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CtFDH PRODUCTION IN PICHIA PASTORIS USING A NOVEL EXPRESSION SYSTEM

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ASSOC. PROF. DR. BARIŞ BİNAY

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

PİCHİA PASTORİS İFADE SİSTEMİ KULLANILARAK CtFDH ÜRETİMİ

BEDRİ BURAK DURAKSOY
YÜKSEK LİSANS TEZİ
KİMYA ANABİLİM DALI

DANIŞMANI
DOÇ. DR. BARIŞ BİNAY

GEBZE
2020

GEBZE TEKNİK ÜNİVERSİTESİ	YÜKSEK LİSANS JÜRİ ONAY FORMU

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JÜRİ

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İMZA/MÜHÜR

ÖZET

Format dehidrogenaz enzimi (FDH), formatın karbondioksite (CO_2) yükseltgenmesini katalizlerken, koenzim olarak NAD^+'ı NADH'a indirgeyerek oluşturmaktadır. Bu nedenle uzun bir süre, NADH bağımlı reaksiyonlar için iyi bir NADH yenileyececisi olarak değerlendirilmiştir. Ayrıca son dönemde FDH için yapılan çalışmalar, bu enzimin aynı zamanda tersinir yönde CO_2'in formata indirgenmesinde de aktivite gösterdiğini rapor etmiştir. FDH'ın bu yeni ozelliklerinin endüstiride uygulanması, enzimin düşük maliyetler ile verimli şekilde üretilmesine bağlıdır. Önerilen çalışmada, *Chaetomium thermophilum* FDH'nin (*Ct*FDH)'un daha yüksek verimde, düşük maliyetler ile üretilmesi için yaygın kullanılan *E. coli* ifade sistemi yerine son dönemde en çok kullanılan *Pichia pastoris* ifade sistemi kullanılmıştır. *Ct*FDH enzimini kodlayan gen pPICZαA plazmidine klonlandıktan sonra, *Pichia pastoris* X-33 hücresine protein ifadesi için aktarılmıştır. *Ct*FDH'ın yüksek protein ifadesini sağlamak için hücre kültürünün büyüme koşulları optimize edilmiştir. Hücre kültürü ortamında, hücre dışına salgılanan *Ct*FDH enzimi affinite kromotografisi yöntemi ile saflaştırılıp, saflığı sodium dodecyl sulfate-polyacrylamide jel'de kontrol edilmiştir. Elde edilen yüksek saflıktaki aktif *Ct*FDH'in miktarı, *P. pastoris* ifade sisteminin yüksek verimde protein üretimi için iyi bir seçenek olduğunu göstermektedir. Endüstriyel seviyede protein üretimi için farklı fermentasyon stratejilerinin tasarlanan protein ifade sistemine adaptasyonu gelecek çalışma olarak düşünülmektedir.

Anahtar Kelimeler: Chaetomium thermophilum format dehidrogenaz, NADH, *Pichia Pastoris* ifade sistemi, endüstriyel biyoteknoloji.

SUMMARY

Formate dehydrogenase enzyme (FDH) catalyzes the oxidation of formate to carbon dioxide (CO_2) and reduces NAD^+ into NADH. Therefore, for a long time, it has been considered as a good NADH regeneration method for NADH dependent reactions. In addition, recent studies for FDH have reported that this enzyme also shows successful activity in the reversible reduction of CO_2 to formate. The industrial application of these novel features of FDH depends on the efficient production of the enzyme at low costs. In the proposed study, the widely preferred *Pichia pastoris* expression system was used instead of the common *E. coli* expression system to produce *Chaetomium thermophilum* FDH (*Ct*FDH) with higher efficiency and low costs. After the gene encoding the *Ct*FDH enzyme was cloned into pPICZαA plasmid, it was transferred to the *Pichia pastoris* X-33 cell for protein expression. The growth conditions of the cell culture have been optimized to ensure high protein expression of *Ct*FDH. In the cell culture medium, the *Ct*FDH enzyme was secreted outside of the cell, and it was purified using the affinity chromatography method. Its purity was confirmed using the sodium dodecyl sulfate-polyacrylamide gel. The obtained amount of highly pure and active *Ct*FDH indicates that the *P. pastoris* expression system is a good option for high yield protein production. The designed protein expression system for an efficient protein production can be adapted for an industrial level production using different fermentation strategies as a future study.

Keywords: Chaetomium thermophilum formate dehydrogenase, NADH, *Pichia Pastoris* expression system, industrial biotechnology.

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I would like to take this moment and give my thanks to my parents, they have been extremely patient with me, and have always believed in my potential. For this I thank them from the bottom of my heart.

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ABBREVIATIONS

Abbrv.		Descriptions
FDH	:	Formate dehydrogenase
*Ct*FDH	:	FDH gene obtained from *Chaetomium thermophilum*
SDS-PAGE	:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
His-Trap	:	Histidine Trap
BMMY	:	Buffered Methanol-complex Medium
BMGY	:	Buffered Glycerol-complex Medium
AOX	:	Alcohol Oxidase promoter
GAP	:	Glyceraldehyde 3-Phosphate Dehydrogenase promoter
Ni-NTA	:	Nickel-nitrilotriacetic acid.
HCDF	:	High Cell Density Fermentation

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1. INTRODUCTION

Humanity has survived many cataclysmic events throughout its existence. Most of these survivals depended also on how much the environment was capable of stabilizing amidst our careless actions. As of the 21'st century the greenhouse emissions caused by human factor has excelled the atmospheric CO_2 concentrations above 400 ppm [1], a level that has not yet been exceeded for the last 400,000 years of Earth's history. These worrying developments are among the culprits of cataclysmic climate events and global health problems plaguing our world. The resulting global temperature change is an increase of approximately 0.85 °C [2] (as of 2018) since 1880. Today the current increase has risen to 0.99 °C [3].

As humans we are dependent on our planet for clean air, nutrition, drinking water and shelter, however with the rapid change in climate patterns caused by our industrial life style has placed all of these issues we are dependent on at risk, causing further unforeseen health risks that will be observable in the upcoming generations. Some might even claim that these cataclysmic changes in the global climate system might undo the advancements made in the last 50 years of the public health system [4]. The effects of climate change can be divided into three categories, the first and direct health impacts of the climate change are the fluctuating frequency and harshness of extreme weather events like heat, drought and heavy rain.

The second impact of climate change is associated through the conciliation of natural systems, for example the alteration of the impact and pattern of vector-, water-, and food borne disease propagation. Current Covid-19 pandemic can be claimed as one of these events, borne in the advent of increasing global temperatures, making it more forgiving for the viral lifespan within the global environment. Final, and the third category is the indirect impact on health caused by social institutions, causing undernutrition as a result of the climatic factors impacting the global food markets, mental health, and even causing violence and conflict.

1.1. Purpose of Thesis

There are many approaches to restore the balance within the climatic system of our planet. One of which is the reduction of atmospheric CO_2 through carbon fixation. Even within this proposed solution there are many alternative ways to reach the common goal of carbon dioxide reduction. One of these approaches is the use of enzymes to lock and fixate carbon by transforming it to another compound as the result of a chemical reduction reaction. Formate dehydrogenase is one such enzyme, which is capable of converting CO_2 into CH_2O_2 while converting NADH into NAD^+. The discovery of the formate dehydrogenases goes back to the mid 1950's, during their initial discovery they were regarded as novel dehydrogenases lacking any conventional applications. An important part of their intrinsic value laid in their ability towards hydride exchange reactions making them invaluable as model compounds. The true aim of this thesis is to first successfully synthesize *Ct*FDH (NAD^+ dependent FDH) in an active form using an efficient expression host. Successful synthesis of this enzyme will not only enable the industry to from new methods to regenerate invaluable co-factors but also, we might be able to curb the increasing CO_2 amounts in our atmosphere with the help of NAD^+ dependent formate dehydrogenases, *Ct*FDH in particular.

2. NAD^+ DEPENDENT FORMATE DEHYDROGENASE (FDH)

The first NAD^+ dependent formate dehydrogenase was obtained from pea seeds by Mathews around 1950's. This form FDHs were able to catalyze the conversion of formate into CO_2 and cause the depletion of NAD^+ to NADH. Due to its capabilities in the regeneration of co-factors, their recognition grew among the scientific society in the 1970's. Around the turn of 21'st century with advent of DNA sequencing technology, these enzymes were found to be among many life forms, distinct and essential. These enzymes are placed into two groups; metal dependent and independent FDHs. The latter, are called NAD^+ dependent FDHs and they require the presence of NAD^+ as a co-factor to drive the reaction forward. This family of dehydrogenases have a wide range of weight spread out from approximately 40 kDa to 100 kDa. The enzyme catalyzes a simple reaction that can potentially help regenerate valuable co-factors as well as help regulate CO_2 emissions.

2.1. Reaction Mechanism

The reaction itself is of a hydride transfer between NAD^+ and formate that depends on breaking an existing carbon-hydrogen bond and forming a new bond [5]. The reaction is occurring as the $C4_N$ of NAD^+ and formate are overlapped orbitally and form a transition state that exists for a short period of time. At this point the nicotinamide ring, which is positively charged attracts the hydride away from formate. This results in the $C4_N$ to acquire two bound hydrogen atoms, while the newly formed nicotinamide ring of NADH is neutralized. Now oxidized, formate has become CO_2 and quickly starts to escape the reaction away from the active site [6]. The reaction itself was first perceived as irreversible [7] however the conversion of CO_2 to formate was later discovered to be possible, albeit the reaction rate was quite low [8]. This is where the importance of the FDH obtained from *Chaetomium thermophilum* comes into focus, as it has been shown in literature [9] to have the most effective catalysis results in reducing CO_2. These enzymes have a wide range of applications within the industry.

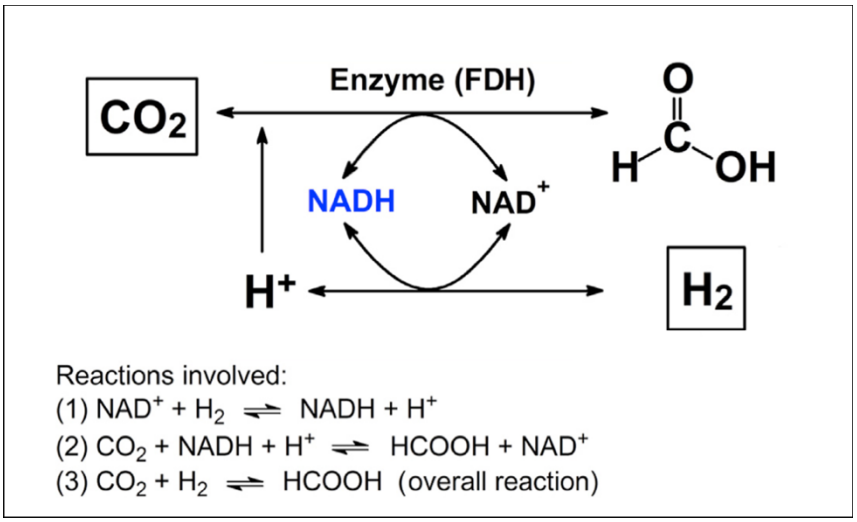

Figure 2.1: Schematic Representation of Coupling Heterogeneous Catalysts Promoting NADH Regeneration in Tandem with Enzymatic Reduction Using CO2 as a Representative Substrate to Produce Formic Acid.

2.1.1. Uses of Formate Dehydrogenase

As members of the oxidoreductases, dehydrogenases are mostly used in the acquisition of optically active compounds via enzymatic synthesis, making them versatile biocatalysts. This is due to their essential role in being stereospecific in the mechanism of hydride transfer. In order to reduce cost and time regenerating NAD^+ and NADH, specific use of the dehydrogenases as opposed to conventional methods has become a viable new synthesis scenario.

The presence of nicotinamide co-factors are indispensable in enzyme catalysis regarding dehydrogenases. Due to their high-value, obtaining and using them in synthetic reactions requires their regeneration. Several methods have been suggested until now, current chemical methods are inept in sustaining the stability of co-factors since these methods require harsh conditions. Other methods such as electrochemical or photochemical reactions require electricity and light energy in order to for the reaction to be carried out. However, these reactions also face the same limitations as the conventional chemical reaction methods. In lieu of all these limitations, several different reactions based on enzymatic catalysis were proposed for the co-factor regeneration. Of all the possible candidates for an enzymatic regeneration mechanism, formate dehydrogenase (FDH) was shown to be the most capable candidate [9]. FDH has shown to have several advantages in regenerating NADH, which includes a suitable thermodynamic equilibrium and the production of CO_2 that

is inert. FDH family has been widely preferred for their forward reaction capabilities, converting $HCOO^-$ into CO_2 and generate NADH in the process.

Their critical role in co-factor regeneration and the synthesis of chiral compounds like the L-tert-leucine has shown an important part of their potential. Studies within recent years [9] have shown that FDHs are also capable of catalyzing the same reaction in reverse, raising a valuable potential of reducing CO_2, while regenerating NAD^+. It is clear that an optimization regarding the methodology for NAD^+ regeneration is required, since only a few NADH oxidases are commercially available. Within the group of FDHs capable of a successful reverse reaction, *Ct*FDH obtained from the *Chaetomium thermophillum* has a wide pH range for catalysis reactions and a moderate thermo-stability that can be vital for regeneration conditions in both directions.

Besides these capabilities, *Ct*FDH is the most capable candidate out of all the FDHs that can actively convert CO_2 and HCO^- directly into formate. It was due to these critical properties that a decision was made to investigate the effective expression and synthesis of *Ct*FDH that aimed to solve the availability of the enzyme and reducing its future costs. Previously, an *Escherichia coli* expression system has been reported to be preferred in order to express FDHs. However, the importance of *P. pastoris* expression can be seen time and time again, with its ability to continuously express target proteins under optimal conditions, capable of extracellular expression along with proteins secreted in their quaternary folded structure. This is the biggest advantage the yeast family has over bacteria.

2.2. Yeasts

Yeasts, as unicellular lower eukaryotic life forms, have filled in the role as extraordinary model organisms to help simulate in-vivo reactions. Yeast models like *Saccharomyces cerevisiae* are utilized to ferment bread, wine, and ale, and are ecologically suitable hosts. As unicellular microorganisms, they can be easily regulated and cultured. The simple switch between the haploid and diploid state makes yeasts preferable for hereditary control. Its closest relative *Pichia pastoris* (also called *Komagataella pastoris*) is as worthwhile and a promising model organism as an expression host.

2.2.1. *Pichia pastoris*

Pichia pastoris, a type of methylotrophic yeast that is as often the most widely utilized expression system for the production of foreign proteins. It has a high development rate and its capacity to develop on a simple, practical medium makes it appropriate for both low and high batch production. *P. pastoris* is similar to the well-studied *Saccharomyces cerevisiae* (a.k.a baker's yeast) with comparative development conditions and resiliencies, and therefore the refined *Pichia pastoris* can be promptly employed by labs without the requirements of any professional gear. When these facts are considered, it became apparent that using *P. pastoris* expression system would help achieve an efficient production of the target recombinant protein. This switch from a prokaryotic to eukaryotic expression system allows us to save time by avoiding cellular breakdown to retrieve expressed proteins, since the expressed proteins are secreted extra-cellularly. Another key advantage is also avoiding the limiting issues of *E. coli*, such as the high cell density limitation in liquid media culture. *P. pastoris* has the capability of continuously producing the target recombinant protein, bypassing the periodic growth, expression and purification of *E. coli*. [10]

2.2.2. *Pichia pastoris* Metabolism

Pichia pastoris, as any other methylotrophic yeast, are capable of harnessing energy through metabolizing methanol as their only carbon source. Any yeast displaying similar properties known to science can be categorized into four groups; *Torulopsis*, *Hansuela*, *Candida* and *Pichia*. This group of yeasts share a common metabolic pathway that consists of a set of specific enzymes. The reaction starts in their peroxisomes, which are specialized organelles, and continues towards subsequent reactions in their cytoplasm. Peroxisomes are critical in the survival of these yeasts since they contain the enzymes necessary for methanol metabolism. These enzymes are alcohol oxidase, catalase and dihydroxyacetone. Rest of the metabolic reactions for the assimilation and dissimilation of methanol takes place in the cytosol [11]. *Pichia pastoris* has two fundamental key advantages over *E. coli* in research labs and modern settings for the production of foreign proteins.

i) *Pichia pastoris* can grow using methanol as its only resource, they can survive under these conditions where most other organisms would perish. The use of methanol makes the setup quite economic and easy to assemble.

ii) *Pichia pastoris* can develop to high cell densities without too much support or attention. Its cell density can quickly reach a point where it can resemble a glue. This is important because using this model organism to carry out high-batch production becomes easier as opposed to using *E. coli* for recombinant protein production [12].

2.3. Recombinant (Foreign) Protein Production

In order to express any recombinant (foreign) protein in P. pastoris, three main key steps must be taken to ensure a possible success, 1) cloning a target gene into an expression vector, 2) transforming the P. pastoris with the expression vector containing the desired gene and 3) detection of the potential strain displaying the desired expression of the foreign gene. There is a trigger mechanism in the methanol metabolism of P. pastoris, which is very dependable, as the enzymes required are only active in the presence of methanol [8.13]. This is known to be the most successful expression system for P. pastoris. Due to this dependability of AOX promoters, which are key players in this metabolic pathway, they have been the most widely preferred promoters. Surely there are other promoters available for the expression of recombinant proteins. GAP (glyceraldehyde 3-phosphate dehydrogenase) is a type of constitutive (non-continuous) promoter, and it is not as preferred as AOX1 since expression under GAP can cause cytotoxic effects on the host cell [13].

Table 2.3: Advantages and disadvantages of using the AOX1 promoter system.

Advantages	Disadvantages
Induction mechanism is simply triggered with the addition of methanol	Two phases of different carbon sources are required while the switch between these phases has to occur at a specific point in time
Recombinant proteins can be produced in large amounts, even if the proteins are toxic to the cell	Methanol is a fire hazard, storage of it in large amounts can raise risks and health hazard concerns
The foreign protein expression is controlled with the tight regulation of its transcription via a repression/de-repression mechanism	Keeping track of the methanol amounts is quite difficult, as online probes can be unreliable at times and off-line measurements are quite complicated.
By using the initial carbon source (glycerol), transcription is repressed thus ensuring a good cell growth before the gene product is abundantly produced.	Main sources for methanol production are often petrochemical in essence, which could make this approach unsuitable to be used for the production of specific food products and additives.

2.3.1. Cultivation Techniques

The preferred cultivation technique often effects the viability of the recombinant proteins as the conditions set by the technique can influence the proteolytic activity of the host cell towards the desired protein product. Cultivating *P. pastoris* in a wide array of pH is possible due to the versatility of the host cell (3.0 to 7.0). The wide pH range does not affect the growth negatively, giving room for setting the pH to inhibit some problematic proteases innately found in the cell. Of course, the selection of a pH value also depends heavily on the choice of recombinant protein and the expression technique [14]. If the desired recombinant protein has a narrow pH window, then the pH condition for expression will be limited. There are however, other compounds that can improve the stability of the desired recombinant protein, such as amino acid-rich compounds like casamino acids and peptone. Just like the pH range, temperature range is also quite influential over the proteolytic activity, lowering the temperature not only eases the metabolic stress on the organism but also helps reduce early apoptosis and preventing the release of proteases into the culture. Li et al. have shown an increase in the yield of herring

antifreeze proteins from 5.3 mg/L to 18.0 mg/L by just reducing the temperature of the process [15].

3. MATERIALS AND METHODS

3.1. Yeast Strain, Plasmid, and Reagents

Chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) 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, USA. The bicinchoninic acid (BCA) Protein Assay Kit was purchased from Thermo Scientific (Waltham, Massachusetts, USA) 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 pPICZα A (provided by BERC Lab, Turkey). The resulting construct was transformed into electrocompetent *P. pastoris* X33 strain (InvitrogenTM) according to the manufacturer's instructions and used for expression of C-terminal 6xHis-Tagged *Ct*FDH. A cell stock of this strain was prepared in 50 % glycerol and stored at -80 °C.

3.2. Cloning of *Ct*FDH Gene into pPICZ*α* A Vector

The fdh gene (approximately 3.7-kb) from Chaetomium thermophilum did not require any codon optimization as both the donor and the host organism share the same kingdom of Fungi and the division Ascomycota. The gene was synthesized (Genscript) based on the amino acid sequence (UniProt accession number: G0SGU4) EcoRI and XbaI sites for cloning were added to the synthesized fdh gene by the following primers:

Table 3.2: Primers used for *Ct*FDH gene cloning.

Forward Primer	5'TCTTCCCCTTCCCCTTTTC3'
Reverse Primer	5'TCCCCACATCCACACATAG3'

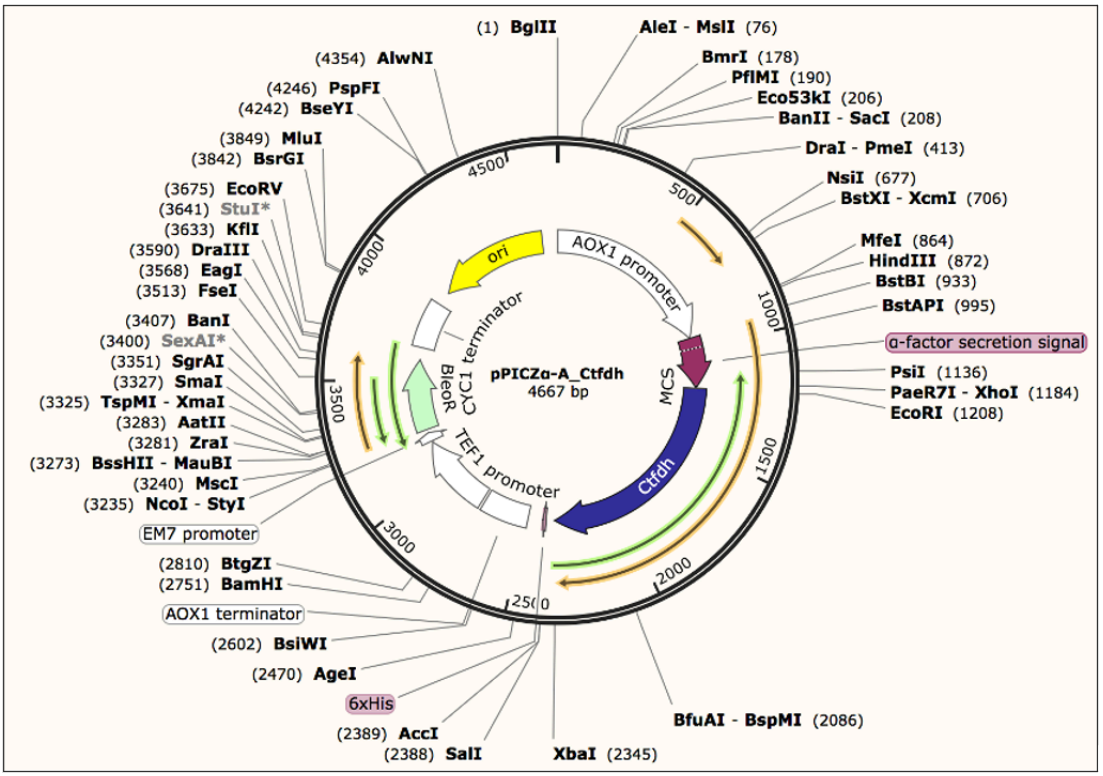

Figure 3.2: Plasmid map of pPICZα A containing the target gene *Ct*FDH.

3.3. Transformation of pPICZαA-*Ct*FDH into *E. coli* (Top10) For Plasmid Amplification

The plasmid containing *Ct*FDH was transformed into chemically competent Top10 competent *E. coli* cells. Tubes containing competent cells were thawed on ice for 10 min. 1-5 µL containing 1-100 ng of DNA was added to the cell mixture and flicked gently 4-5 times to mix DNA and cells. Mixture was placed on ice for 30 min then heat shocked at 42 °C for 30 sec. Mixture was placed on ice for 5 min. 300 µL of room temperature SOC medium was added to the mixture and incubated at 37 °C at 250 rpm for 1 h. Cells were mixed by flicking the tube, followed by the spreading of a 100 µL of the mixture to the selection plates and incubated overnight at at 37 °C. Individual colonies were picked and inoculated into overnight cultures for optimization of the expression conditions.

3.4. Linearization of the pPICZαA-*Ct*FDH

For the transformation to occur, the vector containing the target gene was linearized by restriction enzymes. The restriction enzymes *Sac*I and *Bgl*II were used. Other sites for restriction were also possible, for example the use of *Pme*I was also among the suggested enzymes. It was important to ensure that the gene insert did not contain any of these restriction sites in order to linearize the vector without a faulty nick in the gene insert. For a successful transformation to occur with *P. pastoris* the required linearized DNA needs to be around 5 µg in 5-10 µL sterile water [16].

3.5. Transformation of pPICZαA-*Ct*FDH into *Pichia pastoris* (X-33) for Protein Expression

The linearized pPICZαA-*Ct*FDH construct was transformed into the competent *Pichia pastoris* X-33 via electroporation procedure. BioRAD Gene Puler Xcell Electroporation System was used for the procedure and the parameters for the procedure were as follows; 1500 V, 25 µF, 200 W. Immediately 1 mL of ice-cold 1 M sorbitol was added to the cuvette. The cuvette contents were transferred to a sterile 15-mL tube and were incubated at 30 °C while shaking at 100 rpms for 2 h. 50, 100, and 200 µL of transformed cell were spread on separate plates, labeled as YPDS plates containing 100 µg/mL zeocin. Lower concentrations of cell samples are favorable to give a better selection result with zeocin. Plates were incubated from 3–10 days at 30 °C until colonies formed. After the colonies became visible, 10 distinctive colonies selected and purified to obtain single colonies on fresh YPD plates containing 100 µg/mL zeocin.

3.6. Application of Colony PCR

From the colonies that were successfully grown on the selective media plates containing zeocin, 10 were randomly selected for the Colony PCR method. Each colony sample were picked using sterile toothpicks and resuspended in PCR tubes containing 10 µl of 0.02 M NaOH. These samples were later placed in the PCR and incubated at 99 °C for 10 min. While this process was underway a mastermix for the

PCR reactions was prepared. (The mastermix contains the following ingredients; 0.2 µL $MgCl_2$, 1uL 10X PCR Buffer, 0.2 µL dNTPs (10 mM each), 0.2 µL AOX1 foward primer (100 µM), 0.2 µL AOX1 reverse primer (100 µM), 0.1 µL Taq, 5.3 µL ddH_2O) 9 µL of the master mix solution was aliquoted into fresh PCR tubes. 1 µL of boiled samples were added to the master mix aliquots. The PCR cycle was executed for 30 cycles. After the procedure, the PCR samples were collected and ran in an agarose gel for a visual confirmation.

3.7. The Optimization, Induction and Expression of *Ct*FDH In *Pichia pastoris*

A single colony, which was confirmed with the Colony PCR protocol was selected to be grown in a pre-culture containing 10 mL of YPD broth medium with 100 µg/µL of zeocin placed in a 50 mL falcon. The pre-culture was grown with a control sample of X-33 wild type under 30 °C at 250 rpm and was left for overnight growth. After the pre-culture is grown, the cells were centrifuged at 3000 g for 5 min. The supernatant was discarded and the cell mass was washed in 10 mL of BMGY medium in order to remove zeocin from the cells. Once the cells were washed, they were resuspended in 75 mL of BMGY in a 250 mL sterile Erlenmeyer flask containing 5% (w/v) casamino acids and glycerol feeding with 5% (w/v) casamino acids were used. The cells were placed in a shaker incubator at 22 °C with 300 rpms for 24 h. After the 24 h period the cell concentration was checked to be approximately OD_{600} ~ 5.

Once this was confirmed, the cells were harvested from the BMGY medium, followed by a wash with a 10 mL of BMMY medium in order to remove any glycerol from the cells as this would hinder the methanol pathway and reduce recombinant protein expression. The cell density of OD_{600} value was reduced to 1 before the induction with the Methanol took place. Once the OD_{600} value was set, the washed cells were resuspended in a 75 mL BMMY medium. Initially three different concentrations of methanol inductions were applied to three small pilot flasks. 0.5%, 1% and 2% varying methanol concentrations were used to induce AOX1 promoter in order to trigger the expression of our target gene. The concentration of the methanol is critical as it single handedly is responsible for a successful expression, too low and

the product yield will not be satisfactory or too high and the host organism will fail due to toxic and metabolic stress, plausibly causing an early apoptosis, releasing endo-proteases that further breakdown the product. In order to determine the correct amount of methanol to be used during induction, three separate pilot flasks were induced with 0.5%, 1% and 2% methanol respectively.

The correct amount relates to the optimum percentage of methanol that will help yield the peak amount of target protein by inducing the AOX1 promoter without having adverse toxic effects and unnecessary stress on the host organism, *P. pastoris*. The varying amounts of methanol containing BMMY mediums were prepared according to the protocol. The first 24 h period of the induction, the cells use up the initial amount of methanol within the medium, however after this period, the following 48 h, %2 methanol was added ever 24 h. During this procedure 1ml samples were taken every 6hrs and were later observed in an SDS-gel assay (See Figure 4.3).

3.8. SDS-PAGE analysis

SDS-PAGE electrophoresis (Bio-Rad electrophoresis unit) was performed according to the method of Laemmli 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 de-staining solution (100 mL acetic acid, 300 mL methanol, 600 mL dH_2O) to remove excess dye.

3.9. Purification of Extracellular *Ct*FDH 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.

3.9.1. Determination of Protein Amount by BCA Method

The amount of purified protein to be used in the activity assays is BCA (Bicinchoninic acid) method. 10 µL of the obtained protein samples were placed on a 96-well microplate plate. 200 µL of BCA solution is prepared by mixing 50 volumes of freshly prepared solution A with 1 volume of solution B, and was added onto each sample. Bovine serum albumin (BSA) was used as the defining standards at different concentrations (1-20 µg). Samples were left to incubate for 30 min at 37 °C. They were later measured with a 562 nm absorbance eliza reader (Elx800, Universal Microplate Reader, Biotek). Amounts of protein in the samples were calculated based on the standard curve drawn according to the values obtained from BSA standards.

3.9.2. Activity Determination of Pure *Ct*FDH

The NADH production was assessed at 340 nm, once the purified *Ct*FDH was added to the reaction mixture. The mixture contained 20 mM sodium acetate buffer with a pH of 5.0, + 3.0 mM NAD^+ and 30 mM of sodium formate at 25 °C. The enzyme activity was assayed for 5 minutes, where one-unit enzyme oxidized 1.0 µmole of formate to CO_2 per minute in the presence of NAD, at pH 5.0 at 25 °C.

4. RESULTS AND DISCUSSION

4.1. The Confirmation of Transformation with Colony PCR

The randomly chosen 10 colonies that were obtained from the transformation procedure, these colonies were treated to the Colony PCR procedure described in the material & methods. The PCR products were then run in the agarose gel and were observed in the figure below.

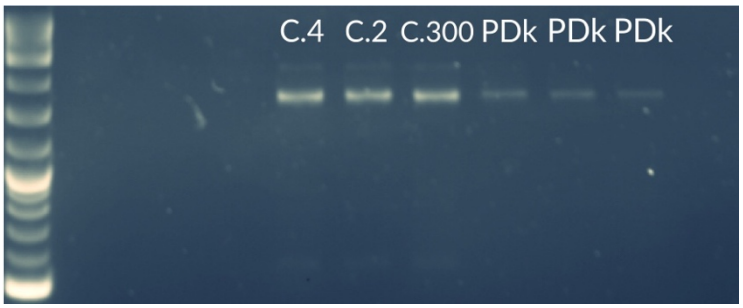

Figure 4.1: Colony PCR Results. The first three wells contain control samples as they show no illumination for, they lack the appropriate plasmid. Wells 4, 5 and 6 contain the target plasmid and genomic material of the host organism, the last three wells contain three more controls containing alternate genomic material.

4.2. Expression of Recombinant *Ct*FDH in *P. pastoris*

A single confirmed colony was selected after the Colony PCR procedure was successful. This colony was later streaked on a YPD plate containing 100 µg/µL zeocin. After a single colony was purified and grown on a dedicated selective plate, another single colony from this plate was selected and grown as a pre-culture in a 10 mL of YPD broth containing 100 µg/µL zeocin. Afterwards, this sample was grown and inducted as described in the material & methods. The growth was observed through the samples taken every 6 h. This proved that the maximum expression efficiency was obtained at 48 h (see figure 4.2).

4.3. Optimization of Expression Conditions

One of the biggest hurdles in this research was the adjustment of expression conditions based on time, temperature and the inhibition of intra/extra-cellular protease activity. Data obtained from executed trials and research literature pointed out that the required fermentation time was 48 h. As the fermentation reached 72 h, a significant drop in the expressed protein amounts were observed. This was possibly due to protease activity and increased amount of stress over the metabolism of the organism caused by methanol minimum media and the strenuous production of the foreign protein. With time, unnecessary stress accumulates. Temperature is another angle in the optimization process. Natural fermentation conditions that the *P. pastoris* favors is around 30 °C, however at this temperature the production is too quick and a possible "bottle-neck" scenario occurs during the production of the foreign protein leading to further stress that results in an early apoptosis of the cells, thus releasing more proteases into the culture media. Dropping the temperature to 15 °C has dramatically improved the protein production. The pH of the reaction also dictates how active the proteases can be. The *P. pastoris* has a wide range of pH preference (3.0-7.0), this allows flexibility of choosing a suitable pH range depending on the target protein aimed to be produced. *Ct*FDH can function within a pH range of 4.5-8.0, so the comparative pH ranges for both the organism and the target protein were not that different. The last variable that was key for optimizing the conditions was the addition of casamino acids. This compound has been previously commended by many other research articles to have suppressed the proteases found within the organism and within the culture media.

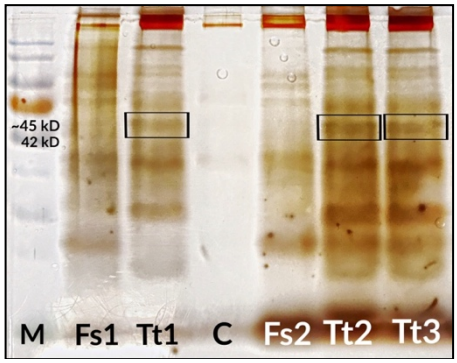

Figure 4.2: Silver staining analysis of *Ct*FDH at 28 °C, pH 6.0 without casamino acids; M: Marker, Fs1 and Fs2: Control supernatant samples from P. pastoris containing Laccase gene; Tt1: Supernatant sample 1 at 24 h; Tt2: Supernatant sample 2 at 48 h; Tt3: Supernatant sample at 72 h; C: Control supernatant sample from wildtype (no-transformation).

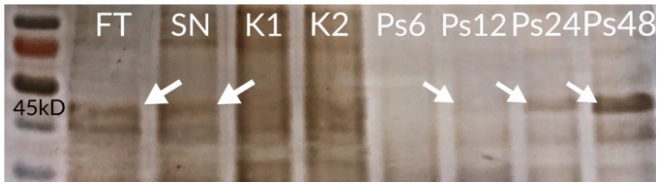

Figure 4.3: Silver staining analysis of purified *Ct*FDH at 25 °C, pH 6.0 with casamino acids; FT: Flowthrough; SN: Supernatant; K1: Control supernatant wild type X33; K2: Control supernatant samples from P. pastoris containing laccase gene; Ps6: Purified sample at 6 h; Ps12: Purified sample at 12 h; Ps24: Purified sample at 24 h. Ps48: Purified sample at 48 h.

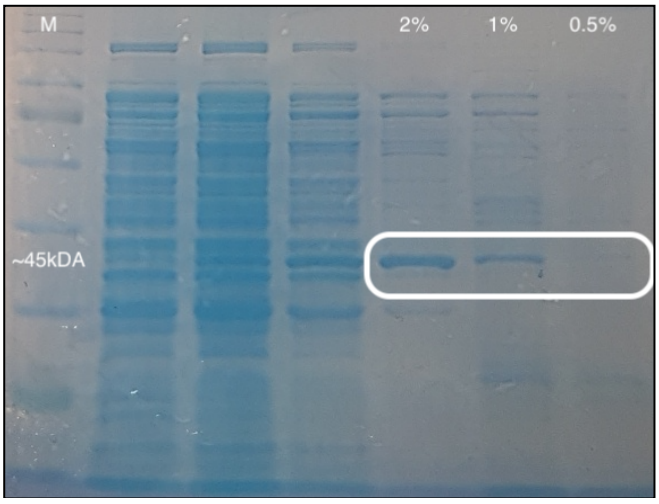

Figure 4.4: SDS results of *Ct*FDH produced under three different methanol concentrations at 18 °C, pH 6.0 with casamino acids; 2% methanol induction, 1% methanol induction, 0.5% methanol induction.

4.4. Secretion and Purification of *Ct*FDH

The chosen host, *P. pastoris* offers a possibility of expressing the target protein (*Ct*FDH) extracellularly due to the presence of α-mating factor fused downstream of AOX1 promoter. This removes all the need of breaking down biomass to harvest the target protein, saving precious time down the line. Since the target protein contains 6xHis-tag at its C-terminal it was possible to purify it using the Ni-NTA His-Trap columns. Both the presence and the molecular weight (~45 kDa) of the target protein were validated by the application of the SDS-PAGE (Figure 2). Results obtained from studies conducted by other groups are also in agreement with our findings regarding our target protein. A gradient spectrum of 10-500 mM of imidazole was setup to achieve an effective purification. Two elution fractions (E2 and E3) containing our target protein were combined and concentrated to obtain a denser amount of the target protein.

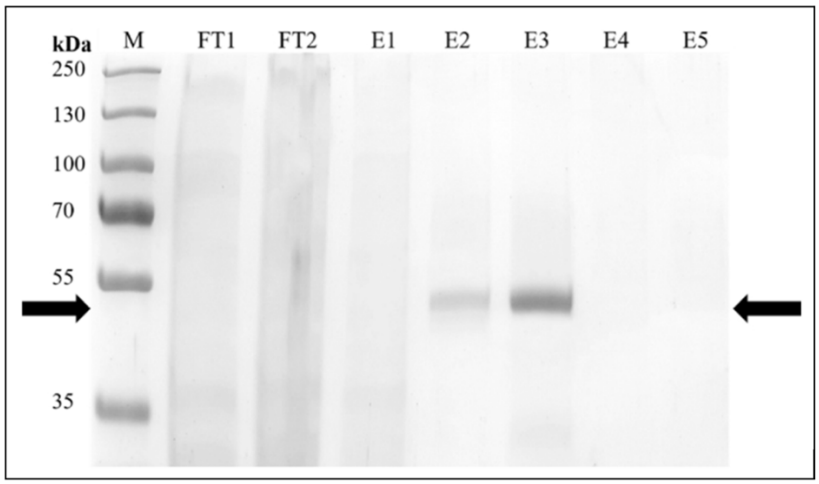

Figure 4.5: SDS-Page analysis of purified *Ct*FDH at 18 °C, pH 6.0, with casamino acids; M: Marker, F1-F2: Flow- through; E1: Elution with buffer A containing 10 mM imidazole; E2: Elution with Buffer A containing 100 mM imidazole; E3: Elution with Buffer A containing 200 mM imidazole; E4: Elution with Buffer A containing 400 mM imidazole; E5: Elution with Buffer A containing 500 mM imidazole.

4.5. Activity Assessment of *Ct*FDH

Of all the known types of FDH, it has been documented that *Chaetomium thermophilum* produces the most competent FDH in converting $HCOO^-$ to CO_2. Pilot studies of shake flask cultivation revealed the optimum percentage of methanol required to effectively induce target protein production (Table 3). Adjusting the right amount of methanol for induction is also crucial in preserving the structure of the target protein, too high and it might dehydrate the protein and possibly alter its structure, thus ultimately risking its functionality. However, this was not the case as the comparative specific activity calculations were roughly the same value. Supernatants from all the shake flask samples were collected and were assessed for activity.

Due their highly diluted states within the supernatant, enzyme concentrations were not large enough to initially cast an analysis. To overcome this issue, all end products including *Ct*FDH found in the supernatant were collected and purified specifically targeting *Ct*FDH. The activity and specific activity of *Ct*FDH expressed via *P. pastoris* host cell can be seen in table 4.6. In the table below, the enzyme activity is given for three varying methanol induction concentrations. Change in the methanol concentrations has shown no significant effect on the enzyme's specific activity however as the concentration rises from 0.5% to 2% a significant increase in the enzyme amount and activity can be observed.

Table 4.5: Activity, concentration and specific activity of *Ct*FDH expressed via *P. pastoris* under different methanol concentrations ranging from 0.5% to 2%.

	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

Table 4.6: Comparative specific activity and concentration results of *Ct*FDH expressed via *P. pastoris* in flask, fermenter and via *E. coli* in flask. * indicates results were obtained from published studies.

Host System	Amount Expressed (mg/L)	Specific Activity (U/mg)
E. coli (Flask)*[17]	~50	0.40
P. pastoris (Flask)	4.36	0.39
P. pastoris (HCDF)*[18]	48.57	0.87

5. CONCLUSION

To summarize the finality of the study, it can be concluded that the implementation of temperature and pH optimization in combination with the use of casamino acids gave the most optimum results for the extracellular expression of *Ct*FDH in a pilot flask fermentation. Having been depended on for decades, the *E. coli* expression system lacks key advantages when compared with *P. pastoris*. As a capable eukaryote, *P. pastoris* is able to finalize the protein structure, and if desired, it can be engineered to secrete target proteins into the culture medium, eliminating the need to harvest by breaking down the cell mass, not only saving time but also preventing target proteins from undue stresses of processes like sonication. If the appropriate conditions are met, and correct optimizations are carried out, production rate can increase 3x as can be seen in the literature, especially if this system is used in a fermenter/bioreactor. There were several problems that affected the expression rate during the trials. The normal expected growth temperature of ~30 °C for the yeast did not work out well for the amount of protein produced. In fact, it was so bad that the production was almost virtually inexistent. So, the temperature was dropped to 28 °C then to 18 °C. At this point we were able to see the difference from the first trials, as there was a substantial increase in the expression rate. Slowing down the metabolism helped reduce the misfolding of the proteins and gave a chance for the metabolic processes to orderly secrete the proteins. Methanol being the inductive compound that activates the AOX1 promoter (found upstream of our target gene and secretion signal), also needed some adjustment. Three different methanol percentages were applied, 0.5%, 1% and 2%. Of these only the 2% has shown substantial effects. It was only now that a tangible amount of protein was being obtained. As we successfully passed one obstacle to the next, another unexpected problem appeared. Even though enough the enzyme was being generated by the organism, the activity and the structural stability was weak. After through research of the organism's metabolic pathways supported by literature, we have figured out that the organism has six main, efficient proteases. To battle with this, we used the casamino acid in the culture solution to inhibit part of proteases. The numbers rose from 0.368 mg/L to 4.36 mg/L. Additionally, reducing the temperature not only eased the metabolic stress but also helped in inhibiting proteolytic activity. Once optimum conditions are

found, the *P. pastoris* expression system can turn into a powerhouse for recombinant protein production. *P. pastoris* is better suited expression system when applied with HCDF (High Cell Density Fermentation) techniques meant for large scale production. Due to its ample and proliferative capabilities *P. pastoris* can reach high cell densities, thus providing an increased protein yield. When cultivated in small stirred reactors the results can reach up to 3-fold [19] higher when compared to the results of Özgün G. et. al. (2015). The research conducted in this thesis is the first of its kind where the *P. pastoris* was used as a host expression system explicitly designed to optimize the production of *Ct*FDH. Considering how feasible it is to upscale the fermentation process, future applications for a larger scale production becomes quite imperative.

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BIOGRAPHY

Bedri Burak DURAKSOY was born in 1985 in Ankara. In 2006 he commenced BSc. in Molecular Biology and Genetics in Murray State University in Kentucky, United States. Due to personal reasons he had to come back to Turkey and transfer to AREL University Molecular Biology and Genetics department. In 2016 he started his MSc. in Chemistry at Gebze Technical University of Natural Sciences department.

APPENDICES

Appendix A: Publications Within the Scope of Thesis Study

Duman Z. E., Duraksoy B. B., Aktaş F., Woodley J. M., Binay B., (2020), "High-level heterologous expression of active Chaetomium thermophilum FDH in Pichia pastoris", Enzyme and Microbial Technology. 137, 109-552.

Appendix B: Additional Media and Protocol Information

- Luria-Bertani (LB) Media

12 g LB [38] were weighed, supplemented with ddH2O to 500 mL and allowed to dissolve. The autoclave was sterilized at 121 ° C for 15 minutes.

- Luria-Bertani (LB) Agar Media

20 g LB Agar was weighed, supplemented to 500 mL with ddH2O and allowed to dissolve. The autoclave was sterilized at 121 ° C for 15 minutes. After cooling to 55 ° C, zeocin was added to give 1 μL / 1 mL ratio. It was divided into Petri dishes in a sterile cabinet. The lids were closed and moved to +4 ° C for future use after being wrapped completely in parafilm.

- BMGY (Buffered Glycerol-complex Medium)

Dissolved 8 g of yeast extract and 16 g peptone in 560 mL water. Autoclaved for 20 minutes on liquid cycle. Followed by the addition of, 80 mL 1 M potassium phosphate buffer, pH 6.0; 80 mL 10X Yeast Nitrogenous Base, 0.16 mL 500X Biotin, 80 mL 10X Glycerol.

- BMMY (Buffered Methanol-complex Medium)

Dissolved 8 g of yeast extract and 16 g peptone in 560 mL water. Autoclaved for 20 minutes on liquid cycle. Followed by the addition of, 80 mL 1 M potassium phosphate buffer, pH 6.0; 80 mL 10X Yeast Nitrogenous Base, 0.16 mL 500X Biotin, 80 mL 10X Methanol.

- Yeast Nitrogenous Base (YNB)

Dissolved 26.8 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 200 mL of water and filter sterilized it. Heated the solution to dissolve YNB completely in water.

- YPD (Yeast Extract Peptone Dextrose Medium) Broth

Dissolved 5 g yeast extract and 12 g peptone in 540 mL of water. Autoclaved them for 20 minutes on liquid cycle. The autoclaved solution was cooled to ~60°C, followed by the addition of 60 ml of 20% dextrose that was previously filter-sterilized.

- YPDS (Yeast Extract Peptone Dextrose Medium with Sorbitol) + Zeocin Plates

5 g yeast extract, 91.1 g sorbitol and 10 g peptone were dissolved in 450 mL of water. 10 g of agar and a magnetic stir bar were added and autoclaved for 20 minutes on liquid cycle. In the meantime, 60 mL of 20% dextrose was prepared and filter-sterilized before use. The autoclaved solution was cooled down to ~ 60°C, and 50 mL of filter-sterilized 20% dextrose was added.

Poured a few plates before adding the antibiotic for YPDS plates without Zeocin. After pouring 4 plates, 500 µl Zeocin from a 100 mg/ml stock solution was added to obtain a final concentration of 100 µg/ml Zeocin in the agar. Left it to stir on a magnetic plate when the antibiotic was being added and the medium was left to

stir for about an additional 2 minutes to equally mix. Poured the media into Petri dishes and covered them with black plastic (to prevent light exposure that can potentially denature Zeocin). The plates were left to dry on bench overnight. YPD plates containing Zeocin were stored at 4°C.

- Silver staining protocol

The gel was fixed with 50% MeOH, 12% Hac, 0.05% formalin and was left overnight. The following day the gel was washed 3 times with 35% EtOH for 20 minutes each wash. Followed by the sensitizing of the gel with 0.02% Na2S2O3 for 2 minutes. After this step the gel is washed with dH2O 3 times, each wash lasting 5 minutes. The gel is then stained using 0.2% AgNO3, 0.076% formalin for 20 minutes. From here on out the gel is washed again using dH2O twice for 1 minute each wash. To develop the gel 6% Na2CO3, 0.05% formalin, 0.0004% Na2S2O3 is added. This step lasts around 10 minutes or when the bands form according to your satisfaction. Once the bands form, this process must be stopped before the bands become too dark to be distinguishable. At this point 50% MeOH, 12% HAc is added for 5 minutes in order to stop the staining and the gel can be left at 4oC in: 1% HAc.

Appendix C: Transformed *P. pastoris* Colonies

Figure C1: Transformed colonies grown on selective YPD plates.