Molecular modeling and dynamics simulation of alcohol dehydrogenase enzyme from high efficacy cellulosic ethanol-producing yeast mutant strain *Pichia kudriavzevii* BGY1-γm

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1. Introduction

One of the greatest challenges of the twenty-first century is to meet the growing demand of energy for transportation and industrial processes. Environment and health concerns, together with non-renewable nature of fossil fuels, have led scientists to search for an alternative, cleaner and sustainable energy source (Dhaliwal et al., 2011). In recent years, the global production of lignocellulosic biomass is around 180 × 10^9 tons/year, of which about 8–20 × 10^9 tons of the primary biomass remains potentially accessible (Ma et al., 2017). Three major structural components of lignocellulosic biomass are lignin (2–40%), hemicellulose (20–30%) and cellulose (30–60%). Lignin is the most recalcitrant phenolic polymer that acts as a cementing agent outside the cellulosic microfibrils. Cellulose, a linear homopolymer of glucose units (500–15,000) linked through β-1,4 glycosidic bonds is a highly crystalline major structural component of the plant cell wall. Hemicellulose, a heteropolymer comprising of C₅ (xylene and arabinose) and C₆ (mannose, galactose and glucose) sugars with 50–200 units, provides a linkage between lignin and cellulose (Oberoi et al., 2012).

The hemicellulose can be hydrolyzed into monomeric pentose sugar units by microbial hemicellulases such as xylanases, arabianases and xyloarabinases, etc. (Gong et al., 2018; Radhika et al., 2011). The cellulases secreted by cellulolytic microbes such as *Cellulomonas* sp., *Trichoderma* sp.,...
Aspergillus sp. and Bacillus sp., etc. are used for enzymatic saccharification and bioconversion of cellulose into fermentable sugars (Aggarwal et al., 2017; El-Shishtawy et al., 2015). Glucose released from the biomass can be fermented into ethanol by yeasts (S. cerevisiae, Candida sp., Kluyveromyces marxianus and Pichia stipitis) or bacteria (Zymomonas mobilis, Lactobacillus sp. and Pediococcus sp. (Ajit et al., 2017; Choudhary et al., 2016; Soleimani et al., 2017). S. cerevisiae is the most commonly employed yeast for industrial alcohol production. However, it has low thermo- and osmo-tolerance, a narrow substrate range and ability to produce ethanol from only C6 sugars, preferably glucose. The C5 sugars released from the hemicellulosic component of lignocellulosic biomass are generally not fermented into ethanol by most of the strains of S. cerevisiae (Hashem et al., 2014). Therefore, a high ethanol yield from saccharified lignocellulosic biomass can be achieved if the fermenting organism is able to produce ethanol from both C5 and C6 sugars. Several species of Pichia such as P. stipitis, P. anomala, P. kudriavzevii and P. pastoris, etc. have recently been reported to ferment xylose into ethanol (Chen et al., 2012; Silva et al., 2011; Yu & Zhang, 2003). Improved strains with enhanced alcohol-producing efficacy have been developed through mutagenesis and genetic engineering techniques (Dong et al., 2020; Ho et al., 1998; Kordowska-Wiater & Targoriski, 2001).

The enzymatic conversion of sugar into ethanol is carried out by the enzyme alcohol dehydrogenase (ADH) synthesized by ethanogenic microbial strains (Fonseca et al., 2008). Pyruvate, an intermediate in the central metabolism of carbohydrates, can be non-oxidatively decarboxylated and converted to acetaldehyde (AD) and carbon dioxide by pyruvate decarboxylase (PDC). Further, AD is eventually reduced to ethanol by ADH enzyme. The structure of ADH has been reported from several organisms like Thermoanaerobacter ethanolicus (PDB ID: 7JNQ), Streptococcus pneumonia (PDB ID: 5YLN), Entamoeba histolytica (PDB ID: 2NVB), Thermoanaerobacter brockii (PDB ID: 1BXZ), Saccharomyces cerevisiae (PDB ID: 4W6Z) and Homo sapiens (PDB ID: 1HDZ), etc. (Goihberg et al., 2008; Hurley et al., 1994; Li et al., 1999; Raj et al., 2014). Here, in this study, we aimed at the identification and exploration of parent (BGY1) and γ mutant strains (BGY1-γm) of the newly isolated C5/C6 sugar fermenting yeast P. kudriavzevii. The in vitro ethanol production by BGY1 and BGY1-γm using glucose and xylose was compared with the common ethanol-producing yeast S. cerevisiae. The high efficacy alcohol dehydrogenase (PkADH) enzyme from P. kudriavzevii BGY1-γ mutant was characterized as ADH-1 type. Three-dimensional model of PkADH mutant was predicted to perform the in silico studies. Molecular docking and dynamics were conducted to evaluate the binding of substrate (AD) and product (ethanol) with PkADH mutant. Overall, results confirmed that substrate and product bind at the active site of PkADH mutant to form a stable protein–ligand complex.

2. Materials and methods

2.1. Yeast strains and culture medium

Newly isolated native yeast strain Pichia kudriavzevii BGY1 previously isolated from Black grape fruit and its gamma mutant (P. kudriavzevii BGY1-γm) developed earlier in the laboratory were used during the present study for in vitro ethanol production from C5/C6 sugars. Saccharomyces cerevisiae, ATCC 9763 procured from MTCC, Chandigarh, India was used as a standard ethanogenic yeast. The yeast cultures were activated and grown at 30 ± 1°C for 48 h in yeast extract peptone dextrose (YPD) agar medium containing (% w/v): yeast extract, 1.0; peptone, 2.0; glucose, 2.0; agar, 2.0 (Gupta et al., 2009).

2.2. Selection of C5/C6 co-sugar fermenting strain

Ethanol-producing (in vitro) capabilities of P. kudriavzevii BGY1 (wild type), P. kudriavzevii BGY1-γm (mutant) and S. cerevisiae were determined qualitatively by plate assay using YPD agar medium amended with glucose (2%, w/v). Active yeast culture (OD = 0.8) and ceric ammonium nitrate reagent in equal volumes were added to the wells made in agar plates with the help of a cork borer. The plates were immediately visualized for the development of intense red color around the wells. The in vitro quantification of ethanol production by the strains was done spectrophotometrically following the method given by Reid and Salmon (1955) with slight modifications. Batch ethanol fermentation was carried out in three sets of flasks containing YPD broth supplemented with either glucose (15%), xylose (15%) or glucose + xylose (7.5% each), inoculated with active yeast cultures individually and incubated at 30 ± 1°C for 72 h (Reid & Salmon, 1955). The samples were withdrawn at 48 and 72 h of fermentation and ethanol contents were measured spectrophotometrically at 486 nm (Pinyou et al., 2011).

2.3. Estimation of alcohol dehydrogenase activity

Intracellular ADH activities of the wild-type and mutant strains of P. kudriavzevii BGY1 and S. cerevisiae were determined using the method given by Vuralhan et al. (2003). YPD broth cultures (48-h old) were centrifuged at 8000 rpm for 10 min (4°C) and the cell pellets were washed twice with 0.1 M phosphate buffer (pH 7.5). The cell extracts were prepared by sonication at 0°C for 2 min at regular interval of 30 s with LABSONIC U-sonicator (133 V, 0.5 repeating cycles per second). The cell lysate was centrifuged at 12,000 rpm for 10 min (4°C) to remove the cell debris. The supernatant was used for protein estimation and analyzed for ADH activity following the method described by Longhurst et al. (1990). The reaction mixture (3.8 ml) contained 1.5 ml sodium pyrophosphate buffer (0.1 M, pH 9.2), 0.5 ml ethanol (2.0 M), 1 ml NAD (0.025 M) and 0.8 ml crude enzyme. The increase in absorbance at 340 nm was recorded at room temperature (25°C) and the absorbance/time curve was plotted. One unit of ADH enzyme activity is ‘the amount of enzyme required to reduce one micromole of NAD’ per min at 25°C under specified conditions’. Specific ADH activity was calculated using the formula given below:

\[
\text{ADH units/mg protein} = \frac{A_{340} \times 6.22 \times mg \text{ protein/ml reaction mixture}}{min}
\]
2.4. PCR amplification and characterization of ADH gene

The high efficacy ADH-1 encoding gene from the mutant strain (*P. kudriavzevi* BGY1-γm) showing higher values for ethanol production during co-fermentation of C5/C6 sugars in batch cultures and ADH activity as compared to the wild-type strain was characterized. For identification and characterization of ADH-1 gene, genomic DNA from the strain BGY1-γm was isolated from overnight grown cultures using a chromosomal Genomic DNA isolation kit (Looke et al., 2011). The target ADH encoding gene (adh-1) was amplified by PCR technique using forward primer PK-ADH-1-FP (5'-CCATTTAGTCGAATACCTAAGAGAATC-3') and the reverse primer PK-ADH-1-RP (5'-ACTGATCTGCTGTTGAGTACGAATG-3'). The PCR cycle parameters were as follows: initial denaturation at 95 °C for the duration of 5 min, followed by 35 cycles at 94 °C for 30 s, at 52 °C for 30 s, at 72 °C for 45 s, and final extension was performed at 72 °C for 7 min and final storage at 4 °C. The PCR amplified product was then purified, sequenced and compared with the ADH gene sequences available in the nucleotide database (NCBI) using the BLAST algorithm. Multiple sequence alignment was carried out with CLUSTAL W and a phylogenetic tree was constructed with the MEGA version 5 program (Tamura et al., 2007; Thompson et al., 1997).

2.5. Molecular modeling

Sequencing results of ADH from *P. kudriavzevi* BGY1-γm (PkADH) showed that it has 99.2% sequence similarity with ADH-I of *P. kudriavzevi* (accession no. XP_020544925.1). Pblast of ADH from *P. kudriavzevi* BGY1-γm revealed that it has sequence identity of 51.4% and query coverage of 98% with the crystal structure of zinc-dependent ADH-2 from *Streptococcus pneumonia*-apo form (PDB ID: 5YLN). Modeler was utilized to generate the comparative 3D model of PkADH mutant using crystal structure of zinc-dependent ADH-2 from *Streptococcus pneumonia*-apo form as a template (Webb & Sali, 2014). Twenty models were predicted and the model with the lowest DOPE score was energy minimized by using Swiss PDB Viewer, as done by Kumar et al. (Guex & Peitsch, 1997; Kumari et al., 2020). Disordered loops were refined using ModLoop, as done by Saini et al. (Fiser et al., 2000; Saini et al., 2021).

The energy minimized model was validated by the Ramachandran plot generated by PROCHECK (Laskowski et al., 1993). Verify-3D, ERRAT, ProSA and ProQ were also used to assess the stereochemical quality of the model, as done by previous studies (Colovos & Yeates, 1993; Dhankhar, Dalal, Kotra, et al., 2020; Eisenberg et al., 1997; Pandit et al., 2018; Saini et al., 2019; Singh et al., 2017, 2019; Wallner & Elofsson, 2003; Wiederstein & Sippl, 2007). Further, the model was also validated by molecular dynamics using GROMOS96 43a1 force field in GROMACS suite on NMRbox platform (Maciejewski et al., 2017; van der Spoel et al., 2005; van Gunsteren et al., 1996). PDBsum was used to generate the topology diagram of the model (Laskowski et al., 2005). Dali server was used for the PkADH mutant structure comparison with other known structures (Holm & Sander, 1993). The multiple sequence alignment of PkADH mutant with another ADH from different organisms was generated and illustrated by using Clustal Omega and ESPript3.0, respectively (Gouet et al., 1999; Sievers et al., 2011).

2.6. Molecular docking

AutoDock Vina 1.1.2 was utilized to find the interactions between protein and ligands, as done by Dalal et al. (Dalal et al., 2019, 2021; Trot & Olson, 2010). AutoDock Tools version 1.5.6 was used for the addition of hydrogen atoms and Kollman charges (–1.97) on the protein (Morris et al., 2009). AD (CID177) and ethanol (CID702) were retrieved from PubChem compound database in the SDF format (Kim et al., 2016). OpenBabel 2.3.1 was utilized to convert all the SDF format of ligands into PDB format (O’Boyle et al., 2011). Gasteiger charges on AD and ethanol were added and were saved in pdbqt formats. The characteristic motifs consisting of conserved Cys40, Ser42, His69, Trp123 and Asp163 were used to create the atomic grid for docking. The atomic potential grid map with a spacing of 0.375 Å was generated by using Autogrid4. The grid box size was 34 Å × 32 Å with dimensions of –10.78, 19.66 and –12.33 (X, Y and Z coordinates). AutoDock Vina 1.1.2 was used for docking and pose with maximum binding energy was analyzed in PyMol and Maestro11.2 of Schrodinger (DeLano, 2002; Schrödinger Release, 2016).

2.7. Molecular dynamics simulation

Molecular dynamics simulation was performed to determine the stability of PkADH-mutant, PkADH-mutant AD and PkADH-mutant ethanol complexes. Protein coordinates and topologies were created using GROMOS96 43a1 force field in GROningen MAchine for Chemical Simulations (GROMACS), hosted on NMRbox computing platform (Maciejewski et al., 2017; van der Spoel et al., 2005; van Gunsteren et al., 1996). Ligands (AD and ethanol) topologies were generated using the PRODRG server, as done by Kumari et al. (Kumari et al., 2021; Schüttelkopf & Van Aalten, 2004). The protein was kept 1.5 nm from the edge of the triclinic box with dimensions (8.98 × 7.95 × 9.03 nm) and volume (644.70 nm³). The systems were solvated in simple point charge (SPC) and counterions (Na⁺) were added using genion tool to maintain the overall neutral charge. Energy minimization was done using the steepest descent algorithm for 50,000 steps with an energy cut of 1 kJ mol⁻¹. The systems were equilibrated for 1 ns using a constant number of particles, volume and temperature (NVT) followed by a constant number of particles, pressure and temperature (NPT). NVT and NPT equilibrations were done using the Parrinello–Rahman barostat pressure coupling method and Berendsen thermostat, respectively (Berendsen et al., 1984; Parrinello & Rahman, 1981). Linear Constraints Solver (LINCS) was used to constrain the covalent bonds and long-range interactions were calculated using the particle mesh Ewald (PME) (Abraham & Gready, 2011). The
final molecular dynamics simulation was run for 100 ns with the generation of coordinates at an interval of 10 ps. The trajectories were used to determine the root mean square deviations (RMSD), radius of gyration (Rg), solvent-accessible surface area (SASA), hydrogen bond numbers and principal component analysis (PCA) of PkADH-mutant, PkADH-mutant AD and PkADH-mutant ethanol complexes.

2.8. MMPBSA binding energy

The binding energies of protein–ligand complexes were calculated using the Molecular Mechanics/Position-Boltzmann Surface Area (MMPBSA) method, as done in previous studies (Dhankhar, Dalal, Mahto, et al., 2020; Gupta et al., 2021; Kumari & Dalal, 2021; Kumari et al., 2014; Malik et al., 2019; Singh et al., 2018, 2020). The binding energies were estimated as:

$$\Delta G_{\text{bind}} = \Delta G_{\text{complex}} - (\Delta G_{\text{protein}} + \Delta G_{\text{ligand}})$$

where $\Delta G_{\text{complex}}$, $\Delta G_{\text{protein}}$ and $\Delta G_{\text{ligand}}$ are the total free energy of protein–RNA complex, protein and ligand in a solvent, respectively.

The free energy of solvation and molecular mechanics potential energies were calculated from the retrieved molecular dynamics trajectories using g_mmpbsa. The combination of polar and non-polar solvation energies gives free solvation energy. In the current study, total of 2000 snapshots extracted at regular interval of 10 ps from the 40–60 ns, 60–80 ns and 80–100 ns have been used to determine the binding energies of PkADH-mutant AD and PkADH-mutant ethanol complexes.

2.9. Statistical analysis of data

Analysis of variance (ANOVA) was done with statistical software using the programmes Stpr2 and Stpr3. All the experiments were conducted in triplicates, and the results are reported in terms of critical difference (CD).

3. Results and discussion

3.1. Selection of C6/C5 co-sugar fermenting strain

The present study was aimed at exploring wild-type and mutant strains of a native ethanogenic yeast with broad-spectrum utilization of sugars. The native strain P. kudriazevii BGY1 was isolated from black grape fruit source using the pure culture techniques and its mutant P. kudriazevii BGY1-γm was developed earlier in the laboratory. Both the strains were tested for their in vitro ethanol-producing capabilities. During qualitative testing by plate assay using glucose-containing medium, all the yeast cultures (P. kudriazevii BGY1, P. kudriazevii BGY1-γ and S. cerevisiae) produced intense red color around the colony as a measure of ethanol production (Figure 1(A)).

The cultures were then evaluated for quantitative estimation of ethanol produced from glucose/xylose sugars during batch fermentation. The in vitro ethanol yield for the yeast strains in YPD broth supplemented with glucose (15%) varied from 9.02% to 13.65% in 48 h (Figure 2(A)). In the case of xylose (15%) fermentation, the ethanol concentration varied from 0.0% (S. cerevisiae) to 10.97% (P. kudriazevii BGY1-γm) in 72 h (Figure 2(B)). While during co-fermentation of both the sugars (7.5% xylose + 7.5% glucose) in batch cultures, the ethanol yield varied from 4.43% to 14.98% at 72 h (Figure 2(C)). Pichia kudriazevii BGY1 and its mutant P. kudriazevii BGY1-γ were capable of fermenting pentose (xylose) sugar. The results indicated that both wild-type and mutant strains of P. kudriazevii BGY1 were capable of co-fermenting C6/C5 sugars while S. cerevisiae was unable to ferment xylose. Glucose (C6) has already been reported as the most preferential sugar source for ethanol production by the industrial ethanol-producing yeast S. cerevisiae limiting its ability to ferment xylose or other C5 sugars. In comparison to the wild-type strain the mutant strain P. kudriazevii BGY1-γ showed higher ethanol titer of 13.65% from glucose in 48 h, 10.97% from xylose in 72 h and 14.98% upon co-fermentation of glucose + xylose in 72 h during batch fermentation. The P. kudriazevii BGY1 strain used in the present study showed higher ethanol yield as compared to the ethanol yield formerly reported for a wild-type strain P. kudriazevii RZ8-1 (Chamnipa et al., 2017). The mutant strain P. kudriazevii BGY1-γ showed better ethanol yield during co-fermentation of glucose + xylose as compared to the ethanol yields obtained with either of the two sugars individually in batch cultures. The mutant strain in the present study was thus found as a superior strain for co-fermentation of C6/C5 sugars as compared to the wild-type strain of P. kudriazevii BGY1 and S. cerevisiae (Sangwan et al., 2020). The results further confirmed mutagenesis as an effective tool for strain improvement of ethanogenic yeast. It could develop a mutant strain with better efficacy for assimilation and ethanol production from the pentose (xylose) sugar. The utilization of different sugars is one of the most common restrictions for the majority of yeasts. The yeast strains used during the present study overcome this limitation as it is able to ferment hexose as well as pentose sugar. Only a few yeasts, such as Candida sp., Metschnikowia sp. and Pichia sp. are reported to have the potential to ferment xylose into ethanol (Hahn-Hägerdal et al., 1994; Selim et al., 2020; Toivola et al., 1984). Several species of Candida and Pichia have emerged as good fermentors of D-xylose (Kim et al., 2010; Selim et al., 2020). The ability to produce ethanol by co-fermenting xylose and glucose sugars in batch culture presents P. kudriazevii BGY1 and its mutant strain as a suitable candidate for bioconversion of the sugars (C6/C5) released from the lignocellulosic biomass.

3.2. Alcohol dehydrogenase enzyme activity

ADH is the key enzyme responsible for ethanol production in ethanogenic yeast and bacteria that is mostly produced intracellularly. Therefore, ADH activity is considered as a measure for determining the ethanol fermenting capability of the organism. The crude intracellular enzyme from the wild-type and mutant strains of P. kudriazevii BGY1 and S. cerevisiae strains showed significant differences in ADH.
enzyme activities that varied from 4.27 to 11.10 U ml\(^{-1}\), as shown in Figure 1(B). The mutant strain \textit{P. kudriazvevii} BGY1-\(\gamma\)m revealed maximum ADH activity (11.10 U ml\(^{-1}\)), which was significantly higher than the ADH activities recorded for the wild-type strain \textit{P. kudriazvevii} BGY1 and \textit{S. cerevisiae} used during the present investigation. The wild-type and mutant yeast strains used during the present study showed better ADH activities as compared to \textit{S. cerevisiae} (4.27 U ml\(^{-1}\)).
Figure 3. Characterization of ADH 1 gene from the mutant strain *Pichia kudriavzevii* BGY1-ym: (A) Separation of ADH-1 gene amplicon on an agarose gel and (B) Phylogenetic tree showing similarity of alcohol dehydrogenase-1 (ADH1) encoding gene from BGY1 mutant with the ADH1 gene sequences from different strains of *Pichia kudriavzevii* available in GenBank database.

Figure 4. Cartoon representation of modeled structure of alcohol dehydrogenase-1 enzyme from the mutant strain of *Pichia kudriavzevii* BGY1 (BGY1-ym) with α helices (cyan color) and β sheets (orange color).
higher ethanol production by the mutant strain is because of transition and transversion mutation in the gene that can damage macromolecules such as DNA, proteins and lipids due to its high frequency, short wavelength and high energy photon that induces the SOS and other might also be correlated to its higher ADH activity. A decline in ethanol yield after 48 h (in case of glucose) and 72 h (in case of xylose) might be due to the feedback inhibition of enzyme activity by the end product (ethanol). It is well reported that the activity of the enzyme ADH is regulated by feedback inhibition and the end product ethanol represses the activity of the enzyme (Taber, 1998).

3.3. Characterization of ADH gene from Pichia kudriavzevii BGY1-γm

Although different types of ADH enzymes have been reported, the key enzyme for fermentative ethanol production is ADH1 type, which reduces AD to ethanol in the presence of NADH, catalyzing the reaction in the forward direction. Apart from the fermentation conditions, the ethanogenic efficacy of a fermenting yeast depends upon its ADH activity, therefore, for a better understanding of the enzyme from the mutant strain *P. kudriavzevii* BGY1-γm, the ADH encoding gene (adh) was characterized. The ADH gene was amplified by PCR technique and was subjected to electrophoresis using 1% agarose gel. The electrophoretic analysis showed 1 kb band for ADH gene (Figure 3(A)).

The ADH encoding gene from the mutant strain was sequenced and compared with other ADH encoding genes available in GenBank and a phylogenetic tree was constructed for determining its relatedness with other ADH genes. The phylogenetic analysis of the ADH gene from BGY1-γm on BLAST search revealed its maximum homology of 99% with the *adh1* gene from different strains of *P. kudriavzevii* such as *P. kudriavzevii* strain CBS573, *P. kudriavzevii* BOH78, *P. kudriavzevii* strain CY902, *P. kudriavzevii* strain CBS5147 and *P. kudriavzevii* strain SJF (Figure 3(B)). Based on its 99% similarity with the ADH-1 gene sequences from...
Figure 6. The root means square deviation (RMSD) of PkADH-mutant and PkADH-mutant ligand complex(s) for the molecular simulation of 100 ns. (A) PkADH mutant (black color), PkADH-mutant acetaldehyde (red color) and PkADH-mutant ethanol (green color). (B) Ligand RMSD of acetaldehyde (red color) and ethanol (green color).

Table 2. Average values of RMSD (protein and ligand), radius of gyration (Rg), solvent accessible surface area (SASA) and intra-hydrogen bond numbers for PkADH-mutant, PkADH-mutant acetaldehyde and PkADH-mutant ethanol complexes for the duration of 100 ns.

| Averages          | PkADH mutant | PkADH-mutant acetaldehyde | PkADH-mutant ethanol |
|-------------------|--------------|---------------------------|----------------------|
| Protein RMSD (nm) | 0.39 ± 0.03  | 0.32 ± 0.02               | 0.33 ± 0.02          |
| Ligand RMSD (nm)  | 2.03 ± 0.02  | 1.98 ± 0.01               | 1.99 ± 0.01          |
| Rg (nm)           | 156.50 ± 5.74| 151.82 ± 3.94             | 152.36 ± 5.01        |
| Intra-protein H bonds | 251.80 ± 8.65 | 259.47 ± 9.01             | 258.79 ± 9.07        |

Figure 7. Radius of gyration (Rg) showing the compactness of PkADH-mutant, PkADH-mutant acetaldehyde and PkADH-mutant ethanol for the span of 100 ns of molecular simulation at 300 K.
other *P. kudriavzevii* strains the ADH gene from the mutant strain *P. kudriavzevii* BGY1-cm was identified as ADH-I type.

### 3.4. Molecular modeling

The homology model of ADH from *P. kudriavzevii* BGY1-cm (*PkADH*) was generated to compare the structural and functional properties of the protein. Ramachandran plot of the model produced by PROCHECK shows that 87.7% residues are in the most favored region, 11.9% are in the additional allowed region, and 0.3% are in the additional disallowed region as shown in (Supplementary Figure S1). Verify-3D result shows that the quality factor of 91.5% of amino acids has an average 3D-1D score greater than or equal to 0.2, as shown in Supplementary Figure S2(A). The predicted model assessed by ERRAT showed a quality factor of 95.7%, as shown in Supplementary Figure S2(B). ProSA predicted the Z-score for the model and its homologous structure of −9.15 and −8.82, respectively, as shown in Supplementary Figure S2(C,D). LG score of 4.64 for the predicted model showed that the model was extremely good. The superposition of the validated model of *PkADH* mutant with the crystal structure of zinc-dependent ADH-2 from *Streptococcus pneumonia*-apo (PDB ID: 5YLN) shows RMSD of 0.12 Å. Further, the RMSD graph of molecular dynamics revealed that the predicted model had no major fluctuation after 29 ns molecular simulation of 100 ns, as shown in Supplementary Figure S3. Overall, all these
validations confirmed that the model is reliable and can be used for further study. The predicted model consists of 14 \( \alpha \) helices and 16 \( \beta \) sheets, as shown in Figure 4.

The Dali server was used to determine the structural homologous of \( \text{PkADH} \). There are 15 structures that possess Z-score of more than 39.3 and RMSD less than 2.1 Å. Protein structures with a Z-score above 42.7 and RMSD less than 1.9 Å were considered to compare with PKAD: zinc-dependent ADH-2 from \( \text{Streptococcus pneumonia} \) - apo form (PDB ID: SYLN), ADH TADH from \( \text{Themus sp. ATN1 (PDB ID: 4CPD)} \), crystal structure of a thermophilic ADH substrate complex from \( \text{Thermoanaerobacter brockii} \) (PDB ID: 1BXZ) and ADH from \( \text{Entamoebahistolotica} \) in complex with cacodylate (PDB ID: 1Y9A). It has been reported from the crystal structure of thermophilic ADH substrate complex from \( \text{Thermoanaerobacter brockii} \) (PDB ID: 1BXZ) that His59 and Asp150 are at the active site and play a vital role in the binding of a substrate (Li et al., 1999). His59 and Asp150 of thermophilic ADH substrate complex from \( \text{Thermoanaerobacter brockii} \) correspond to His69 and Asp163 in \( \text{PkADH} \). Multiple sequence alignment of \( \text{PkADH} \) with the other homologous ADHs was performed and generated using Clustal Omega and Esprit3.0, respectively, as shown in Supplementary Figure S4.

### 3.5. Molecular docking

Molecular docking was used to check the preference of binding of the orientation of ligands with protein. The molecular docking of AD and ethanol with \( \text{PkADH} \) mutant was done using AutoDock Vina. AD and ethanol form three hydrogen bonds with Ser42, His69 and Asp163 of \( \text{PkADH} \) mutant. ADH-1 from \( \text{P. kudriaevzevii BGY1} \) utilizes AD as a substrate and converts it into ethanol. The interacting residues of \( \text{PkADH} \) mutant with AD and ethanol are shown in Figure 5. The binding affinity of substrate and products with \( \text{PkADH} \) mutant are shown in Table 1. Both substrate (AD) and product (ethanol) are bound at the same site of \( \text{PkADH} \) mutant, as shown in Figure 5.

### 3.6. Molecular dynamics simulation

The structural and conformational changes at the atomistic level of ADH from \( \text{P. kudriaevzevii BGY1} \) (\( \text{PkADH} \) mutant) with AD and ethanol were determined using molecular dynamics simulation. The molecular dynamics was done using GROMACS and the results (RMSD, Rg, SASA, hydrogen bond numbers and PCA) were analyzed. Several studies have used molecular dynamics to assess the stability of protein-ligand complexes (Dhankhar, Dalal, Singh, et al., 2020; Kesari et al., 2020; Kumar, Dalal, Kokane, et al., 2020; Kumar, Dalal, Sharma, et al., 2020).

The conformational movements of Cx backbone atoms of \( \text{PkADH} \) mutant and \( \text{PkADH} \)-mutant ligand(s) complexes were determined using RMSD. The lesser RMSD of \( \text{PkADH} \)-mutant AD and \( \text{PkADH} \)-mutant ethanol complexes as compared to
PkADH mutant suggested that PkADH-mutant ligand(s) complexes were more stable than PkADH mutant (Figure 6(A)). The average RMSD of PkADH mutant, PkADH-mutant AD and PkADH-mutant ethanol complexes were 0.39 ± 0.03, 0.32 ± 0.02 and 0.33 ± 0.02 nm, respectively (Table 2). The ligand RMSD of AD and ethanol is shown in Figure 6(B). The average RMSD of AD and ethanol are 0.007 ± 0.002 and 0.009 ± 0.003 nm, respectively, as shown in Table 2. Overall, RMSD results indicated that the binding of AD and ethanol to PkADH mutant form stable complexes.

The radius of gyration was computed to determine the compactness and stability of PkADH mutant and PkADH-mutant ligand(s) complexes during the molecular simulation. The PkADH-mutant ligand(s) complexes showed lesser Rg than PkADH mutant, as shown in Figure 7. The average Rg values of PkADH mutant and PkADH-mutant ligand(s) complexes are shown in Table 2. SASA values signify the area of a protein covered by the solvent molecules. PkADH-mutant AD and PkADH-mutant ethanol complexes exhibit lesser SASA values than PkADH mutant, as shown in Supplementary Figure S5. The average SASA values of PkADH mutant, PkADH-mutant AD and PkADH-mutant ethanol complexes are 156.50 ± 5.74, 151.82 ± 3.94 and 152.36 ± 5.01 nm², respectively, as shown in Table 2. Rg and SaSA values revealed that PkADH-mutant AD and PkADH-mutant ethanol complexes were more stable and compact as compared to PkADH mutant.

The g_hbond tool was used to predict the hydrogen bonds in order to determine the stability of PkADH mutant and PkADH-mutant ligand(s) complexes during the molecular simulation. The PkADH mutant showed intra-protein hydrogen bonds in the range of 230 to 270, as shown in Figure 8(A). PkADH-mutant AD and PkADH-mutant ethanol complexes had a more number of intra-protein hydrogen bonds than PkADH mutant, as shown in Figure 8(B,C). The average

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Table 3. MMPBSA binding free energy of PkADH-mutant acetaldehyde and PkADH-mutant ethanol calculated from the last 20 ns (80–100 ns) from molecular dynamics. Van der Waals, electrostatic, polar solvation, SASA and binding energy of protein–ligand complexes are in kJ/mol.

| Energy (kJ/mol)                  | PkADH-mutant acetaldehyde | PkADH-mutant ethanol |
|----------------------------------|---------------------------|----------------------|
| Van der Waals energy             | −39.50 ± 0.73             | −33.66 ± 1.17        |
| Electrostatic energy             | −36.92 ± 0.81             | −37.63 ± 1.22        |
| Polar solvation energy           | 15.45 ± 1.43              | 14.29 ± 1.48         |
| SASA energy                      | −4.33 ± 0.06              | −4.86 ± 0.07         |
| Binding energy                   | −65.34 ± 1.05             | −61.89 ± 1.41        |

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Figure 10. Principal component analysis (PCA) of PkADH mutant (black color), PkADH-mutant acetaldehyde (red color) and PkADH-mutant ethanol (green color) complexes for the molecular simulation of 100 ns. (A) Eigenvalues versus eigenvector index for the first 20 eigenvectors. (B) 2D projection showing projection on eigenvector 1 (PC1) and projection on eigenvector 2 (PC2) for PkADH mutant and PkADH-mutant ligand(s) complexes.
number of intra-protein hydrogen bonds of PkADH mutant and PkADH-mutant ligand(s) complexes are shown in Table 2.

From Figure 9(A), it can be clearly seen that PkADH-mutant AD complex was stabilized by 2 to 4 hydrogen bonds during the molecular simulation. PkADH-mutant ethanol complex displayed a minimum of 2 hydrogen bonds throughout the molecular simulation (Figure 9(B)). Overall, hydrogen bond analysis revealed that binding of AD and ethanol form stable PkADH-mutant AD and PkADH-mutant ethanol complexes. Further, the superposition of PkADH-mutant AD and PkADH-mutant ethanol complexes at different time intervals (0, 20, 40, 60, 80 and 100 ns) of molecular simulation confirmed that PkADH mutant sustained the interactions with AD and ethanol (Supplementary Figure S6).

Essential dynamics were used to determine the conformational motion between PkADH mutant and PkADH-mutant ligand(s) complexes. The dynamical differences of Cα were predicted along 1092 eigenvectors for PkADH-mutant Native and PkADH-mutant ligand(s) complexes. From Figure 10(A), it can be clearly observed that the first 10 eigenvectors showed 76.01%, 71.31% and 70.85% movements for PkADH mutant, PkADH-mutant AD and PkADH-mutant ethanol complexes, respectively. The first two eigenvectors contributed 51.41%, 48.52% and 47.42% for PkADH mutant, PkADH-mutant AD and PkADH-mutant ethanol complexes, respectively. Therefore, two-dimensional conformational space along the first eigenvector (PC1) and second eigenvector (PC2) were generated for PkADH mutant and PkADH-mutant ligand(s) complexes, as shown in Figure 10(B). PCA along PC1 and PC2 affirmed that binding of ligands to PkADH mutant form higher stable PkADH-mutant AD and PkADH-mutant ethanol complexes as compared to PkADH mutant.

3.7. MMPBSA binding energy

The binding energy calculations of protein–ligand complexes were performed using MMPBSA. The trajectories from 40 to 60 ns, 60 to 80 ns and 80 to 100 ns of molecular dynamics simulation were used to estimate the van der Waals energy, electrostatic energy, polar solvation energy and SASA energies of PkADH-mutant ligand(s) complexes. The binding affinity of PkADH-mutant AD and PkADH-mutant ethanol complexes are $-65.34 \pm 1.05$ and $-61.89 \pm 1.41$ kJ/mol, respectively as shown in Table 3. Further, MMPBSA binding free energy calculated from 40 to 60 ns and 60 to 80 ns also revealed that ligands bound PkADH mutant was stable (Supplementary Table S1). MMPBSA results confirmed that the binding of AD and ethanol resulted in the formation of lower energy stable PkADH-mutant AD and PkADH-mutant ethanol complexes.

4. Conclusion

In the current study, P. kudriavzevii BGY1 isolated from black grapes was identified as high efficacy ethanol-producing yeast with high ADH activity and capability to produce ethanol effectively from both hexose (glucose) and pentose (xylose) sugars. The mutant strain of P. kudriavzevii (BGY1-γm), was found superior for batch fermentation of pentose sugar as well as co-fermentation of C6/CS sugars as compared to its wild-type strain and S. cerevisiae. Based on 99% sequence homology of the mutant (BGY1-γm) ADH encoding gene with the gene sequences from other yeast available in GenBank, the enzyme was identified as ADH-1 type. The molecular docking study revealed that substrate (AD) and product (ethanol) can bind efficiently at the active site of PkADH mutant. Molecular dynamics and MMPBSA studies confirmed that the binding of AD and ethanol with PkADH resulted in the formation of the lower energy PkADH-mutant ligand(s) complexes. The efficacy for ethanol production by the P. kudriavzevii BGY1-γm can be further enhanced after proper optimization and bioreactor studies. The native P. kudriavzevii BGY1 and its mutant strain can be utilized efficiently for bioethanol production from the sugars of lignocellulosic biomass.

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Disclosure statement

No conflict of interest is reported by the authors

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