Pak. J. Bot., 55(4): 1563-1568, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-4(35)

TORULASPORA DELBRUECKII: A NOVEL YEAST ISOLATE FOR L-PHENYLACETYLCARBINOL (L-PAC) PRODUCTION

ZAREENA MUSHTAQ AND HAMID MUKHTAR*

Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan
**Corresponding author; hamidwaseer@yahoo.com*

Abstract

The current study describes the biosynthesis of L-phenylacetylcarbinol (L-PAC) by *Torulaspora delbrueckii* using molasses in shake flasks. The yeast isolates were screened for temperature tolerance, solvent stress and carboligase activity. Solvent resistant and thermotolerant *Torulaspora delbrueckii* yeast was selected which was identified through sequencing of their rDNA R26, R18 and ITs regions. The incubation time of 24hrs, incubation temperature of 30°C, total sugar at 17% and pH of the medium at 5.5 with 10% inoculum size were found to be optimum for maximum production of L-PAC. Urea and Di-amonium Phosphate were selected as the best nitrogen sources for this purpose. The wild strain of *T. delbrukii* was subjected to random mutagenesis through UV, nitrous acid and ethyl methanesulfonate (EMS) treatment. Nitrous acid mutant treated for 40 min was selected after quantitative screening for further optimization. After mutagenesis, optimum pH and incubation time of *Torulaspora delbrueckii* were shifted to 5.0 and 18hrs while other parameters were same as for wild strain. After optimization of process parameters, nitrous acid mutant of *T. delbrukei* produced 7.99 g/l L-Phenylacetyl carbinol L-PAC as compared to 6.44 g/l of L-PAC from the wild strain. Hence, the yield of this strain was enhanced significantly after mutagenesis so the mutant strain of *Torulaspora delbrueckii* can be used in the industry for enhanced production of an important pharmaceutical ingredient, L-PAC.

Key words: Random mutagenesis, Carboligase activity, Pyruvate decarboxylate (PDC), Shake flask fermentation, Optimization.

Introduction

L-Phenylacetylcarbinol (L-PAC) is an intermediate for the commercial production of bronchial dilators and nasal decongestants known as pseudoephedrine and ephedrine. Asthma and chest congestion can also be treated with such alkaloids (Borchardt, 2003). They have a history, over the decades of use for medical purposes as they are effective in reliving symptoms of the flu, common cold, asthma, bronchitis and sinusitis. These chemicals have properties to stimulate central nervous system (CNS) and structural resemblance with amphetamine. Ephedrine and pseudoephedrine are taken as appetite reducers, and agents which eliminate fatigue and drowsiness to improve concentration. Their likely application in obesity control has been reported (Głowacka & Wiela-Hojeńska, 2021).

L-PAC is produced through a process called biotransformation catalyzed by the pyruvate decarboxylase (PDC), a thiamine pyrophosphate (TPP) and Mg^{2+} dependent enzyme. PDC in its natural function in ethanol fermentation, converts TPP bounded pyruvate to acetaldehyde. Simultaneously, acetaldehyde is released and converted to alcohols by alcohol dehydrogenase (ADH). PDC can ligate TPP bounded acetaldehyde to the added benzaldehyde to produce L-PAC. Active PDC is a tetramer consisted of two dimers. These four subunits are identical with a relative molecular mass of 60 kDa. Several yeasts like *Hansenula polymorpha*, *Hansenula anomola* (Suresh *et al*., 2009) *Candida pseudointermedia*, *Issatchenkia orientials*, *Candida pseudointermedia* (Kumar *et al*., 2006) and *Candida utilis* have been used for biosynthesis of L-PAC.

The benzaldehyde added in fermentation medium can be oxidized to benzoic acid or reduced to benzyl alcohol by alcohol dehydrogenase in yeast. Similarly, phenylacetylcarbinol may be reduced to 2-Phenyl-1, 3-propanediol (PAC-diol). This production of by products can exhaust substrates to reduce the yield of the L-phenylacetylcarbinol (US 2013/0309732A1). Therefore random mutagenesis of yeast cells was employed in present study to produce mutant yeast cells with desired properties which can consume substrates efficiently to produce higher amounts of L-PAC. This technique has been used to obtain genes in test tube through a repetitious process aggregating recombinant generation (Labrou, 2010).

Random mutagenesis is undoubtedly a directed evolution plan which induces point mutations randomly into whole genome of the microorganisms. It induces changes through five ways, which includes; (i) transitions, it involves substitution of a pyridine nucleotide by another pyridine or a purine by other purine, (ii) transversions, it involves substitutions of a pyrimidine by a purine. These replacements also include purine by pyrimidine. (iii) deletion, it involves deletion of one or more nucleotide from a genome, (iv) insertions, it involves addition of one or more nucleotide into the genome, (v) inversions, it involves the rotation of double stranded segment of DNA by 180°. This segment may consist of two or longer pair of bases. Combined with an efficient, sensitive screening method to select desired mutants, this technique is a successful strategy to solve many problems in protein engineering.

There are many procedures to produce genetic diversity by random mutagenesis. This can be done by treating yeast with different physical and chemical mutagens. Mostly chemical mutagens are azides and alkylating agents. Chemical and physical mutagenizing agents are considered as “food grade” because they don’t involve the introduction of heterologous DNA or manipulation by recombinant means. For example, nitrous acid (HNO_2) is a chemical mutagen. It causes deamination of cytosine and adenine residues resulting transversion point mutation (A=T to G≡C and G≡C to A=T). Ethyl methansulfonate (EMS) causes alkylation of guanidine

residues, which is incorrectly copied during DNA replication. EMS can be used *In vitro* to treat directly DNA or *In vivo* by using whole cells (Labrou, 2010).

X rays, particle radiation (fast and thermal neutrons), alpha particles, electromagnetic radiations and ultraviolet (UV) light all are physical mutagens (Kodym & Afza, 2003). Ultraviolet light makes precise mutations in the cell and causes triplet mutations (Ikehata & Ono, 2011).UV is one of the most commonly used and a well-known mutagenizing agent (Basavaraju *et al*., 2014). It causes all types of base pair replacements and provides high prepositions of pyrmidine dimmers (Rani *et al*., 2012). Due to ease of handling, low capital cost and various ways of evolution ultraviolet (UV) light, ethyl methansulfonate (EMS) and nitrous acid (HNO_2) were used to induce random mutagenesis in whole cells of *Torulaspora delbrueckii* for enhanced biosynthesis of L-PAC (phenyl acetyl carbinol) through Pryruvate Decarboxylase (PDC).

The main aim of the present research was to isolate a yeast strain with superior qualities such as resistant to solvent stress and high temperature and to enhance the L-PAC yield through random mutagenesis and optimization of fermentation parameters.

Materials and Methods

Isolation of yeasts: Fruit samples with damaged surfaces from different areas of Lahore were collected in sterilized zip lock bags. Samples were washed and rinsed with distilled water. They were then cut into small pieces which were placed on YPDA plates containing (g/L; yeast extract 10, peptone 7.5, glucose 7.5 and agar 2.0) supplemented with antibiotics (8µg /ml ampicillin) and incubated for 72hrs at 30^{o}C. The isolated yeast colonies were sub-cultured until the purified cultures were obtained and stored at 4^{o}C.

Screening of Yeast isolates for PDC production

Qualitative screening: Brady's reagent (2, 4-Dinitrophenylhydrazine abbreviated as 2,4DNPH) reacts with benzaldehyde to produce 2, 4-Dinitrophenyl-hydrazones which are orange in color. To develop a quick method for qualitative screening of L-PAC producing yeasts, 2, 4-dinitrophenylhydrazine (2,4DNPH) was used to detect the unreacted benzaldehyde in YPDA plates supplemented with pyruvate (100 mM), $MgSO_4$ (20 mM) and TPP (1mM) (Suresh *et al*., 2009). Brady's reagent was prepared by dissolving 3 g of 2,4-dinitrophenylhydrazine in a solution of 97 ml methanol and 3 ml concentrated sulfuric acid (Kadam *et al*., 2012).

Yeast isolates were cultured on screening plates at 30^{o}C for 48-72hrs. 3 ml Benzaldehyde was added in all the plates and incubated at 30^{o}C for 3 hrs. 3ml Braday's reagent was poured into the plates and re-incubated on the same temperature. Unreacted benzaldehyde reduces 2, 4-dinitrophenylhydrazine (pale yellow) into orange or red colored dinitrophenylhydrazone. Pale yellow color of Brady's reagent will not be changed if benzaldehyde is consumed to produce L-PAC.

Quantitative screening (Carboligase Assay): L-PAC produced was estimated through carboligase activity of PDC. Fermented broth with whole cells was incubated with 40 mM benzaldehyde and 100 mM pyruvate in carboligase buffer (50 mM MES/KOH, 1.5M Ethanol, 20mM $MgSO_4$, 1mM TPP, pH 6.0) in water bath for 30min at 30^{o}C. To stop the reaction, trichloroacetic acid (10%) was used to precipitate out the proteins including PDC. These proteins were removed by centrifugation at 6000 rpm for 15 min. L-PAC along with other products was quantified through Gas chromatography (Shimadzus QP 2010) as described by Rosche *et al*., (2002).

Screening of isolates for temperature and solvent stress: Yeast isolates were screened for acetaldehyde and benzaldehyde tolerance by method reported by Augustin & Marcel, (2004). YPDA plates were supplemented with 200µl of acetaldehyde and benzaldehyde mixtures (5, 10, 15 and 20%) and incubated at 4^{o}C overnight for maximum diffusion of solvents into the medium. Solvent screening plates were streaked with PDC (L-PAC) producing yeast isolates and incubated at 30^{o}C for 72hrs. Well grown colonies on screening plates were transferred on YPDA slants. For temperature tolerance study, YPDA plates streaked with yeast isolates were incubated at different temperatures (26, 30, 34, 38, 42 and 46^{o}C) for 72hrs (Tsegay, 2016). Colonies with characteristic growth were transferred on YPDA slants incubated at 30^{o}C for 72hrs**.**

Optimization of Process parameters: Nutritional parameters such as total sugar conc. (15-20%), organic nitrogen sources (Yeast extract, Meat extract, Beef extract and Casamino acid) and inorganic nitrogen sources ($(NH_4)_2SO_4$, KNO_3, NH_3NO_2 and $NaNO_3$) were optimized for L-PAC production. Physical parameters influencing the L-PAC production such as, fermentation time (12-72hrs), temperature (28-46^{o}C), initial pH of growth medium (4.5-6.5) and inoculum size (8-18 %) were also optimized for L-PAC production.

Twenty five mL of fermentation medium (total sugars 14%, Urea and $MgSO_4$ (0.1 %) pH adjusted with 1M) was transferred to 250 ml shake flask and inoculated with 8% of 24hrs old inoculum ($160x10^6$ cells/ml). The inoculated flasks were incubated at 180rpm, 30^{o}C to accomplish a cell count $120x10^6$cells/ml. At this stage, benzaldehyde was added into the fermentation medium in slots of 6-8 and the interval between the doses ranged from 15- 60min.

Toluene was used to extract L-PAC produced in the fermentation broth, in a sample to toluene ratio 1:2 in the separating funnel. Extracted L-PAC was estimated by polarimetric method (Khan *et al*., 2012) and optical rotation was documented to calculate L-PAC (g/L) as given below:

$$PAC\ (g/L) = OR*2*1.11$$

where, OR = Optical rotation, 1.11 = Density of PAC and 2= Dilution factor for PAC during the extraction.

Mutagenesis and screening of mutants: UV and chemical (EMS and Nitrous Acid) mutagenesis was done by the methods of Khan *et al*., (2020). Mutants were screened using modified method of Breuer *et al*., 2002.

Ribotyping for Identification of selected yeast: The best L-PAC producing yeast isolates were identified by ribotyping of their rDNA R26, R18 and ITs regions, amplified by LR0R 5' (ACCCGCTGAACTTAAGC) 3', LR7 5' (TACTACCACCAAGATCT) 3', NS1 5' (GTA GTC ATA TGC TTG TCT C) 3', NS8 5' (TCC GCA GGT TCA CCT ACG GA) 3', ITS1 5' (TCC GTA GGT GAA CCT GCG G) 3' and ITS4 5' (TCC TCC GCT TAT TGA TAT GC) 3' primers.

Single colonies from YPDA plates were picked and inoculated in to 50ml rich medium (YPDM; yeast extract 1%, peptone 1%, Dextrose 1%, Malt extract 1%) and incubated in the shaking incubator at 30^{o}C for 24 hrs. DNA was isolated by method reported by Harju *et al*., 2004.

PCR reactions consisted on Taq DNA polymerase (0.75 U) (Fermentas), Taq buffer (1X) with $(NH_4)_2SO_4$ (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 1. 2 mM dNTP mixture (Promega), 0.2 µM of primers (Euro fins MWG operon) and sterile MilliQ-H_2O were added upto the final volume of 30µl. 20ng of DNA was used as the template to perform PCR, by using a EFTaq (So lGent, Korea). Activation of Taq polymerase was done at 95°C for 2min. Thirty five cycles of 95, 55 and 72 °C for 1 min were performed. The last step was a 10-min at 72°C. The filter plate multiscreen (Millipore Corp., Bedford, MA, USA) was used for the purification of amplified products.

PRISM BigDye ^{TM} Terminator v3.1 Cycle sequencing Kit was used for sequencing. The DNA samples and Hi-Di^{TM} formamide (Applied Bio systems, Foster City, CA) were mixed and incubated for 5 min at 95 °C, followed by 5 min on ice. Analysis was done by ABI (3730XL) DNA analyzer (Applied Bio systems, Foster City, CA). Macrogen Inc. Seoul, Korea provided services for sequencing. Samples were stored in insulated jars packed with ice. Specie of yeast was identified using Nucleotide blast (Blastn) of NL-1 sequence (Baere *et al*., 2005).

Results

Seventy eight yeasts colonies were isolated from twenty one different fruit samples including peach, mango, dates, plums, strawberry, banana, apple etc. Twenty isolates were differentially surviving through solvent and temperature stress as given in Table 1. Although, fifteen were L-PAC producing isolates but only one strain (HM 54) was selected for process optimization as it grew with characteristic colonies under solvent and temperature stress (Table 1). It was identified as *Torulaspora delbrueckii* (Fig. 1) on the basis of molecular characterization.

Table 1. Screening of yeast isolates for solvent stress and temperature tolerance.

Sr. No.	Yeasts isolates	A*	B*	C*	D*	E*	F*	G*	H*	I*
1.	HM54	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
2.	HM44	-Ve	+Ve	+Ve	-Ve	-Ve	-Ve	+Ve	+Ve	-Ve
3.	HM21	-Ve	+Ve	+Ve	-Ve	-Ve	-Ve	+Ve	+Ve	-Ve
4.	HM 33,34	-Ve	+Ve	+Ve	-Ve	-Ve	-Ve	+Ve	+Ve	-Ve
5.	HM 70-85	+Ve	+Ve	+Ve	+Ve	-Ve	+Ve	+Ve	+Ve	+Ve

A* = Isolates surviving in presence of Acet. & Benzaldehyde mixture in 5% (v/v); B* = Isolates surviving in presence of Acet. & Benzaldehyde mixture in 10% (v/v); C* = Isolates surviving in presence of Acet. & Benzaldehyde mixture in 15% (v/v); D* = Isolates surviving in presence of Acet. & Benzaldehyde mixture in 20 % (v/v); E*= Growth of yeast isolates at 26^{o}C; F*= Growth of yeast isolates at 30^{o}C; G*= Growth of yeast isolates at 34^{o}C; H*= Growth of yeast isolates at 38^{o}C; I*= Growth of yeast isolates at 42^{o}C

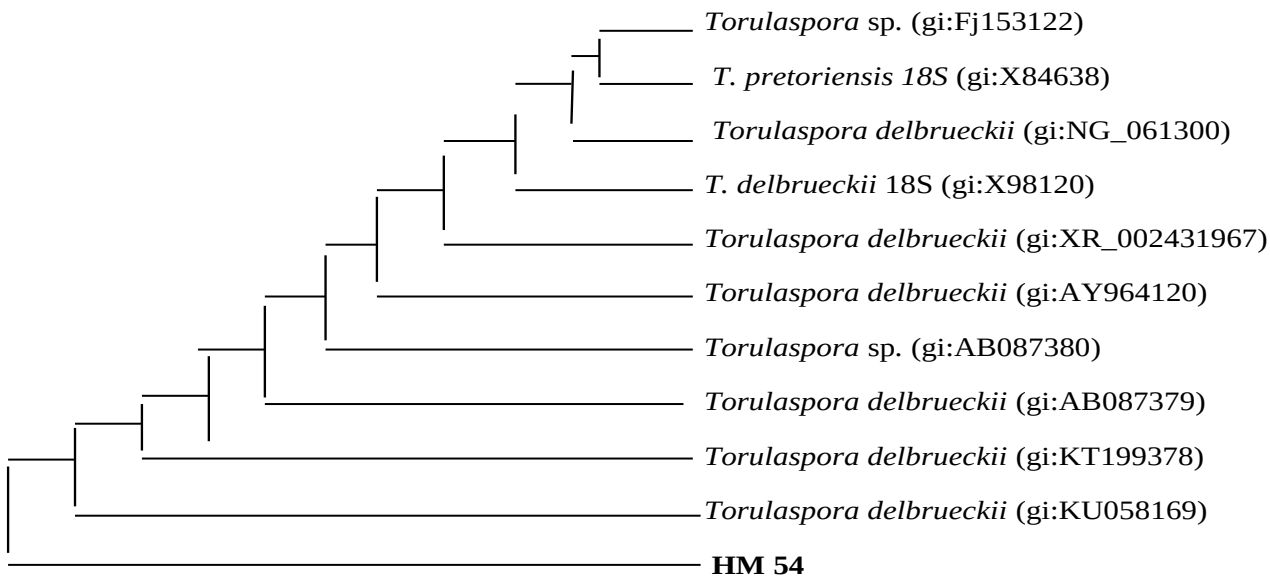

Fig. 1. Phylogenetic Tree of *Torulaspora delbrueckii* (Gen Bank Accession number; MN932368.1 and MN932369.1).

Table 2. Screening of organic and inorganic Nitrogen sources for L-PAC production from *T. delbrukii*.

Organic nitrogen sources	L-PAC (g/L)	Inorganic nitrogen sources	L-PAC (g/L)
Yeast extract	1.11	Di. amonium phosphate	1.99
Meat extract	0.88	Amonium sulphate	0.66
Beef extract	0.77	Ammonium nitrate	0.33
Casamino acid	0.88	Ammonium acetate	0.45
Egg albumin	1.33	Sodium nitrate	0.55
Urea	1.55	Potasium nitrate	0.11

Among the organic and inorganic nitrogen sources urea and di-amonium hydrogen phosphate were proved to be the best nitrogen sources (Table 2) therefore both were used in the further studies. *T. delbrukii* produced 1.55 g/l when urea was used as nitrogen source whereas L-PAC yield was increased to 1.99 when supplemented with di.ammonium hydrogen phosphate as shown in Table 2.

Table 3 shows summary of the optimization of process parameters. Fermentation time for maximum production of L-PAC by *T. delbrukii* was 24hrs where it produced 2.99 g/l of L-PAC which was decreased by increasing the incubation time. *T. delbrukii* produced maximum L-PAC at 30°C that is 3.22 g/l which was reduced to 1.88 g/l at 34°C. The optimum level of total sugars for L-PAC production by *T. delbrukii* was 17% which resulted in 4.11 g/l of L-PAC yield. It reduced to 2.33 at 18% total sugars and continued to reduce with increasing total sugars. L-PAC production increased with the increasing pH of the medium upto 5.5 at which maximum L-PAC was 5.55 g/l. Later on L-PAC was reduced to 3.11 at pH 6.0 and 2.66 at pH 6.5. As far as inoculum size is concerned, 10% inoculum size was found best which produced 5.77 g/l of L-PAC as shown in Table 3.

Chemicaal and physical mutagens were used to reduce the production of byproducts and to make the cells resistant to higher concentrations of benzaldehyde so that higher amounts of L-PAC can be produced. In case of *T. delbrukii*, nitrous acid treatment for 40min was the most effective mutagenesis giving a strain with good carboligation activity thus producing higher amounts of L-PAC (6.22, g/l) (Fig. 2). Nitrous acid mutant of *T. delbrukii* showed 11.83 % higher L-PAC yield in comparison to the wild strain. All the other mutagenic treatments did not show any promising results. The mutant strain of *T. delbrukii* was subjected to optimization through shake flask fermentation. Optimum incubation time was found to be 18hrs while it was 24 hrs in case of wild starin of *T. delbrukii* as shown in Fig. 3. Mutagenesis produced the strain with reduced sugar consumption by 0.1 %. After 22 hrs of incubation L-PAC was reduced to 3.99 and 2.22 g/L from wild and mutant, respectively. While the Sugar consumption was increased to 4.8 and 4.9 % for wild and mutant, simultaneously lowered L-PAC yield showed that the sugar was used for cell mass or other product formation.

As far as pH of medium is concerned nitrous acid mutant produced 7.99 g/L PAC at 5.0 which was reduced to 5.33 at pH 5.5 which was optimum pH for wild strain of *T. delbrukii* as shown in Fig. 4. While the wild strain produced maximum L-PAC 6.44 g/l at pH 5.5.

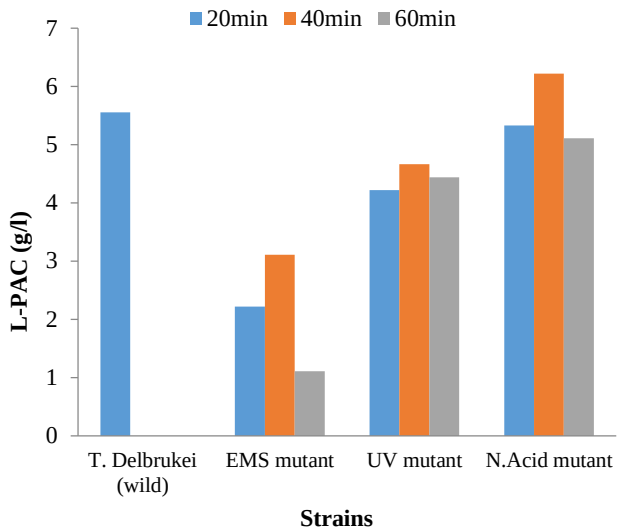

Fig. 2. Selection of best Mutant of *T. delbrukii* for L-PAC production.

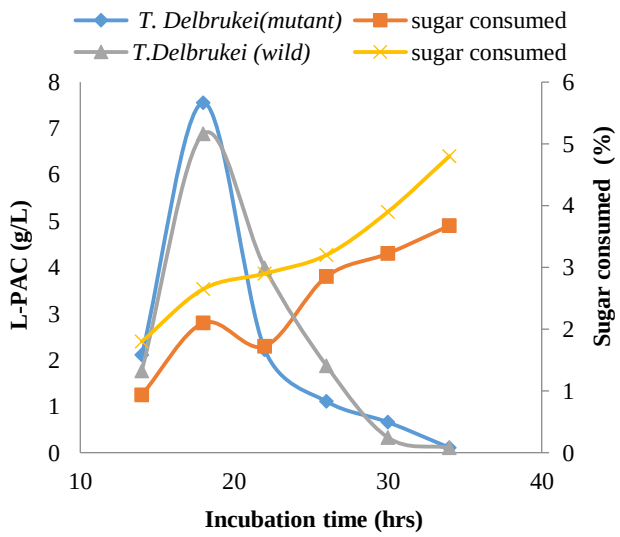

Fig. 3. Optimum incubation time for L-PAC production from Nitrous acid mutant of T. delbrukii.

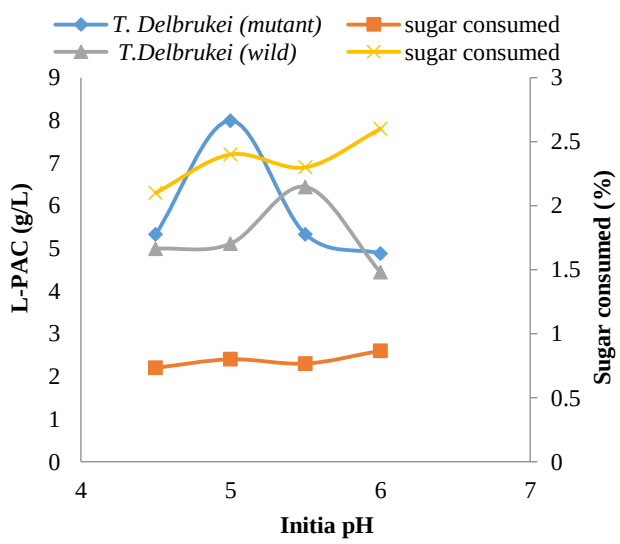

Fig. 4. Optimum pH for L-PAC production from Nitrous acid mutant of *T. delbrukii*.

Table 3. Summary of optimization of fermentation conditions for L-PAC production from *T. delbrukii*.

Strains	Incubation time (hrs)					
	12	**24**	**36**	**48**	**60**	**72**
	0.44 ± 0.02	*2.99 ± 0.20	3.55 ± 0.12	1.22 ± 0.14	0.44 ± 0.16	0.22 ± 0.14
	Temperature (^{o}C)					
	28	**30**	**34**	**38**	**42**	**46**
	0.00 ± 0.12	*3.22 ± 0.10	1.88 ± 0.11	0.66 ± 0.09	No growth	No growth
	Total sugars (%)					
	15	**16**	**17**	**18**	**19**	**20**
T. delbrukii	0.88 ± 0.21	1.11 ± 0.10	*4.11 ± 0.07	2.33 ± 0.13	1.44 ± 0.06	0.22 ± 0.04
	Initial pH of fermentation Media					
	4.5	**5.0**	**5.5**	**6.0**	**6.5**	
	0.66 ± 0.13	2.22 ± 0.04	*5.55 ± 0.02	3.11 ± 0.13	2.66 ± 0.09	
	Inoculum size (%)					
	8	**10**	**12**	**14**	**16**	**18**
	3.99 ± 0.10	*5.77 ± 0.12	4.44 ± 0.08	3.22 ± 0.07	2.44 ± 0.12	1.11 ± 0.09

*Optimum results

In present study, the mutant strain of *T. delbrukii* produced maximum L-PAC (7.55 g/l) after 18hrs of incubation while the wild strain produced highest L-PAC (2.44 g/l) after 24hrs of incubation so the incubation time for *T. delbrukii* was reduced after mutagenesis with improved L-PAC yield which is very significant for any industrial process. Shukla & Kulkarni, (2002) reported 18hrs as optimum incubation time for L-PAC production by *Sachharomyces cerevisiae*.

In our study, *T. delbrukii* produced L-PAC 3.22 g/l at 30^{o}C and the production was reduced at temperature less than 30^{o}C. Many other workers like Miguez *et al*., (2012) has reported 0.6 g/l L-PAC produced by mesophilic yeasts at 30^{o}C while Khan *et al*., (2012) reported 4.15 g/l L-PAC from *Candida utilis* at the same temperature. Therefore, it can be said with certainty that 30^{o}C is the best incubation temperature for most of the yeasts involved in L-PAC production. Husain *et al*., (2012) screened yeast isolates for solvent stress and reported *S. cerevisiae* GCU-36 as best L-PAC producing mesophilic yeast isolate with 2.6 g/l L-PAC at 30±2^{o}C. Khan *et al*., (2012) reported production of L-PAC was 1.5 times more at 30^{o}C as compared to 20^{o}C and inferred that the enzymatic activity and metabolic functions of the yeasts are at maximum at 30^{o}C.

Discussion

Yield of *T. delbrukii* was 4.11 at 17% total sugars while at higher sugar concentration, *T. delbrukii* produced 5.55 g/l L-PAC with a medium pH of 5.5. After mutagenesis, optimum pH for L-PAC was 5.0 which might be due to the slight change in the metabolism of the mutant strain due to mutagenesis which has been brought out in the wild starin through nitrous acid treatment. Some other researchers have also reported nitrous acid as best mutagen to enhance carboligase activity and L-PAC production (Shakula & Kulkarni., 2002). Khan *et al*., (2012) reported optimum pH 6.0 with a maximum amount of L-PAC at this pH i.e., 3.11 g/l. Leksawasdi *et al*., (2003) and Chen *et al*., (2005) also reported pH 6.0 as optimum for L-PAC production. It was assumed that any change in pH can cause inhibition of PDC or its metabolic pathways consequently decreased solubility of the substrates in the medium causes decrease in L-PAC production. Production of L-PAC was greater in comparison to all the previous research work as reported above. Many other researchers reported random mutagenesis as an effective tool to produce mutants with desired properties and higher yields (Demirkan *et al*., 2018). Mutant of *T. delbrukii produced* 7.99 g/l L-PAC during present studies. Some researchers reported very low yield of L-PAC such as Doostmohammadi *et al*., (2016) reported only 2.4 g/l from petite mutants of *Saccharomyces cerevisiae* while Khan *et al.,* (2012) reported maximum yield of L-PAC as 4.15 g/l. Therefore *T. delbrukii* is an appealing strain for economical production of L-PAC as it is mutagenized to get significantly higher quantities of L-PAC.

Conclusions

It is established from this study that the mutant of *T. delbrukii* is the potential strain to produce L-PAC (7.99 g/L) using industrial waste (molasses) as substrate in shake flask with 11.3% increase from wild strain. This production can be increased in a benchtop fermenter with more control over the process parameters. Moreover, this strain is producing significantly higher concentrations of L-PAC after random mutagenesis in contrast to wild strains or previous mutagenized strains. Its ability to survive under solvent stress and higher temperature make it more striking strain for commercial production.

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(Received for publication 28 February 2022)