High-yield production of active recombinant S. simulans lysostaphin expressed in E. coli in a laboratory bioreactor

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High-level heterologous expression of active *Chaetomium thermophilum* FDH in *Pichia pastoris*

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Abstract

Nowadays, the use of formate dehydrogenase (FDH, EC 1.17.1.9) is well established as a means of NADH regeneration from NAD^+ via the coupled conversion of formate into carbon dioxide. Recent studies have been reported that specifically *Chaetomium thermophilum* FDH (*Ct*FDH) is the most efficient FDH catalyzing this reaction in reverse (i.e. using CO_2 as a substrate to produce formate, and thereby regenerating NAD^+). However, to date the production of active *Ct*FDH at high protein expression levels has received relatively little attention. In this study, we have tested the effect of

batch and high cell density fermentation (HCDF) strategies in a small stirred fermenter, as well as the effect of supplementing the medium with casamino acids, on the expressed level of secreted *Ct*FDH using *P. pastoris*. We have established that the amount of expressed *Ct*FDH was indeed enhanced via a HCDF strategy and that extracellular protease activity was eliminated via the addition of casamino acids into the fermentation medium. On this basis, secreted *Ct*FDH in an active form can be easily separated from the fermentation and can be used for subsequent biotechnological applications.

Keywords

*Ct*FDH, *P. pastoris* expression system, HCDF strategy, Casamino acid addition

Introduction

Nicotinamide cofactors are of great importance in enzyme catalysis. Since they are expensive to provide in stoichiometric amounts to synthetic reactions, they are usually regenerated. To date several strategies have been proposed and developed for nicotinamide cofactor regeneration. Although chemical methods are favorable, they often lack the required specificity for the formation of active cofactors and require relatively harsh reaction conditions (frequently leading to a loss of cofactor stability). Electrochemical or photochemical methods need electrical or light energy, respectively, to conduct the regeneration of cofactors but have the same limitations as chemical methods. As a consequence, several enzymatic approaches for cofactor regeneration have been proposed [1,2]. Formate dehydrogenase (FDH) has been reported as the most suitable enzyme, amongst those tested, due to several advantages in the regeneration process of NAD(P)H, including a favorable thermodynamic equilibrium and the production of an inert by-product, CO_2 [3–5].

In this way, FDHs are used most frequently to catalyze the oxidation of formic acid into CO_2 while coupled to the reduction of $NAD(P)^+$ to NAD(P)H. FDHs have therefore found a broad range of applications for cofactor regeneration in the synthesis of optically active chiral compounds such as L-*tert*-leucine [6–11]. Recently studies have been reported that FDHs can also be used in reverse and thereby also have the potential to reduce CO_2, while regenerating $NAD(P)^+$, although methods to drive the equilibrium would be required [4,12–15]. Indeed, effective methods for $NAD(P)^+$ regeneration are still needed, since only a few NADH oxidases are commercially available [16,17]. Amongst the reported FDHs, *Chaetomium thermophilum* FDH (*Ct*FDH) has a wide catalytic range of operational pH and shows moderate thermo-stability, which is important for regeneration conditions in either direction [12]. Moreover, *Ct*FDH is one of the most efficient of the known FDH enzymes capable of reducing directly CO_2 and HCO_3^- to formate [12–14,18]. These two properties motivated us to investigate the effective expression and synthesis of *Ct*FDH with the ultimate goal of making the enzyme more available and at a reduced cost.

Previously, an *Escherichia coli* expression system has been reported to be preferred in order to express FDHs. Nevertheless, we considered the secretory expression system of *Pichia pastoris* (*P. pastoris*) as a useful alternative. By switching to a eukaryotic expression system not only do we gain time by eliminating the strenuous cell breakdown step that is necessary for releasing target proteins produced intracellularly by *E. coli* but also avoid factors such as high cell density limit of *E. coli* in liquid culture [19]. *P. pastoris* is capable of continuously producing the target recombinant protein dismissing the periodic growth, expression and purification of *E. coli* [20]. *P. pastoris* (also known as *Komagataella pastoris*), is a methylotrophic single-cell yeast that is frequently used as a heterologous protein expression system due to rapid growth, coupled with ease

of high cell density fermentation, the possibility of extensive post-translational modification, as well inexpensive and straightforward growth requirements [21,22].

In this study, different fermentation strategies such as batch and High Cell Density Fermentation (HCDF) to produce higher amounts of *Ct*FDH in an active form were tested using BMGY (Buffered Glycerol-complex Medium) and BMMY (Buffered Methanol-complex Medium). In preliminary work, the pPICZαA with 6xHis-Tag *Ct*FDH was obtained and transformed in the *P. pastoris* X33 expression system. Initially, the Invitrogen™ *P. pastoris* Expression Kit protocol was used in a batch strategy. Subsequently, an HCDF strategy was used in order to enhance the yield of the protein. Finally, the addition of casamino acids into the fermentation medium combined with the HCDF strategy was tested and this approach was found to enhance both the amount and the specific activity of recombinant *Ct*FDH [23,24].

Materials and methods

Yeast strain, plasmid, and reagents

Chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, ABD) unless indicated otherwise. Casamino Acids (Bacto casamino acids: acid-hydrolyzed casein, low sodium chloride, and iron concentrations) Cat #223050 were purchased from Becton, Dickinson and Company, Franklin Lakes, New Jersey, ABD. The bicinchoninic acid (BCA) Protein Assay Kit was purchased from Thermo Scientific (Waltham, Massachusetts, ABD) and the His-Trap column was purchased from GE Healthcare (Chicago, Illinois, USA). All measurements were performed in triplicate. An expression cassette encoding an α-factor signal sequence for secretion, the *fdh* gene from *Chaetomium thermophilum* with a 6xHis-Tag (UniProt accession number: **G0SGU4)** was cloned into the *p*PICZα A (provided by BERC Lab, Turkey). The resulting construct was transformed into chemically competent *P. pastoris* X33 strain (Invitrogen™) according to the manufacturer's

92 instructions and used for expression of C-terminal 6xHis-Tagged *Ct*FDH [25]. A cell stock of this
93 strain was prepared in 50 % glycerol and stored at -80 °C.

94 ***Fermentation strategies***

95 Fermentation of *P. pastoris* cells for extracellular *Ct*FDH expression was carried out by using a
96 batch and a high cell density fed-batch fermentation (HCDF) strategy in a 3 L jacketed bench-top
97 stirred tank fermenter (STR) (Applikon, Schiedam, The Netherlands). The fermenter was equipped
98 with a temperature probe, a sparger, dissolved oxygen (DO) probe and pH probe.

99 YPD broth (Yeast extract peptone dextrose broth, 10 g/L yeast extract, 20 g/L peptone and 20 g/L
100 dextrose) and BMGY (Buffered glycerol-complex medium, 10 g/L yeast extract, 20 g/L peptone, 40
101 ml/L glycerol, 1.34 % (w/v) YNB, $4x10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate buffer
102 pH 6.0) medium were used for pre-culture and the inoculum for the fermenter, respectively. A
103 single colony of transformed *P. pastoris* X33/pPICZα A cells was pre-grown overnight in a 100 mL
104 shake flask filled with 10 mL YPD broth at 28 °C and agitated at 180 rpm. Inoculum for the
105 fermenter was initiated in four 500 mL baffled shake flasks filled with 50 mL BMGY (1 % yeast
106 extract, 2 % peptone, 1.34 % YNB, 4 % glycerol, $4x10^{-5}$ % biotin and 100mM potassium phosphate
107 buffer pH 6.0) and subsequently inoculated (5 mL) into pre-cultures at 28 °C and agitated at 230
108 rpm until the cell concentration reached approximately 1.5 g dcw/L.

109 ***Batch strategy***

110 Recombinant *Ct*FDH was expressed extracellularly by *P. pastoris* X33 using the expression
111 procedure in the Invitrogen™ *Pichia* Expression Kit User Guide. The induction stage of the batch
112 operation of transformed *P. pastoris* cells was initiated by inoculating the fermenter with 10 % of 2
113 L BMMY (Buffered methanol-complex medium, 10 g/L yeast extract, 20 g/L peptone, 1.34 % (w/v)
114 YNB, $4x10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate buffer pH 6.0, 2 % methanol) which

was prepared and sterilized in the 3 L STR and incubated for 48 hours according to shake flask studies **(Figure S1)**. The fermentation parameters of the fermenter were as follows: temperature 18 °C, airflow 1 volume of air per volume of fermentation broth per minute (vvm) and the dissolved oxygen (DO) at 60 %, controlled in cascade mode with an agitation speed between 400-800 rpm and pH 6.0 (maintained via the addition of 3 M KOH and 3 M H_3PO_4, as required). The culture was induced every 24 hours with methanol (2% v/v). The 0.5% (v/v), 1% (v/v) and 2% (v/v) methanol had been previously tested for the induction stage in shake flask studies (**Figure S2, Table S1**) and the optimum methanol concentration was identified as 2% (v/v). 1 mL samples were collected at 0, 6, 18, 24 and 48 hours from induction to measure the activity, dry cell weight (dcw) and thereby calculate biomass yield on substrate (Y_{XS}) (Eq S1). The supernatant part of the fermentation broth was separated by centrifugation at 4500 rpm (30 minutes) and stored at -80 °C for further analysis.

High cell density fermentation (HCDF) strategy

Recombinant *P. pastoris* fermentation comprised of three phases: Glycerol batch phase (phase I), glycerol fed-batch phase (Phase II) and methanol induction phase (Phase III). In this study, two different HCDF strategies were carried out in order to enhance active *Ct*FDH secretion. This was developed according to the Invitrogen™ Pichia fermentation process guidelines with some modifications [26]. The HCDF1 of *P. pastoris* was started with Phase I for initial cell growth in BMGY prepared and sterilized in the 3 L STR. Phase I lasted for 28 hours by controlling the DO and Phase II was commenced for further cell growth by feeding a glycerol solution (40% (v/v)) glycerol, 1.34 % (w/v) YNB, $4x10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate buffer pH 6.0) at a rate of 1.44 to 12.96 mL/(L.h). When the cell concentration reached approximately 45 g dcw/L, glycerol feeding was stopped and left for a further 4 hours (to allow the cells to consume residual glycerol in the reaction environment). DO spikes were followed in order to determine

whether glycerol was consumed from the culture environment before adding methanol [26]. Throughout Phases I and II, the temperature was maintained at 28 °C and pH was controlled at 6.0 by using 3 M KOH and 3 M H_3PO_4. The DO was maintained above 60 % air saturation by supplying filter-sterilized air and automatic adjustment of the agitation speed between 400 and 800 rpm. The methanol induction phase (Phase III) was started by the addition of methanol (2.6 mL/L.h – 7 mL/L.h) and proceeded for 48 hours, during which time the temperature was maintained at 18 °C and the pH was controlled at 6.0 by using 3 KOH and 3 M H_3PO_4. The DO was maintained above 60% air saturation by supplying filter-sterilized air and automatic adjustment of the agitation speed between 400 and 800 rpm. The same fermentation parameters with HCDF1 were used for HCDF2 for Phase I,II and III, BMGY containing 5% (w/v) casamino acids [27] acid and glycerol feeding with 5% (w/v) casamino acids were used during Phase I and Phase II respectively. Centrifugation was applied at the end of Phase III to recover the supernatant and stored at -80 C for further analysis. 1 mL samples were collected at 0, 24, 28, 46, 52, 55, 66, 72, 84, 90 hours and 114 hours of the reaction in order to measure the activity, cell concentration (g dcw/L and hence calculate Y_{XS} (Eq S1).

Purification of secreted CtFDH from P. pastoris

The stored supernatant part of the culture was passed through a 0.45 µm filter, and samples were then loaded on a His-trap column (after equilibration with buffer A (20 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole pH 7.4)). 6xHis-tagged *Ct*FDH was purified with an optimized TAGZyme purification system (QIAGEN) by eluting with buffer A containing different imidazole concentrations (100, 200, 400 and 500 mM). Collected fractions were analyzed by SDS-PAGE in order to determine fractions bearing pure *Ct*FDH which were subsequently combined. Protein samples were then concentrated in ultracentrifuge tubes (MM Amicon Ultracel-30K). The buffer

was exchanged with NaH_2PO_4 (20 mM, pH 7.0) using a PD-10 desalting column (Amersham Biosciences, Little Chalfont, United Kingdom) at 4 °C and the purified protein was stored at 4 °C for further analysis. The same purification protocol was applied for both fermentation strategies. The protein concentration was measured with a BCA Protein Assay Kit and the product yield on biomass (Y_{PX}) was calculated for both strategies via **Eq S2**.

SDS-PAGE analysis

SDS-PAGE electrophoresis (Bio-Rad electrophoresis unit) was performed according to the method of Laemmli [28] by using 12 % SDS-polyacrylamide gels to determine the purity and approximate molecular mass of the enzyme. All proteins run on the polyacrylamide gel were stained with 0.2% (w/v) Coomassie brilliant blue R-250 for 1 h, then washed with the SDS-PAGE destaining solution (100 mL acetic acid, 300 mL methanol, 600 mL dH_2O) to remove excess dye.

Activity determination of pure CtFDH

The purified enzyme activity was tested by monitoring the formation of NADH at 340 nm after adding the purified *Ct*FDH to the reaction mixture having 20 mM sodium acetate buffer pH 5.0 and 3.0 mM NAD^+, 30 mM sodium formate at 25 °C, during 5 minutes was assayed as previously described [12]. One-unit enzyme oxidized 1.0 μmole of formate to CO_2 per minute in the presence of ß-NAD, at pH 5.0 at 25 °C.

Results and Discussions

Cell growth profile of batch and HCDF strategy

Recombinant *Ct*FDH was successfully expressed extracellularly through bench-top culturing of *P. pastoris* X33 in a batch and HCDF manner. The cell growth profile indicated that transformed *P. pastoris* cells were able to use methanol as a carbon source with the tested fermentation parameters **(Figure 1)**. However, the cell density reached 7.3 g dcw/L at the end of 48 hours induction with 2%

184 (v/v) methanol **(Figure 1-a)**. On the other hand, the capacity to grow in a minimal medium at a high
185 density of *P. pastoris* cells facilitated the cellular yield through an HCDF strategy (HCDF1 and
186 HCDF2) during Phase I and Phase II that demonstrated the efficiency of applying HCDF strategy in
187 comparison to the batch strategy on the bioreactor **(Figure 1-b)** [29]. Since the temperature was
188 decreased from 28 °C to 18 °C at the beginning of Phase III, the growth rate of *P. pastoris* cells
189 diminished and the adaptation into the new environment took approximately 6 hours [30]. As
190 shown in **Figure 1-b**, applying HCDF strategy of *P. pastoris* contributed to the cellular yield which
191 results in 44.9 g dcw/L and 46.9 g dcw/L for HCDF1 and HCDF2 respectively. The biomass yields
192 on carbon for both strategies were calculated and presented in **Table 1**. The results clearly indicate
193 the biomass yield of *P. pastoris* cells was improved about 20-fold in the presence of glycerol in a
194 stirred tank fermenter. The yield of biomass obtained on methanol was lower than that attained with
195 glycerol [31]. Even though the Y_{XS} of Phase II of both HCDF1 and HCDF2 were lower than the
196 batch induction phase, the amount of obtained enzyme was higher than that expressed using the
197 batch strategy. Therefore, enhancing the cell concentration via the fed-batch glycerol fed-batch
198 stage improved the yield of secreted *Ct*FDH.

199 ***Secretion of Ct*FDH *and its purification***

200 The preferred *P. pastoris* as a host system provided a high-yield extracellular protein production via
201 fusion product to the secretion signal of the α-mating factor that eliminates the step of harvesting
202 the disruption of the yeast cells [29]. The purification of secreted recombinant *Ct*FDH with C-
203 terminal 6XHis-tag was conducted using immobilized metal affinity chromatography (IMAC). The
204 performed SDS procedure **(Figure 2)** following purification confirmed the presence and the
205 molecular weight of the target protein FDH as ~45 kDa. Also, the expected protein size was
206 correlated with reported results [32]. An imidazole gradient from 10 to 500 mM was applied in

order to optimize the purification procedure. The fractions (E2 and E3) containing the pure protein were pooled together, concentrated and gave a *Ct*FDH yield of 48.6 mg from a liter culture (HCDF2) with over 90 % purity based on SDS-PAGE analysis. The purity of the *Ct*FDH was sufficient for further experiments.

Activity measurement of CtFDH expressed via different strategies

FDH from *Chaetomium thermophilum* has been reported as one of the most efficient known FDH enzymes in converting formate to CO_2. The results of shake flask studies were summarized in Table S1 which represented that the amount of secreted *Ct*FDH enhanced as an increased concentration of methanol. Moreover, the highest methanol concentration had not any adverse effect on the activity of *Ct*FDH since the calculated specific activities were approximately the same. The activities were assayed during the expression of all strategies from collected supernatants. However, since they were highly diluted due to being in the supernatant, their concentration was not ample enough to get a satisfactory reading. Therefore, at the end of fermentation, all produced *Ct*FDH was collected and assayed. The calculated activity, specific activity, the obtained amount of pure *Ct*FDH, the specific product yield and the previously reported results are presented in **Table 2**. The efficiency of two expression systems, *E. coli* and *P. pastoris* for producing FDH was tested in previously reported studies [12,18,20,32]. Amongst these the most promising one was obtained as ~50 mg/L with 0.40 U/mg specific activity by *E. coli*, which is approximate 10-fold higher than obtained from *P. pastoris* cultured in a shake flask [20,32]. In this study, the acquired pure *Ct*FDH via HCDF2 showed the best activity **(Figure S3)** at about 7.5-fold and 11-fold more than the batch and HCDF1 strategy respectively. This clearly shows the benefits of enhanced expression via an appropriate fermentation strategy.

A summary of the performance of the different strategies with respect to protein synthesis is presented in **Table 2**. The conducted bioreactor studies showed that the highest amount of *Ct*FDH obtained was 48.6 mg/L via HCDF2. The activity and specific activity of *Ct*FDH were enhanced about 1.5-fold through the addition of casamino acids into the fermentation medium, repressing extracellular protease activity as suggested by other studies [23,33,34]. On the other hand, while the addition of casamino acid had no significant effect on Y_{PX} of HCDF1 and HCDF2, applying the HCDF strategy resulted in a 2-fold increase in product yield on biomass (Y_{PX}). Additionally, space-time yield (U/L per day) was calculated for batch, HCDF1 and HCDF2 as 1.35, 5.84 and 8.85 U/L per day respectively. HCDF strategy implementation and the addition of casamino acid had a dramatic effect on the space-time yield.

Although the amount of enzyme in this study was approximately the same as the expressed *Ct*FDH via *E. coli*, the secreted enzyme showed approximately 2-fold higher specific activity [32]. These results showed that our obtained *Ct*FDH is far more active than the previously reported version. For further improvement on the yield of *Ct*FDH as follows; strain improvement can be performed for elimination of vacuolar proteases [35], lower pH values facilitate declining neutral extracellular protease effect on enzyme [36], using sorbitol as a co-substrate in methanol induction phase provides the higher cell density of *P. pastoris* and reduces the specific protease production [37], decreasing the airflow rate by replacing air with pure O_2 in order to decrease the enzyme inactivation effect of gas bubbles [38].

Conclusion

In this study, the results demonstrated that HCDF of *P. pastoris* with BMMY medium containing casamino acids is the best methodology for *Ct*FDH secretion in a stirred-tank bioreactor. Although the *E. coli* expression system has been well-studied, the *P. pastoris* expression system to secrete

protein into media facilitated the elimination one of the downstream process steps of breaking down the cell mass to release the desired proteins as it would have been necessary if the expression was carried in *E. coli*. Additionally, high-cell density growth of *P. pastoris* provided higher protein yield in STR and facilitated about 3-fold higher *Ct*FDH than reported by Özgün G. et al., (2015) [18], about the same amount of *Ct*FDH with *Cl*FDH [39] and obtaining 2-fold more than *Lb*FDH [40]. This work is the first in which *P. pastoris* expression system was used as a host expression system for expression of FDH enzyme in STR which The Given the ease of upscaling fermentation of *P. pastoris*, these findings will open-up possibilities for larger-scale production of recombinant *Ct*FDH enzyme.

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High-level heterologous expression of active *Chaetomium thermophilum* FDH in *Pichia pastoris*

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Abstract

Nowadays, the use of formate dehydrogenase (FDH, EC 1.17.1.9) is well established as a means of NADH regeneration from NAD^+ via the coupled conversion of formate into carbon dioxide. Recent studies have been reported that specifically *Chaetomium thermophilum* FDH (*Ct*FDH) is the most efficient FDH catalyzing this reaction in reverse (i.e. using CO_2 as a substrate to produce formate, and thereby regenerating NAD^+). However, to date the production of active *Ct*FDH at high protein expression levels has received relatively little attention. In this study, we have tested the effect of

batch and high cell density fermentation (HCDF) strategies in a small stirred fermenter, as well as the effect of supplementing the medium with casamino acids, on the expressed level of secreted *Ct*FDH using *P. pastoris*. We have established that the amount of expressed *Ct*FDH was indeed enhanced via a HCDF strategy and that extracellular protease activity was eliminated via the addition of casamino acids into the fermentation medium. On this basis, secreted *Ct*FDH in an active form can be easily separated from the fermentation and can be used for subsequent biotechnological applications.

Keywords

*Ct*FDH, *P. pastoris* expression system, HCDF strategy, Casamino acid addition

Introduction

Nicotinamide cofactors are of great importance in enzyme catalysis. Since they are expensive to provide in stoichiometric amounts to synthetic reactions, they are usually regenerated. To date several strategies have been proposed and developed for nicotinamide cofactor regeneration. Although chemical methods are favorable, they often lack the required specificity for the formation of active cofactors and require relatively harsh reaction conditions (frequently leading to a loss of cofactor stability). Electrochemical or photochemical methods need electrical or light energy, respectively, to conduct the regeneration of cofactors but have the same limitations as chemical methods. As a consequence, several enzymatic approaches for cofactor regeneration have been proposed [1,2]. Formate dehydrogenase (FDH) has been reported as the most suitable enzyme, amongst those tested, due to several advantages in the regeneration process of NAD(P)H, including a favorable thermodynamic equilibrium and the production of an inert by-product, CO_2 [3–5].

In this way, FDHs are used most frequently to catalyze the oxidation of formic acid into CO_2 while coupled to the reduction of $NAD(P)^+$ to NAD(P)H. FDHs have therefore found a broad range of applications for cofactor regeneration in the synthesis of optically active chiral compounds such as L-*tert*-leucine [6–11]. Recently studies have been reported that FDHs can also be used in reverse and thereby also have the potential to reduce CO_2, while regenerating $NAD(P)^+$, although methods to drive the equilibrium would be required [4,12–15]. Indeed, effective methods for $NAD(P)^+$ regeneration are still needed, since only a few NADH oxidases are commercially available [16,17]. Amongst the reported FDHs, *Chaetomium thermophilum* FDH (*Ct*FDH) has a wide catalytic range of operational pH and shows moderate thermo-stability, which is important for regeneration conditions in either direction [12]. Moreover, *Ct*FDH is one of the most efficient of the known FDH enzymes capable of reducing directly CO_2 and HCO_3^- to formate [12–14,18]. These two properties motivated us to investigate the effective expression and synthesis of *Ct*FDH with the ultimate goal of making the enzyme more available and at a reduced cost.

Previously, an *Escherichia coli* expression system has been reported to be preferred in order to express FDHs. Nevertheless, we considered the secretory expression system of *Pichia pastoris* (*P. pastoris*) as a useful alternative. By switching to a eukaryotic expression system not only do we gain time by eliminating the strenuous cell breakdown step that is necessary for releasing target proteins produced intracellularly by *E. coli* but also avoid factors such as high cell density limit of *E. coli* in liquid culture [19]. *P. pastoris* is capable of continuously producing the target recombinant protein dismissing the periodic growth, expression and purification of *E. coli* [20]. *P. pastoris* (also known as *Komagataella pastoris*), is a methylotrophic single-cell yeast that is frequently used as a heterologous protein expression system due to rapid growth, coupled with ease

of high cell density fermentation, the possibility of extensive post-translational modification, as well inexpensive and straightforward growth requirements [21,22].

In this study, different fermentation strategies such as batch and High Cell Density Fermentation (HCDF) to produce higher amounts of *Ct*FDH in an active form were tested using BMGY (Buffered Glycerol-complex Medium) and BMMY (Buffered Methanol-complex Medium). In preliminary work, the pPICZαA with 6xHis-Tag *Ct*FDH was obtained and transformed in the *P. pastoris* X33 expression system. Initially, the Invitrogen™ *P. pastoris* Expression Kit protocol was used in a batch strategy. Subsequently, an HCDF strategy was used in order to enhance the yield of the protein. Finally, the addition of casamino acids into the fermentation medium combined with the HCDF strategy was tested and this approach was found to enhance both the amount and the specific activity of recombinant *Ct*FDH [23,24].

Materials and methods

Yeast strain, plasmid, and reagents

Chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, ABD) unless indicated otherwise. Casamino Acids (Bacto casamino acids: acid-hydrolyzed casein, low sodium chloride, and iron concentrations) Cat #223050 were purchased from Becton, Dickinson and Company, Franklin Lakes, New Jersey, ABD. The bicinchoninic acid (BCA) Protein Assay Kit was purchased from Thermo Scientific (Waltham, Massachusetts, ABD) and the His-Trap column was purchased from GE Healthcare (Chicago, Illinois, USA). All measurements were performed in triplicate. An expression cassette encoding an α-factor signal sequence for secretion, the *fdh* gene from *Chaetomium thermophilum* with a 6xHis-Tag (UniProt accession number: **G0SGU4)** was cloned into the *p*PICZα A (provided by BERC Lab, Turkey). The resulting construct was transformed into chemically competent *P. pastoris* X33 strain (Invitrogen™) according to the manufacturer's

instructions and used for expression of C-terminal 6xHis-Tagged *Ct*FDH [25]. A cell stock of this strain was prepared in 50 % glycerol and stored at -80 °C.

Fermentation strategies

Fermentation of *P. pastoris* cells for extracellular *Ct*FDH expression was carried out by using a batch and a high cell density fed-batch fermentation (HCDF) strategy in a 3 L jacketed bench-top stirred tank fermenter (STR) (Applikon, Schiedam, The Netherlands). The fermenter was equipped with a temperature probe, a sparger, dissolved oxygen (DO) probe and pH probe.

YPD broth (Yeast extract peptone dextrose broth, 10 g/L yeast extract, 20 g/L peptone and 20 g/L dextrose) and BMGY (Buffered glycerol-complex medium, 10 g/L yeast extract, 20 g/L peptone, 40 ml/L glycerol, 1.34 % (w/v) YNB, $4x10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate buffer pH 6.0) medium were used for pre-culture and the inoculum for the fermenter, respectively. A single colony of transformed *P. pastoris* X33/pPICZα A cells was pre-grown overnight in a 100 mL shake flask filled with 10 mL YPD broth at 28 °C and agitated at 180 rpm. Inoculum for the fermenter was initiated in four 500 mL baffled shake flasks filled with 50 mL BMGY (1 % yeast extract, 2 % peptone, 1.34 % YNB, 4 % glycerol, $4x10^{-5}$ % biotin and 100mM potassium phosphate buffer pH 6.0) and subsequently inoculated (5 mL) into pre-cultures at 28 °C and agitated at 230 rpm until the cell concentration reached approximately 1.5 g dcw/L.

Batch strategy

Recombinant *Ct*FDH was expressed extracellularly by *P. pastoris* X33 using the expression procedure in the Invitrogen™ *Pichia* Expression Kit User Guide. The induction stage of the batch operation of transformed *P. pastoris* cells was initiated by inoculating the fermenter with 10 % of 2 L BMMY (Buffered methanol-complex medium, 10 g/L yeast extract, 20 g/L peptone, 1.34 % (w/v) YNB, $4x10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate buffer pH 6.0, 2 % methanol) which

was prepared and sterilized in the 3 L STR and incubated for 48 hours according to shake flask studies **(Figure S1)**. The fermentation parameters of the fermenter were as follows: temperature 18 °C, airflow 1 volume of air per volume of fermentation broth per minute (vvm) and the dissolved oxygen (DO) at 60 %, controlled in cascade mode with an agitation speed between 400-800 rpm and pH 6.0 (maintained via the addition of 3 M KOH and 3 M H_3PO_4, as required). The culture was induced every 24 hours with methanol (2% v/v). The 0.5% (v/v), 1% (v/v) and 2% (v/v) methanol had been previously tested for the induction stage in shake flask studies (**Figure S2, Table S1**) and the optimum methanol concentration was identified as 2% (v/v). 1 mL samples were collected at 0, 6, 18, 24 and 48 hours from induction to measure the activity, dry cell weight (dcw) and thereby calculate biomass yield on substrate (Y_{XS}) (Eq S1). The supernatant part of the fermentation broth was separated by centrifugation at 4500 rpm (30 minutes) and stored at -80 °C for further analysis.

High cell density fermentation (HCDF) strategy

Recombinant *P. pastoris* fermentation comprised of three phases: Glycerol batch phase (phase I), glycerol fed-batch phase (Phase II) and methanol induction phase (Phase III). In this study, two different HCDF strategies were carried out in order to enhance active *Ct*FDH secretion. This was developed according to the Invitrogen™ Pichia fermentation process guidelines with some modifications [26]. The HCDF1 of *P. pastoris* was started with Phase I for initial cell growth in BMGY prepared and sterilized in the 3 L STR. Phase I lasted for 28 hours by controlling the DO and Phase II was commenced for further cell growth by feeding a glycerol solution (40% (v/v)) glycerol, 1.34 % (w/v) YNB, $4x10^{-5}$ % (w/v) biotin and 100 mM potassium phosphate buffer pH 6.0) at a rate of 1.44 to 12.96 mL/(L.h). When the cell concentration reached approximately 45 g dcw/L, glycerol feeding was stopped and left for a further 4 hours (to allow the cells to consume residual glycerol in the reaction environment). DO spikes were followed in order to determine

whether glycerol was consumed from the culture environment before adding methanol [26]. Throughout Phases I and II, the temperature was maintained at 28 °C and pH was controlled at 6.0 by using 3 M KOH and 3 M H_3PO_4. The DO was maintained above 60 % air saturation by supplying filter-sterilized air and automatic adjustment of the agitation speed between 400 and 800 rpm. The methanol induction phase (Phase III) was started by the addition of methanol (2.6 mL/L.h – 7 mL/L.h) and proceeded for 48 hours, during which time the temperature was maintained at 18 °C and the pH was controlled at 6.0 by using 3 KOH and 3 M H_3PO_4. The DO was maintained above 60% air saturation by supplying filter-sterilized air and automatic adjustment of the agitation speed between 400 and 800 rpm. The same fermentation parameters with HCDF1 were used for HCDF2 for Phase I,II and III, BMGY containing 5% (w/v) casamino acids [27] acid and glycerol feeding with 5% (w/v) casamino acids were used during Phase I and Phase II respectively. Centrifugation was applied at the end of Phase III to recover the supernatant and stored at -80 C for further analysis. 1 mL samples were collected at 0, 24, 28, 46, 52, 55, 66, 72, 84, 90 hours and 114 hours of the reaction in order to measure the activity, cell concentration (g dcw/L and hence calculate Y_{XS} (Eq S1).

Purification of secreted CtFDH from P. pastoris

The stored supernatant part of the culture was passed through a 0.45 μm filter, and samples were then loaded on a His-trap column (after equilibration with buffer A (20 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole pH 7.4)). 6xHis-tagged *Ct*FDH was purified with an optimized TAGZyme purification system (QIAGEN) by eluting with buffer A containing different imidazole concentrations (100, 200, 400 and 500 mM). Collected fractions were analyzed by SDS-PAGE in order to determine fractions bearing pure *Ct*FDH which were subsequently combined. Protein samples were then concentrated in ultracentrifuge tubes (MM Amicon Ultracel-30K). The buffer

was exchanged with NaH_2PO_4 (20 mM, pH 7.0) using a PD-10 desalting column (Amersham Biosciences, Little Chalfont, United Kingdom) at 4 °C and the purified protein was stored at 4 °C for further analysis. The same purification protocol was applied for both fermentation strategies. The protein concentration was measured with a BCA Protein Assay Kit and the product yield on biomass (Y_{PX}) was calculated for both strategies via **Eq S2**.

SDS-PAGE analysis

SDS-PAGE electrophoresis (Bio-Rad electrophoresis unit) was performed according to the method of Laemmli [28] by using 12 % SDS-polyacrylamide gels to determine the purity and approximate molecular mass of the enzyme. All proteins run on the polyacrylamide gel were stained with 0.2% (w/v) Coomassie brilliant blue R-250 for 1 h, then washed with the SDS-PAGE destaining solution (100 mL acetic acid, 300 mL methanol, 600 mL dH_2O) to remove excess dye.

Activity determination of pure CtFDH

The purified enzyme activity was tested by monitoring the formation of NADH at 340 nm after adding the purified *Ct*FDH to the reaction mixture having 20 mM sodium acetate buffer pH 5.0 and 3.0 mM NAD^+, 30 mM sodium formate at 25 °C, during 5 minutes was assayed as previously described [12]. One-unit enzyme oxidized 1.0 μmole of formate to CO_2 per minute in the presence of ß-NAD, at pH 5.0 at 25 °C.

Results and Discussions

Cell growth profile of batch and HCDF strategy

Recombinant *Ct*FDH was successfully expressed extracellularly through bench-top culturing of *P. pastoris* X33 in a batch and HCDF manner. The cell growth profile indicated that transformed *P. pastoris* cells were able to use methanol as a carbon source with the tested fermentation parameters **(Figure 1)**. However, the cell density reached 7.3 g dcw/L at the end of 48 hours induction with 2%

(v/v) methanol **(Figure 1-a)**. On the other hand, the capacity to grow in a minimal medium at a high density of *P. pastoris* cells facilitated the cellular yield through an HCDF strategy (HCDF1 and HCDF2) during Phase I and Phase II that demonstrated the efficiency of applying HCDF strategy in comparison to the batch strategy on the bioreactor **(Figure 1-b)** [29]. Since the temperature was decreased from 28 °C to 18 °C at the beginning of Phase III, the growth rate of *P. pastoris* cells diminished and the adaptation into the new environment took approximately 6 hours [30]. As shown in **Figure 1-b**, applying HCDF strategy of *P. pastoris* contributed to the cellular yield which results in 44.9 g dcw/L and 46.9 g dcw/L for HCDF1 and HCDF2 respectively. The biomass yields on carbon for both strategies were calculated and presented in **Table 1**. The results clearly indicate the biomass yield of *P. pastoris* cells was improved about 20-fold in the presence of glycerol in a stirred tank fermenter. The yield of biomass obtained on methanol was lower than that attained with glycerol [31]. Even though the Y_{XS} of Phase II of both HCDF1 and HCDF2 were lower than the batch induction phase, the amount of obtained enzyme was higher than that expressed using the batch strategy. Therefore, enhancing the cell concentration via the fed-batch glycerol fed-batch stage improved the yield of secreted *Ct*FDH.

Secretion of Ct*FDH *and its purification

The preferred *P. pastoris* as a host system provided a high-yield extracellular protein production via fusion product to the secretion signal of the α-mating factor that eliminates the step of harvesting the disruption of the yeast cells [29]. The purification of secreted recombinant *Ct*FDH with C-terminal 6XHis-tag was conducted using immobilized metal affinity chromatography (IMAC). The performed SDS procedure **(Figure 2)** following purification confirmed the presence and the molecular weight of the target protein FDH as ~45 kDa. Also, the expected protein size was correlated with reported results [32]. An imidazole gradient from 10 to 500 mM was applied in

207 order to optimize the purification procedure. The fractions (E2 and E3) containing the pure protein
208 were pooled together, concentrated and gave a *Ct*FDH yield of 48.6 mg from a liter culture
209 (HCDF2) with over 90 % purity based on SDS-PAGE analysis. The purity of the *Ct*FDH was
210 sufficient for further experiments.

211 ***Activity measurement of CtFDH expressed via different strategies***

212 FDH from *Chaetomium thermophilum* has been reported as one of the most efficient known FDH
213 enzymes in converting formate to CO_2. The results of shake flask studies were summarized in Table
214 S1 which represented that the amount of secreted *Ct*FDH enhanced as an increased concentration of
215 methanol. Moreover, the highest methanol concentration had not any adverse effect on the activity
216 of *Ct*FDH since the calculated specific activities were approximately the same. The activities were
217 assayed during the expression of all strategies from collected supernatants. However, since they
218 were highly diluted due to being in the supernatant, their concentration was not ample enough to get
219 a satisfactory reading. Therefore, at the end of fermentation, all produced *Ct*FDH was collected and
220 assayed. The calculated activity, specific activity, the obtained amount of pure *Ct*FDH, the specific
221 product yield and the previously reported results are presented in **Table 2**. The efficiency of two
222 expression systems, *E. coli* and *P. pastoris* for producing FDH was tested in previously reported
223 studies [12,18,20,32]. Amongst these the most promising one was obtained as ~50 mg/L with 0.40
224 U/mg specific activity by *E. coli*, which is approximate 10-fold higher than obtained from *P.*
225 *pastoris* cultured in a shake flask [20,32]. In this study, the acquired pure *Ct*FDH via HCDF2
226 showed the best activity **(Figure S3)** at about 7.5-fold and 11-fold more than the batch and HCDF1
227 strategy respectively. This clearly shows the benefits of enhanced expression via an appropriate
228 fermentation strategy.

A summary of the performance of the different strategies with respect to protein synthesis is presented in **Table 2**. The conducted bioreactor studies showed that the highest amount of *Ct*FDH obtained was 48.6 mg/L via HCDF2. The activity and specific activity of *Ct*FDH were enhanced about 1.5-fold through the addition of casamino acids into the fermentation medium, repressing extracellular protease activity as suggested by other studies [23,33,34]. On the other hand, while the addition of casamino acid had no significant effect on Y_{PX} of HCDF1 and HCDF2, applying the HCDF strategy resulted in a 2-fold increase in product yield on biomass (Y_{PX}). Additionally, space-time yield (U/L per day) was calculated for batch, HCDF1 and HCDF2 as 1.35, 5.84 and 8.85 U/L per day respectively. HCDF strategy implementation and the addition of casamino acid had a dramatic effect on the space-time yield.

Although the amount of enzyme in this study was approximately the same as the expressed *Ct*FDH via *E. coli*, the secreted enzyme showed approximately 2-fold higher specific activity [32]. These results showed that our obtained *Ct*FDH is far more active than the previously reported version. For further improvement on the yield of *Ct*FDH as follows; strain improvement can be performed for elimination of vacuolar proteases [35], lower pH values facilitate declining neutral extracellular protease effect on enzyme [36], using sorbitol as a co-substrate in methanol induction phase provides the higher cell density of *P. pastoris* and reduces the specific protease production [37], decreasing the airflow rate by replacing air with pure O_2 in order to decrease the enzyme inactivation effect of gas bubbles [38].

Conclusion

In this study, the results demonstrated that HCDF of *P. pastoris* with BMMY medium containing casamino acids is the best methodology for *Ct*FDH secretion in a stirred-tank bioreactor. Although the *E. coli* expression system has been well-studied, the *P. pastoris* expression system to secrete

protein into media facilitated the elimination one of the downstream process steps of breaking down the cell mass to release the desired proteins as it would have been necessary if the expression was carried in *E. coli*. Additionally, high-cell density growth of *P. pastoris* provided higher protein yield in STR and facilitated about 3-fold higher *Ct*FDH than reported by Özgün G. et al., (2015) [18], about the same amount of *Ct*FDH with *Cl*FDH [39] and obtaining 2-fold more than *Lb*FDH [40]. This work is the first in which *P. pastoris* expression system was used as a host expression system for expression of FDH enzyme in STR which The Given the ease of upscaling fermentation of *P. pastoris*, these findings will open-up possibilities for larger-scale production of recombinant *Ct*FDH enzyme.

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Tables

Table. 1 Calculated biomass yield on glycerol and methanol for glycerol batch phase and induction phase of batch strategy; Phase I, Phase II and Phase III of HCDF strategy.

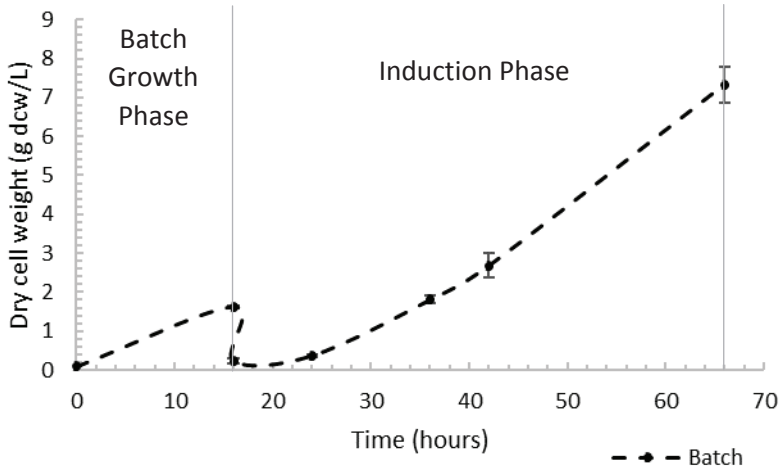
	Phase	Yxs ($g.g^{-1}$)
Batch	Glycerol Batch	0.04 ± 0.00
	Induction	0.17 ± 0.01
HCDF1	Phase I	0.79 ± 0.00
	Phase II	0.19 ± 0.00
	Phase III	0.02 ± 0.00
HCDF2	Phase I	0.84 ± 0.01
	Phase II	0.09 ± 0.00
	Phase III	0.04 ± 0.00

Table. 2 Final cell concentration, activity, concentration, specific activity of *Ct*FDH and product yield on the biomass of previously published results and conducted studies.

	Shake Flask (*E. coli*, *Ct*FDH), [32]	Shake Flask (*P. pastoris*, *Cb*FDH), [20]	Batch	HCDF 1	HCDF 2
Enzyme activity (U/L)	-	-	3.71 ± 0.09	27.75 ± 0.08	42.06 ± 1.80
Recovered enzyme concentration (mg/L)	~50	~9	5.77 ± 0.07	37.19 ± 0.63	48.57 ± 0.15
Enzyme specific Activity (U/mg)	0.40	0.13	0.64 ± 0.00	0.78 ± 0.01	0.87 ± 0.06
Y_{PX} (mg/g)	-	-	0.79 ± 0.01	1.56 ± 0.02	1.58 ± 0.04
Cell Concentration (g dcw/L)	-	-	7.33 ± 0.47	44.88 ± 0.35	46.91 ± 0.07

Figures

a)



b)

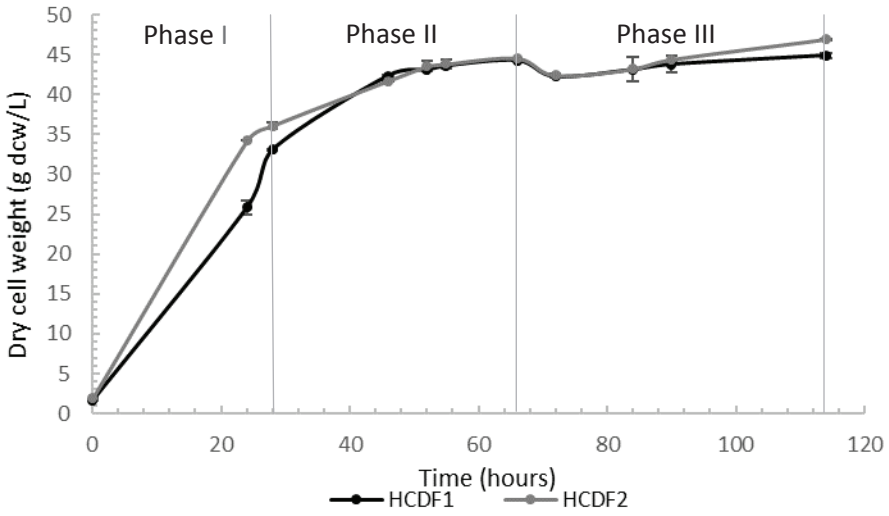


Figure 1. Cell density profiles of batch and HCDF strategies of *P. pastoris* culture: **a)** dcw values of batch growth strategy (black dashed line: Batch), **b)** dcw measurements of HCDF strategy; **Phase I:** Glycerol Batch phase, **Phase II:** Glycerol Fed-batch phase, **Phase III:** Methanol fed-batch phase (black line:HCDF1, grey line: HCDF2).

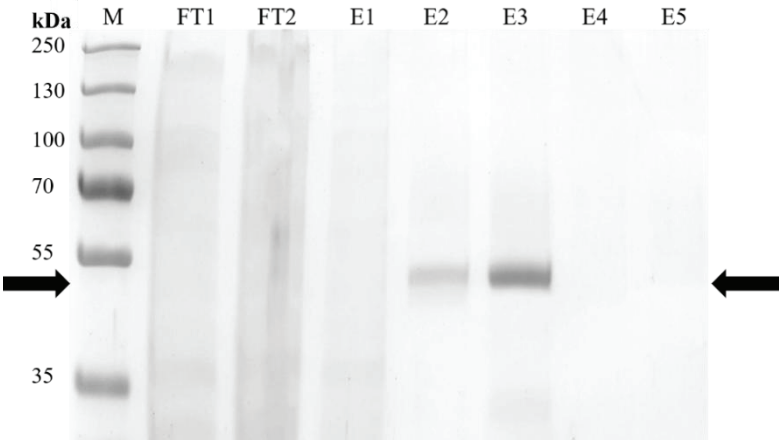


Figure.2 SDS-Page analysis of purified *Ct*FDH; M: Marker, **F1-F2:** Flow-through; **E1:** Elution with buffer A containing 10 mM imidazole; **E2:** Elution with Buffer A containing 100 mM imidazole; **E3:** Elution with Buffer A containing 200 mM imidazole; **E4:** Elution with Buffer A containing 400 mM imidazole; **E5:** Elution with Buffer A containing 500 mM imidazole.

High-level heterologous expression of active *Chaetomium thermophilum* FDH in *Pichia pastoris*

Supporting Information

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Supporting Information

Equation.S1 Theoretical yield of produced biomass from the consumed substrate.

$$YXS = \frac{g\ cells\ produced\ (\ g\ dcw.L-1)}{g\ glycerol/methanol\ consumed\ (g.L-1)}$$

Equation.S2 Theoretical yield of produced product from produced biomass.

$$YPX = \frac{mg\ product\ produced\ (\ mg.L-1)}{g\ cells\ produced\ (\ g\ dcw.L-1)}$$

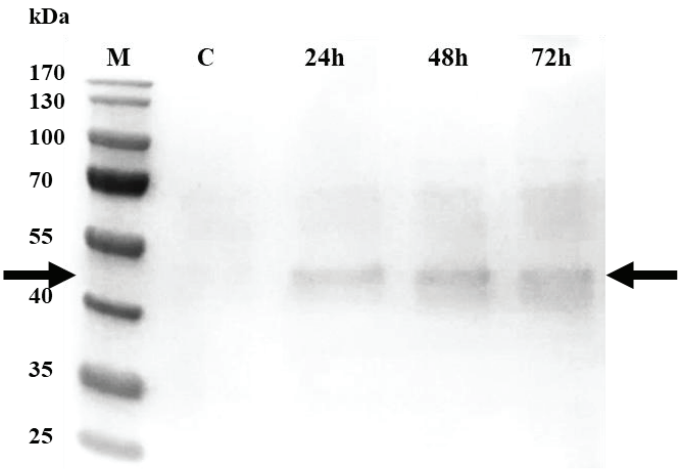

Figure.S1 SDS Page analysis of methanol induction phase on shake flask at different time points. M: Marker**; C:** Control; **24h-48h-72h:** 2% (v/v) methanol induction on shake flask.

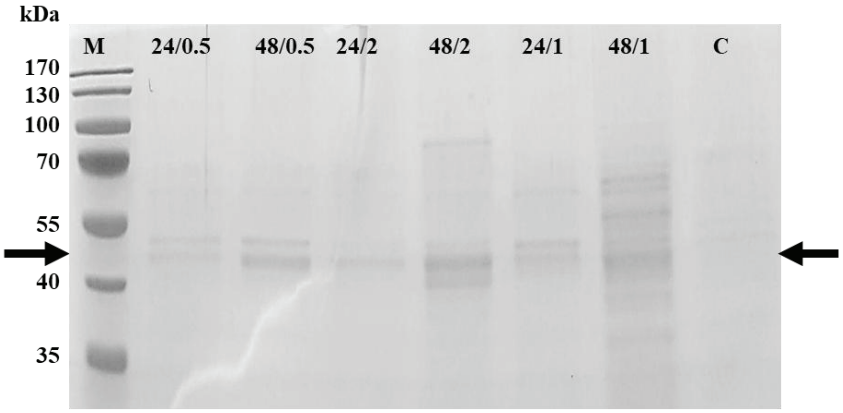

Figure.S2 SDS Page analysis of methanol induction phase with different methanol concentration on shake flask. M: Marker; **24/0.5-48/0.5:** 0,5% (v/v) methanol induction at 24h and 48 h; **24/2-48/2:** 2% (v/v) methanol induction of colony at 24 h and 48 h; **24/1-48/1**: 1% (v/v) methanol induction of colony at 24 h and 48 h; **C:** Control induced with 2% (v/v) methanol at 48 h.

Table.S1 Activity, concentration and specific activity of *Ct*FDH expressed via *P. pastoris* with different methanol concentrations.

	Methanol Induction Concentration (v/v)		
	0.5%	**1%**	**2%**
Enzyme activity (U/mL)	0.04 ± 0.00	0.09 ± 0.01	0.14 ± 0.01
Enzyme activity (U/L)	0.13 ± 0.00	0.31 ± 0.01	0.46 ± 0.02
Recovered enzyme concentration (mg/L)	1.74 ± 0.01	2.29 ± 0.02	4.36 ± 0.07
Enzyme specific Activity (U/mg)	0.31 ± 0.00	0.40 ± 0.02	0.39 ± 0.01

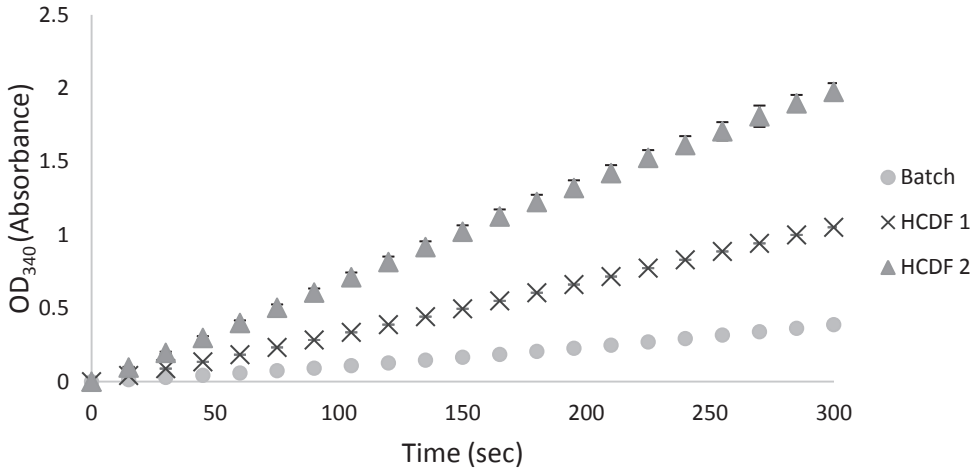

Figure.S3 Enzyme activity assay graph of purified *Ct*FDH enzyme measuring the conversion of NAD^+ to NADH during the oxidation of sodium formate to CO_2 at pH 5.0.