Functional characterization of alcohol dehydrogenase from Crabtree-negative yeast *Komagataella (Pichia) pastoris*

Alcohol dehydrogenases (ADHs) are fermentative enzymes involved in the inter-conversion of acetaldehyde and ethanol; they are present in multiple copies in Crabtree-positive yeasts. Some have a very narrow substrate range such as acetaldehyde and ethanol, others have a broader substrate range which includes medium and long chain aldehydes and alcohols[1]. They have also been reported in Crabtree-negative yeasts such as *Kluyveromyces lactis*[2], *Scheffersomyces stipitis*[3], *Wickerhamomyces anomalus*[4], *Ogataea angusta*[5,6], *Komagataella* (*Pichia*) *pastoris*[7], etc.

ADHs play an important role in redox balance, as the reactions catalysed by them are redox reactions coupled with $NADH/NAD^+$ conversions. The fermentative nature of *K. pastoris*[8], report of ethanol formation[9] and available genome sequence[7] have indicated the presence of ADH in *K. pastoris*. One of the ADHs (XM_002491337.1) was studied in detail. There are five other ADHs described in the *K. pastoris* genome. Four of them (XM_002493969.1, XM_002492172.1, XM_002489969.1 and XM_002492524.1) are NADP/NADPH-dependent and have broad substrate specificity and one (XM_002491163.1) is a bifunctional enzyme with ADH and glutathione-dependent formaldehyde dehydrogenase.

Details of the materials and methods have been described elsewhere[10].

ADH activity was determined according to the method described by Wang *et al.*[11]. Metabolite measurement was carried out in Aminex HPX 87H column (Bio-Rad) as described[12].

Presence of ethanol in yeasts has been reported as a result of either excess carbon source (Crabtree effect), or difference in the aeration levels (Pasteur effect), or both. To verify the conditions under which ethanol was being formed in *K. pastoris* varying amounts of carbon source and aeration were used. Since ethanol formation has also been reported in non-fermentative glycerol, we used both glucose and glycerol as carbon sources at a concentration of 2% and 10% respectively. Different aeration levels were used by growing the cells at 50 rpm (low aeration), 250 rpm in normal flask and 250 rpm in baffled flask. With 10% glycerol at low aeration (50 rpm) the growth was negligible and hence it was not included in the data.

In the case of extremely low aeration (50 rpm) the growth was inhibited, more with higher percentage of carbon source (Table 1). In the case of baffled flasks, negligible ethanol was observed with 2% and 10% carbon source (Table 1) until 30 h, after which ethanol accumulated, due to partial anaerobic conditions generated as a result of high cell densities. In normal flasks as well as those at 50 rpm, ethanol formation was visible around 12 h in 2% and 10% glucose, suggesting that ethanol formation in *K. pastoris* is due to Pasteur effect (low aeration). With 2% carbon source, the residual ethanol was utilized at the 48 h stage when the primary carbon source was completely used, indicating the diauxic growth pattern in *K. pastoris*.

Glycerol is a non-fermentative carbon source. Nevertheless, low level of ethanol (0.3–0.5 gl^{-1}) formation was observed, similar to the values (0.1 gl^{-1}) reported earlier[13]. These levels were sufficient for AOX (alcohol oxidase) repression.

K. pastoris ADH was expressed and its protein product validated through a complementation experiment. Ciriacy[14] had constructed a deletion mutant of *Saccharomyces cerevisiae* Δadh (1, 2, 3) (MC-892-1C) which lacked three important *ADH* genes (ADH 1, 2, 3) and also lacked any detectable ADH activity. It had a slow growth phenotype on glucose as carbon source due to disturbed NADH/NAD ratio. Further, in the presence of glucose, if the antibiotic antimycin A is used, the strain is not able to grow. This is because antimycin A blocks the respiratory chain and in the absence of ADH, fermentation of pyruvate to ethanol does not take place. Therefore, in such a case there is no route for NADH oxidization. This defect could be complemented by any functional ADH activity. With this objective *KpADH* (XM_002491337.1) was cloned in a constitutive expression vector pRS426-GPD along with a His-tag. Δadh (1, 2, 3) was transformed with GPD-*KpADH* and the transformants were selected on the basis of URA prototrophy.

A single transformant was grown in the defined media and various dilutions of the cells were spotted on agar plates (YPD or defined media lacking uracil) containing antimycin A at a concentration of 1 μg/ml. As can be seen from the Figure 1, while the parent Δadh 1, 2, 3 did not grow on plates containing antimycin A, the strain transformed with *KpADH* was viable with no apparent growth defect. KpADH was able to complement the defect of Δadh 1, 2, 3, thereby indicating the presence of functional ADH.

To further confirm the presence of KpADH protein, the positive transformants in the above complementation experiment were grown in a defined media without uracil and HIS-tagged protein was purified. The expected molecular weight of the monomer based on the protein sequence was calculated to be 38 kDa. A single purified band with the expected size was observed, confirming the expression of KpADH in the transformed Δadh (1, 2, 3).

ADH activity in the parent strain Δadh (1, 2, 3) and the recombinant expressing KpADH was determined. No activity was found in the parent strain, whereas ADH activity of 0.115 ± 0.004 units/mg was found in the recombinant, further confirming the functionality of KpADH (Figure 2). With propanol as a substrate lesser extent of activity (0.044 ± 0.007) was observed, whereas with butanol no activity was detected. This suggests that the alcohol specificity of the identified ADH is limited to ethanol.

The recombinant strain expressing KpADH produced more ethanol at all time points. This confirms that KpADH could produce ethanol under *in vivo* conditions (Figure 2).

The functionality of one of the alcohol dehydrogenases of *K. pastoris* has been established based on its ability to complement the ADH-based growth defect in a *S. cerevisiae* Δadh (1, 2, 3) strain, by the presence of ADH activity and enhanced ethanol formation in the strain transformed with the *KpADH* gene. KpADH was annotated as a putative mitochondrial ADH (based on homology with *S. cerevisiae* ADH III) in the genome database by De Schutter *et al.*[7]. However, no

Table 1. Optical density (OD_{600} nm) measurement and ethanol concentration of *K. pastoris* grown with varying glucose and glycerol concentration and aeration

	Glucose								Glycerol							
	OD (600 nm)				Ethanol concentration (g L^{-1})				OD (600 nm)				Ethanol concentration (gL^{-1})			
Flask	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h
B-200-2	6.7 ± 0.8	29.3 ± 4.6	27.5 ± 0.7	24.1 ± 3.7	0.2 ± 0.0	0.4 ± 0.1	0.45 ± 0.1	0.43 ± 0.1	10.0 ± 0.7	39.2 ± 1.3	39.3 ± 1.2	38.1 ± 1.1	0	0.5 ± 0.1	0.50 ± 0.0	0.4 ± 0.2
E-200-2	6.3 ± 0.6	22.4 ± 1.6	29.6 ± 0.2	23.9 ± 1.8	0.50 ± 0.0	3.3 ± 2.2	2.03 ± 0.4	0.4 ± 0.0	8.3 ± 0.1	24.4 ± 2.1	29.8 ± 0.3	26.4 ± 1.6	0	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.1
E-50-2	2.4 ± 0.0	10.0 ± 0.8	9.1 ± 0.5	9.0 ± 0.0	1.5 ± 0.1	8.4 ± 0.0	8.0 ± 0.5	7.6 ± 0.5	0.5 ± 0.1	1.2 ± 0.1	1.8 ± 0.1	1.3 ± 0.1	0	0.4 ± 0.2	0.3 ± 0.0	0.3 ± 0.0
B-200-10	5.8 ± 0.7	31.9 ± 0.3	51.3 ± 3.9	53.0 ± 1.4	0	0.9 ± 1.1	1.7 ± 0.1	8.8 ± 0.1	1.7 ± 0.3	6.8 ± 0.6	10.1 ± 1.1	7.9 ± 1.0	0	0	0	0
E-200-10	4.8 ± 0.0	29.5 ± 0.4	26.6 ± 4.7	37.5 ± 6.1	0	19.8 ± 6.7	24.6 ± 0.2	29.3 ± 0.0	1.5 ± 0.3	5.5 ± 0.0	8.0 ± 0.8	6.7 ± 0.5	0	0	0	0
E-50-10	2.0 ± 0.2	7.61 ± 0.1	13.3 ± 1.3	15.3 ± 0.3	0.95 ± 0.1	13.35 ± 6.6	16.9 ± 1.7	24.9 ± 2.0	NA	NA	NA	NA	NA	NA	NA	NA

B, Baffled flasks (for excessive aeration); E, Erlenmeyer flasks (normal conditions). Each letter is followed by a number which denotes the rpm followed by percentage of glucose. The values are mean of duplicates (two different flasks).

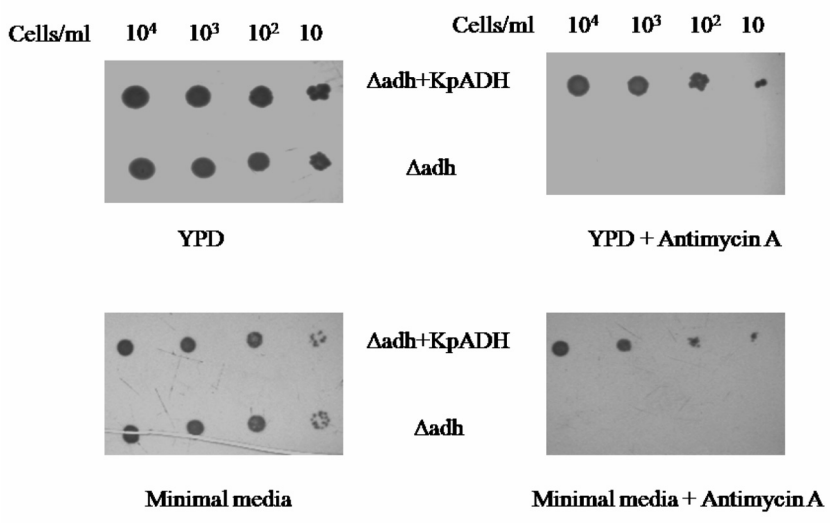

Figure 1. Images of cell growth during complementation experiments with Δadh (1, 2, 3) transformed with KpADH. Numbers on the top of the figure are cells/ml. Defined media consists of glucose, Yeast Nitrogen Base and complete amino acid mix (without any dropout).

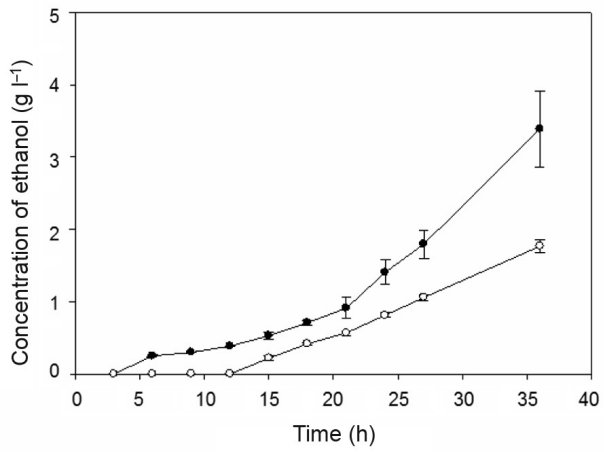

Figure 2. Graph displaying ethanol production in the Δadh (1, 2, 3) (○) and KpADH transformed strain (●). The defined media for Δadh (1, 2, 3) contain complete amino acid mix, whereas that of KpADH transformed strain was grown in defined media lacking uracil for maintenance of selection pressure. The values are mean of duplicates (measurement from two different flasks.)

mitochondrial signal sequence could be detected in the protein sequence of KpADH (using the software MitoProt II). Hence it is suggested that KpADH is a cytosolic ADH. Similar to the *S. cerevisiae* cytosolic ADH I and mitochondrial ADH III, KpADH is capable of producing ethanol with NADH as cofactor. All the other ADHs in the *K. pastoris* genome are medium-chain and NADP-dependent (as annotated in the genome database). This leads to the hypothesis that the gene identified in the present study could be the major enzyme responsible for ethanol production in *K. pastoris* under hypoxic conditions. As it has been observed that ethanol production in *K. pastoris* takes place only under hypoxic conditions, therefore the major role of KpADH could be in maintaining redox balance in *K. pastoris* under oxygen limited conditions. It has been observed that the accumulated ethanol also gets assimilated rapidly[9]. As there are no other annotated NAD-dependent ADHs in the *K. pastoris* genome database, it is possible that the same enzyme catalyses both forward and reverse reactions, thus maintaining a dynamic balance between the oxidized and reduced forms of NAD. The reversible nature of the enzyme ensures that whatever ethanol is formed during the hypoxic conditions is quickly assimilated once sufficient aeration is available.

Conflict of interests: The authors declare that they have no conflict of interests.

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ACKNOWLEDGEMENTS. V.U. was supported by the Council of Scientific and Industrial Research, Government of India. The work was partially funded by the Board of Research in Nuclear Sciences, India.

Received 4 February 2016; revised accepted 11 July 2016

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