	13.0±0.25	0.28	0.49±0.25
250	14.16±0.10	0.34	0.94±0.57

Comparison in different concentration of glucose in growth media was investigated with reference to cell growth rate, enzymes activities and subsequent product formation during selection of a growth cum fermentation medium. During seed development, The effect of glucose concentration (1.25-13.25 g/100 ml). The results are shown in Table 4.8. Comparatively higher values of optimum sp growth rate (0.36 h-1) were found in YEM medium. Significantly ($p \leq 0.05$) higher value of Pyruvate decarboxylase (0.93 U/mg protein) and Dry biomass (18.0 g/l) were also found in the same YEM .

Table 4.8. Effect of different concentration of glucose

Component (w/v) Glucose	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. growth rate, μ (h-1)	Product Specific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
1.25	7.14±0.26	0.11	0.52±0.07
3.25	10.10±0.25	0.20	0.62±0.02
5.25	13.20±0.20	0.24	0.68±0.07
7.25	18.0±0.25	0.36	0.93±0.05
9.25	6.11±0.20	0.13	0.80±0.05
13.25	7.14±0.26	0.15	0.30±0.07

Selection of Yeast extract

Comparison in different concentration of Yeast extract in growth media was investigated with reference to cell growth rate, enzymes activities and subsequent product formation during selection of a growth cum fermentation medium. During seed development, the effect of Yeast extract concentration (0.10-1.50 g/100 ml).The results are shown in Table 4.9. Comparatively higher values of optimum sp growth rate (0.34 h-1) were found in YEM medium. Significantly ($p \leq 0.05$) higher value of Pyruvate decarboxylase (0.63 U/mg protein) and Dry biomass (14.0 g/l) were also found in the same YEM.

Table 4.9. Effect of different concentration of Yeast extract

Component (w/v) Yeast extract	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. growth rate, μ (h-1)	Product Specific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
0.10	3.14±0.16	0.05	0.10±0.07
0.30	6.10±0.15	0.10	0.12±0.02
0.60	11.20±0.10	0.14	0.13±0.07
0.90	14.0±0.25	0.34	0.63±0.05
1.20	6.11±0.10	0.23	0.50±0.05
1.50	3.14±0.16	0.15	0.48±0.07

Selection of Malt extract

Comparison in different concentration of Malt extract in growth media was investigated with reference to cell growth rate, enzymes activities and subsequent product formation during selection of a growth cum fermentation medium. During seed development, the effect of Malt extract concentration (1.25-13.25 g/100 ml).The results are shown in Table 4.10. Comparatively higher values of optimum sp growth rate (0.36 h-1) were found in YEM medium. Significantly ($p \leq 0.05$) higher value of Pyruvate decarboxylase (0.65 U/mg protein) and Dry biomass (11.0 g/l) were also found in the same YEM.

Table 4.10. Effect of different concentration of Malt extract

Component (w/v) Malt extract	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. growth rate, μ (h-1)	Product Specific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
1.25	1.14±0.22	0.15	0.19±0.01
3.25	4.10±0.21	0.20	0.32±0.02
5.25	3.20±0.02	0.24	0.40±0.07
7.25	11.0±0.25	0.36	0.65±0.01
9.25	6.11±0.10	0.13	0.60±0.04
13.25	7.14±0.16	0.15	0.50±0.01

Selection of $(NH_4)_2SO_4$

Comparison in different concentration of $(NH_4)_2SO_4$ in growth media was investigated with reference to cell growth rate, enzymes activities and subsequent product formation during selection of a growth cum fermentation medium. During seed development, the effect of $(NH_4)_2SO_4$ concentration (0.10-1.50g/100ml). The results are shown in Table 4.11. Comparatively higher values of optimum sp growth rate (0.24 h-1) were found in YEM medium. Significantly ($p \leq 0.05$) higher value of Pyruvate decarboxylase (0.70 U/mg protein) and Dry biomass (2.71 g/l) were also found in the same YEM .

Table 4.11. Effect of different concentration of $(NH_4)_2SO_4$

Component (w/v) $(NH_4)_2SO_4$	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. growth rate, μ (h-1)	Product Specific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
0.10	1.14±0.26	0.20	0.63±0.02
0.30	1.80±0.25	0.21	0.65±0.03
0.60	2.0±0.20	0.21	0.52±0.04
0.90	2.11.±0.25	0.23	0.63±0.03
1.20	2.71±0.20	0.24	0.70±0.05
1.50	2.14±0.26	0.23	0.61±0.03

Selection of Na_3PO_4

Comparison in different concentration of Na_3PO_4 in growth media was investigated with reference to cell growth rate, enzymes activities and subsequent product formation during selection of a growth cum fermentation medium.The results are shown in Table 4.12. Comparatively higher values of optimum sp growth rate (0.24 h-1) were found in YEM medium. Significantly ($p \leq 0.05$) higher value of Pyruvate decarboxylase (0.72 U/mg protein) and Dry biomass (2.91 g/l) were also found in the same YEM .

Table 4.12. Effect of different concentration of Na_3PO_4

Component (w/v) Na_3PO_4	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. growth rate, μ (h-1)	ProductSpecific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
0.05	1.24±0.13	0.18	0.53±0.12
0.15	1.50±0.15	0.19	0.55±0.13
0.30	2.12±0.20	0.21	0.51±0.04
0.45	2.31.±0.15	0.22	0.62±0.13
0.60	2.91±0.10	0.24	0.72±0.15
0.75	2.44±0.16	0.21	0.61±0.03

Selection of KH_2PO_4

Comparison in different concentration of KH_2PO_4 in growth media was investigated with reference to cell growth rate, enzymes activities and subsequent product formation during selection of a growth cum fermentation medium.The results are shown in Table 4.13. Comparatively higher values of optimum sp growth rate (0.24 h-1) were found in YEM medium. Significantly ($p \leq 0.05$) higher value of Pyruvate decarboxylase (0.72 U/mg protein) and Dry biomass (2.01 g/l) were also found in the same YEM.

Table 4.13. Effect of different concentration of KH_2PO_4

Component (w/v) KH_2PO_4	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. growth rate, μ (h-1)	Product Specific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
0.05	1.04±0.13	0.20	0.51±0.12
0.15	1.10±0.15	0.21	0.65±0.13
0.30	1.12±0.20	0.22	0.61±0.04
0.45	2.01.±0.15	0.27	0.72±0.13
0.60	1.91±0.10	0.24	0.70±0.15
0.75	1.44±0.16	0.22	0.64±0.03

Selection of $CaCl_2$

Comparison in different concentration of $CaCl_2$ in growth media was investigated with reference to cell growth rate, enzymes activities and subsequent product formation during selection of a growth cum fermentation medium. During seed development, the effect of $CaCl_2$ concentration (0.005-0.075g/100 ml).The results are shown in Table 4.14. Comparatively higher values of optimum sp growth rate (0.25 h-1) were found in YEM medium. Significantly ($p \leq 0.05$) higher value of Pyruvate decarboxylase (0.69 U/mg protein) and Dry biomass (2.41 g/l) were also found in the same YEM.

Table 4.14 Effect of different concentration of $CaCl_2$

Component (w/v) $CaCl_2$	Growth phase		Fermentative phase
	Dry biomass formed (g/l)	Sp. growth rate, μ (h-1)	Product Specific enzyme activities (U/mg protein)
			Pyruvate decarboxylase
0.005	1.0±0.11	0.15	0.41±0.01
0.015	1.30±0.16	0.16	0.45±0.02
0.030	1.42±0.10	0.18	0.51±0.03
0.045	1.81.±0.25	0.21	0.52±0.06
0.060	2.41±0.19	0.25	0.69±0.05
0.075	1.24±0.11	0.22	0.54±0.08

Optimization of preculture

Growth curves of *Hansenula polymorpha-CNC 1* were studied using different concentration changes in media (pH 5.0) at 34^0C. Comparatively higher values of Pyruvate decarboxylase were found in whole cell PDC for (Shake flask 2.36±0.16 U/mg protein), (Fermentor 2.94±0.11 U/mg protein). The results of cell growth at different pH (5.0) and temperatures (34^0C) are depicted in Table. Maximum Pyruvate decarboxylase production the results of glucose (7.25 g/100 ml), the results of (0.90g/100 ml) the results of Malt extract (7.25 g/100 ml) the results of $(NH_4)_2SO_4$ (1.20 g/100 ml) the results of Na_2PO_4(0.60 g/100 ml) the results of $CaCl_2$ (0.060 g/100 ml) the results of Agitation (250 rpm) . An increase or decrease in the values of process parameters resulted in the decline of maximum production.

Table 4.15 Optimization of fermentation conditions

Component Pyruvate decarboxylase Extract	Fermentative phase Specific enzyme activities (U/mg protein) Pyruvate decarboxylase
Partially purified PDC	1.89±0.13
Crude PDC	1.23±0.13
Whole cell PDC	2.36±0.16

- Each value is an average of three parallel replicates. ±SD indicates standard deviation from the mean value.

Discussion

The influence of the difference in PDC preparations on production of phenylacetylcarbinol for a partially purified, a crude extract and a whole cell PDC preparation. According to **Pamment *et al.*,** (**1997**), added acetaldehyde may react with ingredients of a medium making it nonavailable to the microorganisms. In this study the isolate CNC-1 with hyper pyruvatedecarboxylase producing ability seemed to be suitable with reference to the both, genetic as well as physiological aspects.

In this study the yeast strain **CNC-1** was identified based on biochemical characterization by using API Candida strip. The result which was compared the similarity percentages of sugar acidification and/or enzymatic reactions patterns with reference strain. The reactions produced during incubation were revealed by spontaneous color changes. After 24 h of incubation at 37°C, the reactions were read visually and *Pichia sp* is considered to represent the anamorphic form of *Hansenula polymorpha* because the type strains show significant. Asci are persistent and form 4 spheroidal ascospores, ovoidal to elongate, (2.7-4.2) x (5.6-10.1) µm, single or in pairs, budding cells are present and pseudomycelia are developed. All ascospores observed were reniform and aggregated upon release from asci ; four ascospores were produced per ascus. The asci ruptured very readily, so much *so* that quite often asci were not seen when large numbers of ascospores were detected.

In previous study the spore viability of diploids was high and facilitates efficient tetrad and random spore analysis. Tetrad analysis is possible for *H. polymorpha* CBS4732 and has been used successfully to study the mechanisms of plasmid integration **(Bogdanova *et al.*, 1995).** Some differences between sporulating cultures, originating from the CBS4732 and NCYC495 genetic background, probably exist. The ascus membrane of NCYC495 was shown to be resistant to enzymatic digestion. Furthermore, the strong adhesion between ascospores made their separation very difficult (**Gleeson** and **Sudbery, 1988**). We have not had these problems with CBS4732 ascospores, which could be easily detected without prior enzymatic treatment (**Bogdanova *et al.*, 1995**).

In previous studies of growth curves of mutant *S. cerevisiae* GCUN/V/N-1, a bit lengthy lag followed by stationary phase was observed in all the growth media (inoculums 1% v/v). A higher specific activity of PDC (0.65 U/mg protein) was found before fermentation when seed was developed using 10% (v/v) inoculum. The lenghthy lag phase was an indication of slow initial metabolic rate of the strain. The onset of stationary growth phase in growth curves might be the

consequence of depletion of sugars in the medium (**Murray** and **Phillips, 1982).** Furthermore, the use of 1% instead of 10% (v/v) inoculum (**Shukla** and **Kulkurni, 2002**) would result in higher induction of PDC and accumulation of pyruvate because of more cell growth recycling. The higher biomass formation (12.36 g/l) and specific growth rate (0.23 h-1) in urea/molasses medium (UMB) might be due to the readily available nutrients and better mass transfer rate. In the present study, a higher PDC induction using 10% (v/v) inoculum for seed development was on account of re-freshness of the cells.

In the studies effect of agitation rate maximal Pyruvate decarboxylase (0.94 U/mg proteins) accompanied by Dry biomass (14.16 g/l) and Sp. Growth rate (0.34 µ (h-1)) was noticed at 250 rpm. Whereas, the second highest Pyruvate decarboxylase production (0.49 U/mg proteins) was found at 200 rpm. However, Dry biomass and Sp. Growth rate were found to be 0.28 µ (h-1) and 13.0g/l, respectively. Time course studies and kinetic characterizations using shake flasks marked the PDC producing potential of the strain. The enhancement in PDC production fell in the recommended range (2-3 folds) for industrial use of the yeast culture. Moreover, growth-cum fermentation medium containing different concentration of growth medium were prepared and inoculated the yeast (CNC-1) strain in conical flask separately, incubated in the room temperature for 48-72 hrs and measure the optical density at 660 nm.

The organism was grown in selective growth medium by varying Glucose, Yeast extract, Malt extract, pH, Temperature, Agitation, $(NH_4)_2SO_4$, KH_2PO_4, Na_3PO_4, $CaCl_2$ by changing one factor at a time keeping the other factors constant. . The presence of cell membrane components contributed to the excellent performance of whole cell PDC in comparison to partially purified PDC. It was also apparent that surfactants, biologically occurring or synthetically manufactured, enhanced PDC.

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