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Multi-enzymatic synthesis of acetoin from ethanol in one pot reactor

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ABSTRACT

Commercially known as acetoin, 3-hydroxy-2-butanone is a valuable bio-based chemical, whose current production relies strongly on fossil feedstocks, with this becoming more and more limited, finding a biobased synthesis of natural acetoin using biotechnology is crucial. This study addresses this challenge by presenting an approach to acetoin production through a renewable source employing a multi-enzymatic system consisting of pyruvate decarboxylase (ZpPDC), an alcohol oxidase (PcAOX), and catalase. A significant acetoin concentration of 27.63 mM was achieved, starting from an initial concentration of 50 mM ethanol. This marks a substantial advancement over the previous systems based on using ZpPDC, an alcohol dehydrogenase (ScADH), and a NADPH-oxidase (SpNOX), which required additional cofactors.

Production, purification, and characterization of PcAOX were successfully carried out. PcAOX was characterized by pH, temperature, and substrate concentration. Finally, the multi-enzymatic system was tested reaching 98% conversion and 100% product yield of, being twice as fast as the previous multi-enzymatic system involving ZpPDC, ScADH, and SpNOX.

1. INTRODUCTION

Enzymes have been used without even knowing of them, they were used in making bread and cheese, beer brewing, and winemaking [1]. At present time 60% of the total enzyme market is accounted for the food and detergent industry. Offering a sustainable and efficient alternative to accelerate biological and synthetic reactions, enzymes have revolutionized many diverse fields in chemistry. They are widely used in the industry, accelerating the rates of chemical reactions without being consumed in the process [1]. While they have been used in organic synthesis since the early 20th century, it was not until recently, with some exceptions, such as the synthesis of beta-lactam antibiotics, that it has become widely used in industrial processes [2]. It is due to their high enantioselectivity and the advances in recombinant DNA technology that now make the identification and cloning of target genes in host-production organisms such as *E. coli* and chemical synthesis cost-effective, that producing enzymes has become more commercially done and less expensive to use. [2]

Enzymes are known for being nature catalysts, they are derived from renewable resources and are biocompatible, biodegradable, non-hazardous, and non-toxic, which makes them highly applicable. In comparison to conventional organic synthesis, enzymatic synthesis is typically faster and more selective, the usage of enzymes eliminates the need for functional-group activation and protection and deprotection steps, which results in purer products and a shorter synthesis [2]. Another advantage that enzymatic reactions have over conventional ones is they can be conducted under mild conditions which makes them more resource and energy efficient.[3]

The use of enzymes has become increasingly popular in the industry due to their compliance with 10 out of the 12 fundamental principles of Green Chemistry. [4][5] While single enzymes have contributed to these goals, their limitations in substrate range and product diversity have spurred interest in multi-enzymatic systems.[5] These systems involve coupled reactions catalyzed by different enzymes, enabling sequential transformations until the desired product is obtained.[6]. Beyond simplifying processes by eliminating intermediate purification steps, multi-enzymatic systems offer the advantage of one-pot reactor production. This not only reduces energy consumption but also lowers overall process costs, further enhancing their appeal for sustainable and cost-effective chemical synthesis.

These processes exhibit notable benefits compared to single reactions, such as enhanced atom economy, step-saving, and consequently, heightened yield and efficiency.[6] The use of enzymes as catalysts in cascade systems contributes to a more environmentally benign process. However, challenges arise due to variations in enzymes' pH and optimum temperatures, necessitating the development of strategies to overcome these constraints. Hence, the characterization of the enzymes before their usage becomes fundamental. This includes identifying the optimal conditions, including factors such as pH, temperature, substrate specificity, and cofactor requirements. This detailed understanding allows for tailored applications by revealing the enzyme's behavior under diverse conditions, ensuring optimal performance within the targeted processes.[7]

The group of Bioprocess Engineering and Applied Biocatalysis of UAB has been recently exploring the production of acetoin by multi-enzymatic systems. Acetoin (3-hydroxy-2-butanone) is a pale yellowish liquid whose current supply is largely from chemical synthesis, commonly derived from fossil feedstocks that become scarcer as time progresses[8], its commercial prices range from €97,000 - €1,530,000 per ton[9]. The synthesis is also mired by environmental concerns, safety hazards, and the requirements for radical reactions.[8] Hence the extensive effort of producing natural acetoin using biotechnology.[10] Some of these green routes are the bacterial acetoin formation pathway involving three key enzymes, 2-acetolactate synthase (ALS), 2-acetolactate decarboxylase (ALDC), and 2,3-butanediol dehydrogenase (BDH) catalyzing the three reactions from pyruvate to acetoin and 2,3-butanediol, respectively as shown in Figure 1.[10]

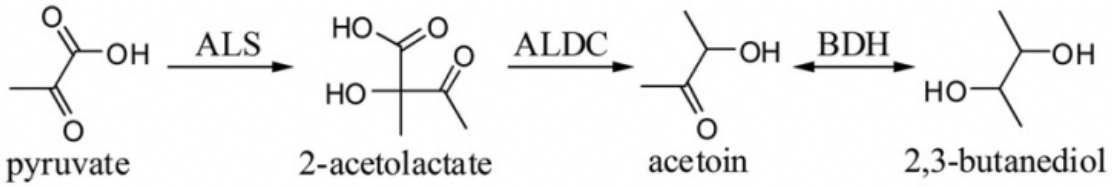

Figure 1. Bacterial acetoin formation pathway from pyruvate involves three key enzymes, 2-acetolactate synthase (ALS), 2-acetolactate decarboxylase (ALDC), and 2,3-butanediol.

The current record of the highest titer of acetoin fermentation is 75.2 g/L achieved by *Serratia Marcescens* H32 incorporating a NOX gene encoding a water-forming NADH oxidase. However, this process needs the addition of the cofactor NAD^+/NADH, thereby compromising the desired cost-effectiveness. [11]

The group of Bioprocess Engineering and Applied Biocatalysis has developed a multi-enzymatic system that utilizes various enzymes to produce acetoin starting from ethanol.

The decarboxylase pyruvate from *Zymobacter Palmae* (ZpPDC) has demonstrated a high capacity to catalyze the aldolic condensation of two molecules of acetaldehyde into acetoin[12]. To replace acetaldehyde, due to its high reactivity and toxicity[13], ethanol is utilized, via an alcohol dehydrogenase from *Saccharomyces cerevisiae* (ScADH), coupled with a third enzyme NAD(P)H oxidase from *Streptococcus pyogenes* (SpNOX) to produce the cofactor regeneration, from NADH into NAD^+, this system is shown in Figure 2. This process although it needs less, still needs the addition of the cofactor NAD^+/NADH, thereby affecting the desired cost-effectiveness.

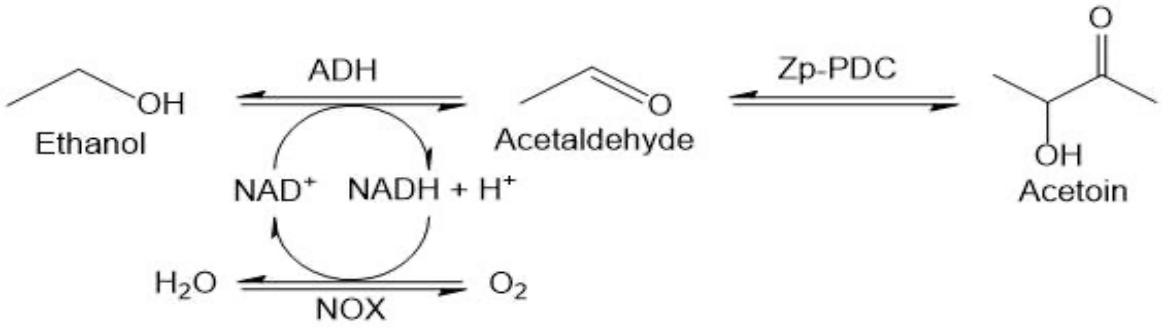

Figure 2. Multi-enzymatic reaction to produce acetoin from ethanol, with cofactor regeneration. Pyruvate decarboxylase from Zymobacter palmae (ZpPDC), alcohol dehydrogenase from Saccharomyces cerevisiae (ScADH), and NAD(P)H oxidase (SpNOX) from Streptococcus pyogenes.

A new route based on the usage of an alcohol oxygenase from *Phanerochaete chrysosporium* (PcAOX) has been proposed (Figure 3). This enzyme demonstrates the capacity to oxidate ethanol into acetaldehyde without the need for cofactors like NAD^+/NADH, offering a more cost-effective process over the long term[12][14]. This new modified multi-enzymatic system could replace ScADH with PcAOX avoiding the use of cofactor and offering a more cost-effective process over the long term.[12][15] However, this substitution produces H_2O_2 as a byproduct. To address this, coupling this reaction with a catalase from bovine liver, will grant the use of H_2O_2 and convert it into O_2, this is highly beneficial since this O_2 is a limiting factor in the reaction.[15]

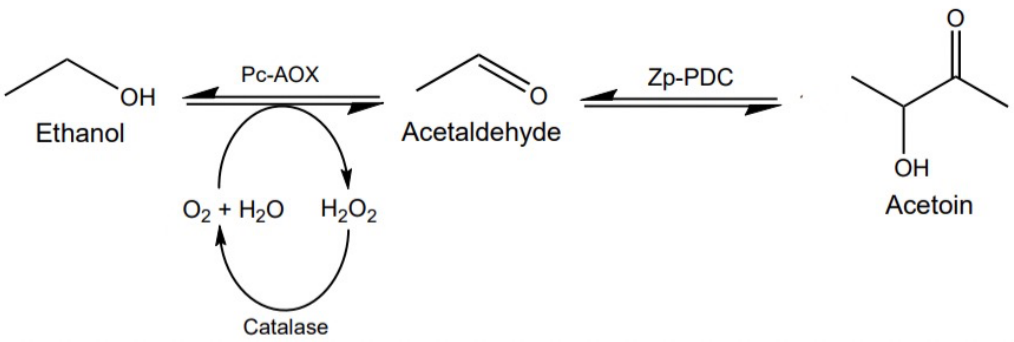

Figure 3. Multi-enzymatic reaction to produce acetoin starting from ethanol, with H2O2 transformation into O2. Pyruvate decarboxylase from Zymobacter palmae ZpPDC, alcohol oxidase from Phanerochaete chrysosporium PcAOX, and an H_2O_2 reducer catalase (CAT)

2. OBJECTIVES

The present work focuses on the production and purification of PcAOX to enable the characterization of its catalytic performance under different pH, temperature, and substrate concentrations. This characterization is essential for identifying suitable working conditions for all enzymes within the multi-enzymatic system. Additionally, a thorough comparison and evaluation of disruption techniques, specifically Sonication and French Press, will be undertaken to identify the most effective method for preserving enzymatic activity and protein content during PcAOX production. Lastly, a comprehensive investigation into the substrate's role in PcAOX activation and its potential inhibitory effects at elevated concentrations will contribute to a better understanding of the enzymatic reaction.

Finally, the system's efficiency will be tested and compared to data from the previously established ADH-based route.

3. MATERIALS AND METHODS

3.1. REAGENTS

All the reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of analytical grade if not stated elsewhere.

3.2. CULTIVATION CONDITIONS

3.2.1. Media Composition

Luria-Bertrani (LB) medium, containing 10 $g \cdot L^{-1}$ peptone, 5 $g \cdot L^{-1}$ yeast extract, 10 $g \cdot L^{-1}$ NaCl, and 0.1 $mg \cdot mL^{-1}$ ampicillin was used for pre-inoculum preparations, shake-flasks inoculums, and bioreactor cultivation.

3.2.2. Shake flask cultures in LB.

Pre-inoculums were prepared in 250 mL shake flasks, containing 25 mL of LB (Luria Bertrani) medium, 0.1 $mg \cdot mL^{-1}$ ampicillin, and cryostrocks from the transformed strain of *E. coli* BL21-AI pBAD-wtPcAOX3. This was left overnight for growth at 37°C and 130 rpm.

The inoculum was then prepared from the pre-inoculum in two separate 1 L shake flasks. The pre-inoculum was transferred calculating the required volume to obtain an initial OD_{600} of 0.2 in the new flasks that contained 100 mL of LB medium and 0.1 $mg \cdot mL^{-1}$ ampicillin. Protein expression was induced when OD_{600} was around 1.0 by adding arabinose at a final concentration of 0.2%, following the plasmid transformation protocol[14]. Temperature was decreased from 37ºC to 30ºC during 18 hours of induction. Growth was also performed at 37°C and 130 rpm.

3.2.3. Batch culture in bioreactor.

Batch cultures were performed in a 1.5 L reactor, previously adjusted to the optimal conditions of 37ºC and a pH of 7. The first phase of the batch was performed by transferring 200 mL of inoculum to 1.3 L of LB medium.[14] The growth conditions were maintained at 37°C, 450 rpm, and a pH of 7, which was done through the addition of H_2SO_4 at 2M and NH_4OH at 15%. Oxygen saturation was maintained at 50% by supplying air at a flow rate of 1.5 $L \cdot min^{-1}$ and with cascade agitation from 450 to 1150 rpm. If the cascade was not

enough to maintain the pO_2 levels, pure oxygen was to be added with a maximum flow rate of 0.5 $L \cdot min^{-1}$.

When OD_{600} reached 1.32, the protein induction was initiated by adding arabinose at a final concentration of 0.2%. The induction time was 18.7 hours.

3.3. ENZYME PURIFICATION

3.3.1. Cell disruption by sonication

Cells were resuspended in lysis buffer (50 mM potassium phosphate pH 7.5) until OD_{600} = 40 was reached and mechanically disrupted by sonication using Vibracell® model VC50 (Sonic & Materials®, Newton, CT, USA) at 4ºC (5 s ON, 10 s OFF, 70% amplitude, total time of 5 min). Finally, cell debris was removed by centrifugation at 13400 rpm for 10 min. The pellets were discarded, and the supernatant was stored at -20ºC.

3.3.2. Cell disruption by French press

The biomass samples were suspended in lysis buffer, 50mM potassium phosphate buffer at pH 7.5, mechanical cell disruption was achieved employing One Shot Cell Disrupter (Constant Systems®, Daventry, United Kingdom). For *Escherichia coli* samples, the pressure used was 1.7 kbar. The cell debris was then removed via centrifugation at 6000g for 15 minutes at 4°C, and the pellets were discarded.

3.3.3. PcAOX purification by affinity chromatography

PcAOX purification was done through affinity chromatography due to the enzyme's HisTag affinity to the nickel groups on the Ni^{2+}-agarose. An FPLC AKTA Pure (Cytiva, USA) was used. Cells were resuspended in lysis buffer (50 mM potassium phosphate pH 7.8, 400 mM NaCl, 100 mM KCl, 20 mM imidazole, and 20 μM FAD^{+}) and disrupted by sonication as described in section 3.3.1.[16]

Initial equilibration of the column was performed with lysis buffer at a flow rate of 4 $mL \cdot min^{-1}$ until the chromatogram indicated stable conductivity and UV signals. The flow-through was collected in 50 mL fractions. Washing was carried out at 10 $mL \cdot min^{-1}$ for four column volumes using lysis buffer, and the washing flow-through was collected in 50 mL fractions.

Finally, in the elution step, a linear gradient from 0% to 100% of (50 mM KH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8, and 20 µM FAD^+) was applied at 5 $mL \cdot min^{-1}$ during 4 column volumes, collected in 15 mL fractions. These samples were analyzed for enzyme activity and protein concentration.[10][14]

3.3.4. Desalting

The eluted PcAOX fractions, purified through Ni^{+2} -agarose affinity chromatography with a linear gradient, were desalted with dialysis tubing cellulose membrane from Sigma-Aldrich® (St. Louis, MO, USA). Before use, the dialysis membrane was rinsed with distilled water after being removed from the ethanol storage solution. Following this, each elution was added to the membrane and introduced into the desalting elution. The prepared dialysis membranes were then immersed into 1 L of desalting buffer (50 mM potassium phosphate buffer, pH 7.5, 20 µM FAD) and incubated overnight at 4°C and stirred mechanically.[12]

3.4. ANALYTICAL METHODS

3.4.1. Biomass concentration

Growth was followed by optical density measurements at 600 nm (OD_{600}) using a HACH D3900 (Hach®, Loveland, CO, USA). Biomass concentration was calculated from OD_{600} values, with one OD_{600} equivalent to 0.3 g L^{-1}. [17]

3.4.2. Total Protein Content

The quantification of the total intracellular protein content was conducted according to the Bradford method, employing the Coomassie Protein Assay Reagent Kit from Thermo Scientific®, with bovine serum albumin (BSA) as the standard. These assays were executed within 96-well microplates, measuring the absorbance at 595 nm using a SPECTROstar Nano Microplate Reader (BGM LABTECH, Germany).

For calibration purposes, a concentration range of BSA was prepared, spanning from 0 to 0.5 $mg \cdot mL^{-1}$. The analyses were carried out in duplicate for accuracy and reliability.

3.4.3. SDS-PAGE

The SDS-PAGE electrophoresis technique was employed to distinguish the purified enzyme from other intracellular proteins within the cell lysates. Initially, 15 µL of each

sample was mixed with 5 µL of loading buffer, consisting of Laemmli Buffer (4X) and β-mercaptoethanol in a 10:1 ratio.

After incubating the samples at 95°C for 5 minutes, they were promptly placed on ice. Simultaneously, 5 µL of Coomassie Blue Molecular Weight Marker was loaded. Subsequently, 15 µL of each sample was loaded onto a 12% Mini-PROTEAN® TGX gel, and the electrophoresis was conducted in a denaturing running buffer 1X at 120 V for approximately 60 minutes.

Following electrophoresis, the gel was stained with InstantBlue Coomassie Protein Stain (Bio-Rad®). Images of the gel were captured using a Gel Doc EZ Imaging System (Bio-Rad®), and the data were analyzed using Image Lab™ 6.0 Software (Bio-Rad®).

3.4.4. Acetoin, acetaldehyde, and ethanol analysis by gas chromatography

Samples of the reaction (100 µL) were added to a mixture of 200 µL of deionized water and 20 µL of concentrated HCl, to stop the reaction and precipitate the enzymes for its analysis in gas chromatography. These samples were centrifuged at 13400 rpm for 5 minutes. Then the supernatant was filtered at 0.45µm and stored in a chromatography vial adapted to 0.5 mL. These samples were analyzed on an Agilent 7890 gas chromatography (GC) with an FID detector (Santa Clara, CA, USA). An HP-INNOWAX formed by polyethylene glycol (PEG) column was used, with He, at a flow rate of 4.6 $mL \cdot min^{-1}$ as the mobile phase. First 1 µL of the sample was taken and the injector, at 250°C, was injected into the column. The temperature program was set as follows: Initial temperature 35°C (0 MIN), gradient 15°C·min^{-1} until 250ºC. Ethanol, acetaldehyde, and acetoin were detected on an FID detector at 250ºC at a flow rate of 40 $mL \cdot min^{-1}$ of hydrogen gas, 450 $mL \cdot min^{-1}$ ffer of air, and 25.4 $mL \cdot min^{-1}$ of Nitrogen gas. Retention times of acetoin, acetaldehyde, and ethanol were 7.83, 1.74, and 3.38 min, respectively.[18]

3.5. ENZYME CHARACTERIZATION

3.5.1. PcAOX enzymatic activity assay

PcAOX enzymatic activity was assessed by coupling the oxidation of ethanol to acetaldehyde, catalyzed by PcAOX, with the oxidation of 4-aminopyridine and 3,5-dichloro-2-hydroxybenzensulfonic acid (DHBS) into a quinoid product at 25ºC and, 42.7 mM Potassium phosphate buffer at pH 7.5, that produced a pink hue tracked at 515 nm,

with an extinction coefficient of 26 $mM^{-1} \cdot cm^{-1}$, via Horseradish peroxidase (HRP). (Figure 4).

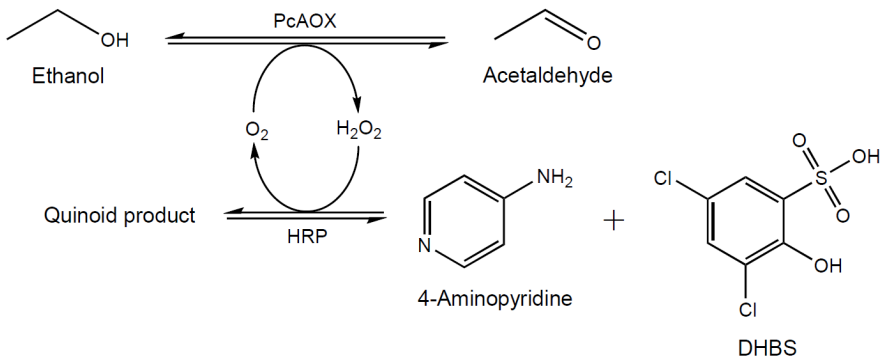

Figure 4. Coupled reaction of PcAOX and HRP enzymatic reactions for the determination of the enzymatic activity of PcAOX. PcAOX enzymatic activity assay is followed at 515 nm by the formation of a quinoid product (ε_{515} = 26 $mM^{-1} \cdot cm^{-1}$)

3.5.2. PcAOX activity towards temperature and pH

For the pH study of PcAOX activity, a 1.5 mL cuvette was employed at 25ºC, containing the components outlined in section 3.5.1. The pH was varied within the range of 4 to 10, simultaneously adjusting the buffers used. Buffer solutions with a pH equal to the pKa value of the acid offer the greatest capacity, and, accordingly, citrate buffer (42.7 mM) was used with respective pKa values of 3.13, 4.76, and 6.40 for testing pH 4, 5, 6, and 6.5. For pH 7, 7.5, and 8, potassium phosphate buffer (42.7 mM) with a pKa of 6.82 was employed. Finally, bicarbonate buffer (42.7 mM) with a pKa of 10.3 was utilized for pH 8.5, 9, and 10.[19]

As for the temperature, this was executed in a 1.5 mL cuvette with the same components as described in section 3.5.1, modifying the temperature values ranged from 20, 30, 35, 40 45, 50, and 60ºC.

3.5.3. PcAOX stability towards pH

Enzyme stability was determined by preparing a 20 % $v \cdot v^{-1}$ solution of the purified enzyme in the buffers mentioned in section 3.5.1., at different pHs ranging from 4 to 10, with a final volume of 2 mL and maintaining the solution at 25ºC and 350 rpm agitation.

Sampling was taken periodically; enzyme activity was measured for every sample by duplicate following the procedure described in section 3.5.1.

3.5.4. Optimal ethanol concentration

For the ethanol concentration study, enzymatic activity was measured according to section 3.5.1 using different ethanol concentrations ranging from 2 mM to 5 M.

3.6. ENZYMATIC REACTION CONDITIONS

3.6.1. PcAOX, ZpPDC, and Catalase coupled enzymatic reaction.

The multi-enzymatic reaction was conducted within a 20 mL syringe equipped with a filter. A 5 mL of reaction medium was prepared containing final concentrations of 250 mM Tris-Base Buffer, 50 M Ethanol, 1 mM TPP, 1 mM $MgCl_2$, 100 $U \cdot mL^{-1}$ ZpPDC (lyophilized), 10 $U \cdot mL^{-1}$ PcAOX(purified), 200 $U \cdot mL^{-1}$ commercial Catalase purchased from Sigma Aldrich® (St. Louis, MO, USA). Optical Oxygen & Temp Meter Firesting®-O_2 sensors were used to monitor oxygen levels and temperature in real-time. The temperature was maintained at 25°C, using a bath and magnetic stirring. Additionally, the pH of the reaction medium was adjusted and maintained at 7.5 throughout the reaction with a pH-STAT using a solution of NaOH at 2M.

4. RESULTS AND DISCUSSION

4.1. SHAKE FLASK PRELIMINARY EXPERIMENTS

Initially, preliminary assays were performed to verify the correct expression of the PcAOX enzyme. For that, the inoculum was prepared as detailed in section 3.2.2, followed by 18 hours of induction, after which cellular disruption was achieved through sonication as outlined in section 3.3.1. Afterward, an SDS-PAGE electrophoresis gel was prepared to visualize protein separation, with a specific focus on the targeted enzyme. The PcAOX enzyme with a molecular weight of 72.6 kDa is fused with SUMO region (11.3 kDa) and a histidine tag (0.8 kDa), The His-SUMO fusion is known to enhance the expression level and solubility of partially insoluble proteins a characteristic that is crucial for maintaining the protein stability based on the findings of Nguyen et. al[14]. The gel analysis of sonicated samples clearly evidenced the expression of the His-SUMO-PcAOX protein, measured at 84.7 kDa Intriguingly, an unknown intense band consistently observed at 37 kDa was present in all samples, although this band did not recur in subsequent studies (Figure 5). Following enzymatic activity assays outlined in section 3.5.1, an activity value of 9.95 $U \cdot mL^{-1}$ was determined.

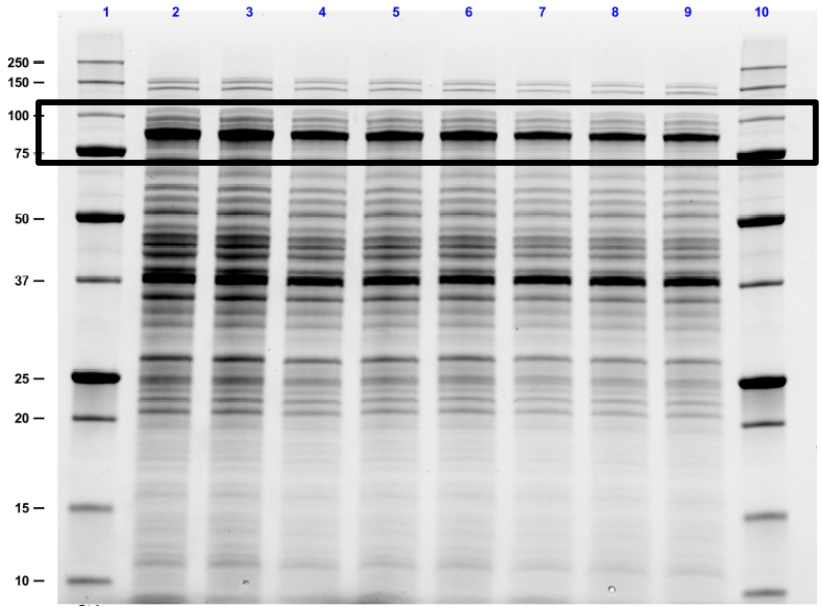

Figure 5. SDS-PAGE electrophoresis gel showing disrupted samples from PcAOX production culture. Lane 1 and 10: BioRad Precision Plus Molecular Weight Marker. Lane 2-9: Pellet and supernatant from different colonies lysate. His-SUMO-PcAOX band is highlighted in black at 84.7 kDa.

4.2. PcAOX PRODUCTION IN BIOREACTOR

Once the preliminary shake flask production was done, the next step was the production of PcAOX in a 5-L Bioreactor, following the methodology mentioned in section 3.2.3. In Figure 5 biomass concentration and the specific activity profile throughout time in the bioreactor culture are depicted. The arabinose was added to the culture to a final concentration of 0.2% once an OD of 1.32 was reached.

As depicted in Figure 6, the biomass reached a final concentration of 2.20 gDCW·mL^{-1} and a specific activity of 711 U·gDCW^{-1}. Moreover, it was observed that the expression was well-regulated since it was only after the induction took place that the enzymatic activity was detected as can also be seen in Figure 7, thus indicating that the expression is well regulated.

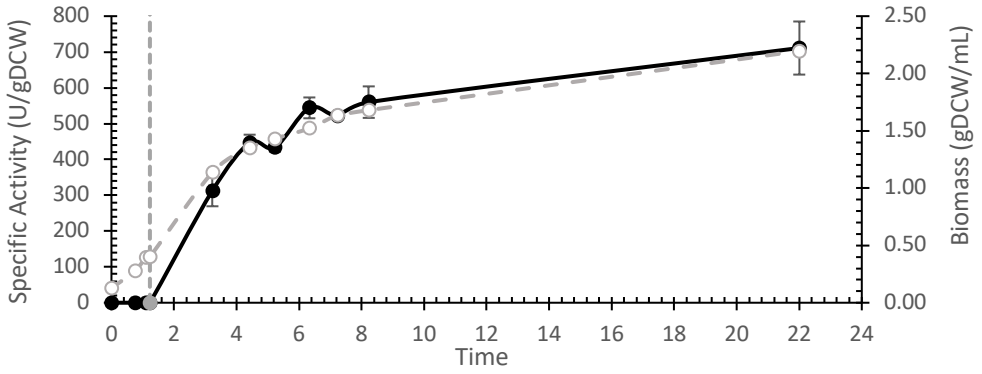

Figure 6. Batch culture for PcAOX production in a 5L-bioreactor using the E. coli strain (BL21 AI) in 1.3 L of LB medium at 37ºC and pH of 7. The moment of the arabinose pulse (1 hour) is indicated by a vertical dotted line. Specific activity (●) and biomass concentration (○) are represented in front of time.

Following sample disruption using a one-shot disruptor as described in section 3.3.2, an SDS-PAGE electrophoresis gel was employed shown in Figure 7. This gel distinctly reveals the presence of the enzyme PcAOX fused with the His-SUMO tag measured at 84.7 kDa with a percentage of the PcAOX band is of 22.5%. This band is highlighted in black for clarity. It is worth noting that the protein shows no basal expression, absent in both the pre-induction sample and the sample obtained immediately after induction. The progressive increase in the color intensity of the band over time indicates a gradual increase of the protein content throughout the fermentation.

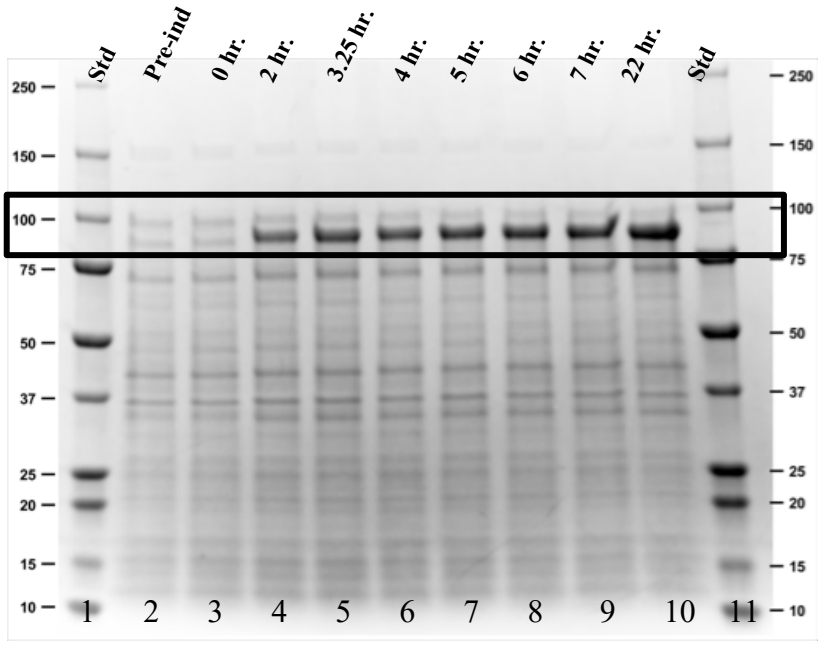

Figure 7. SDS-PAGE electrophoresis gel showing disrupted samples from PcAOX production culture. Lane 1: Bio-Rad Precision Plus Molecular Weight Marker. Lane 2 – 10: Supernatant from 9 samples were taken, since 0.5 mg/mL could not be reached, 0.3 mg/mL was set instead. Lane 11: Bio-Rad Precision Plus Molecular Weight Marker. PcAOX-SUMO-HisTag band is highlighted in black at 84.7 kDa.

4.3. METHOD OF DISRUPTION

After completing 1.5 L bioreactor PcAOX production, the biomass underwent disruption using a one-shot disruptor, leading to the recovery of supernatants utilized for both enzymatic activity assays and protein quantification. The final cell lysate of PcAOX exhibited a value of 82.5% less protein content than that obtained in the 100 mL shake flasks. A significant discrepancy in protein content, 0.95 and 5.45, respectively, was observed, with the only difference being the method of disruption—sonication for the shake flasks and a one-shot cell disrupter for the 1.5 L reactor. Due to this, another disruption was decided to be done, this time through a continuous flow cell disruptor to potentially augment the protein content and specific activity and find out if the disrupting method had a role in the decrease.

Table 1 illustrates the enzymatic activity and protein content obtained from shake flask production, 1.5 L reactor, and lysate pre-purification following another round of disruption via continuous flow cell disruptor, a notable threefold increase in enzymatic activity and protein content was observed, highlighting the disruptive step as a crucial factor contributing to the initially low protein content and enzymatic activity in the 1.5 L reactor production.

Table 1. *Comparison of Enzymatic Activity [U·mL^{-1}] and Protein Content [mg·mL^{-1}] Obtained from Production in Shake Flask disrupted through sonication, 1.5 L Reactor through French press, and Lysate before purification disrupted via continuous flow cell disruptor.*

	Shake Flasks	**1.5 L Reactor**	**Lysate pre-purification from 1.5 L reactor**
Disruption Method	**Sonication**	**One-shot cell disruptor**	**Continuous flow cell disruptor**
Enzymatic Activity [U/mL]	9.95	2.73	11.01
Protein Content [mg/mL]	5.45	0.95	4.48

4.4. PcAOX PURIFICATION BY AFFINITY CHROMATOGRAPHY USING Ni^{+2}-AGAROSE MATRIX

Starting from the lysate mentioned above, affinity chromatography was used to purify PcAOX, due to the presence of HisTag in the enzyme that binds by affinity to the nickel groups of the support (Ni^{+2}-agarose) after a desalting step is done.

Table 2 displays the PcAOX enzymatic activity and total protein content data obtained from various samples during PcAOX purification, lysate, flowthrough wash, and elutions that showed the highest values in activity and concentration before (E3, E4, E5) and after desalting (E3D, E4D, E5D), out of them being E4 the elution with the highest values, and thus what will be used in future experiments. After the affinity chromatography purification step, 82.2% of the protein content initially from the shake flasks was maintained in the lysate purified. Notably, the post-purification enzymatic activity of the lysate surpassed that obtained from shake flask production by 17.8%. An exceptional 2.55-fold increase in the enzymatic activity of the elution concentrate fraction compared to the lysate purified was observed, possibly attributable to the addition of the FAD^{+} cofactor to the lysate and the supplementary disruption step.

Table 2. *Enzymatic activity and protein concentration of PcAOX following its purification via affinity chromatography using Ni^{+2}Agarose matrix and desalting process.*

	Sample	ENZYMATIC ACTIVITY [U/mL]	[U]	CONCENTRATION [mg/mL]	[mg]	SPECIFIC ACTIVITY [U/mg]
AFFINITY CHROMATOGRAPHY	Lysate	11.01	1365.34	4.48	555.94	2.46
	Flowthrough	1.22	150.72	2.39	296.79	0.51
	Wash	0.97	126.08	0.73	95.31	1.32
	E3	14.63	146.32	2.23	22.31	6.56
	E4	39.10	391.00	5.02	50.23	7.78
	E5	19.53	195.29	3.08	30.84	6.33
DESALTING	E3D	19.56	195.65	2.19	21.90	8.93
	E4D	51.44	514.38	4.20	41.99	12.25
	E5D	21.58	215.78	2.77	27.71	7.79

The elution 4 concentrated registered 50.23 mg of protein before desalting and 41.99 mg after desalting. Interestingly, a 31% increase in enzymatic activity was noted post-desalting, potentially explained by the overnight submersion of the sample in FAD^{+}, as detailed in section 3.3.4.[14] The sum of specific activity in all elutions reached 28.97 $U \cdot mg^{-1}$ after desalting., resulting in a yield of 56.4% enzymatic activity and a purification level of 6.8.[20]

Finally, SDS-PAGE electrophoresis gel was executed to validate the successful purification of PcAOX, in accordance with section 3.4.3. Figure 8 provides substantiating evidence for the observations delineated in Table 2. Lanes 4-9 exclusively depict a singular intense band corresponding to PcAOX, 87.4 kDa.[14] Notably, Lane 8, representing elution 4 after desalting, exhibits a more pronounced band compared to Lane 5, representing the elution before desalting, in concordance with chromatographic results.

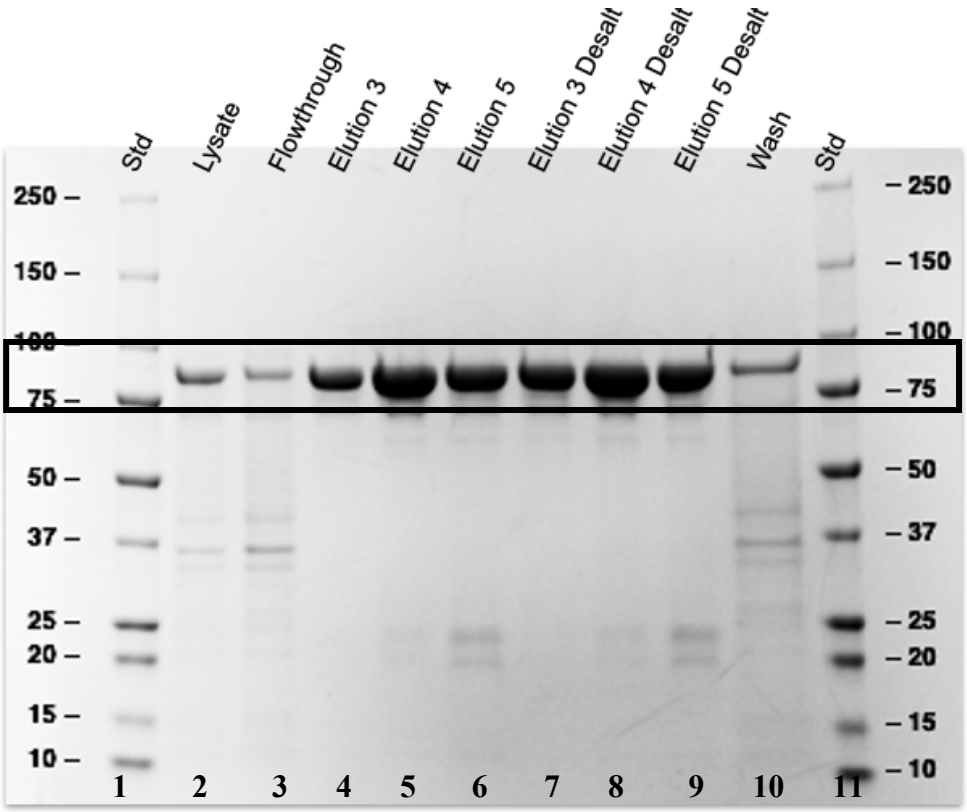

Figure 8. SDS-PAGE electrophoresis of samples obtained in the ion exchange chromatography and desalting, highlighting the PcAOX band within a black box at 84.7 kDa.

4.5. CHARACTERIZATION OF PcAOX

Characterizing enzymes is a crucial step in optimizing their performance, especially within multi-enzymatic systems. Understanding an enzyme's behavior under all conditions allows for the utilization of different strategies that ensure it functions efficiently in combination with other enzymes. This comprehensive understanding is essential for working under optimum conditions, thereby maximizing the production of valuable compounds such as acetoin.

Given that the system involves two additional enzymes, ZpPDC, and catalase, it is imperative to consider their optimal conditions as well. ZpPDC, for instance, exhibits optimal activity at pH 6 [21], although it remains active up to pH 8. Catalase, however, shows maximum activities at pH 7 and 7.5 [22]. Therefore, ensuring that all enzymes operate in their respective optimal conditions is crucial for achieving optimal performance in the multi-enzymatic system.

4.5.1. PcAOX activity towards pH

The pH characterization assay enlightened the enzyme's behavior across different pH conditions, showcasing distinctive patterns in the enzymatic activity assay. As detailed in Figure 8, the enzymatic activity exhibits an upward trend from pH 5 to pH 7, reaching its peak at pH 7. Subsequent deviation from this optimal pH range led to a gradual decline in enzymatic activity, demonstrating a 5% reduction at pH 7.5, an 11% decrease at pH 8, and a stark decline at pH 9 and 10.

These outcomes emphasize the enzyme's dependency on specific pH conditions for optimal functionality, that is an optimum at pH 7 and a pH range from 6.5 to 8 with activities higher than 89%.

These differ slightly from the findings of Nguyen et al. [14], where the optimal pH of PcAOX values was assessed using methanol oxidation rates. In their study, the enzyme depicted a distinct preference for neutral to basic conditions, showcasing an optimal pH value of 9 rather than 7. PcAOX retained over 75% of its activity between pH 7 and pH 10. Notably, activity sharply declined below 50% outside the pH range of 6 to 11.

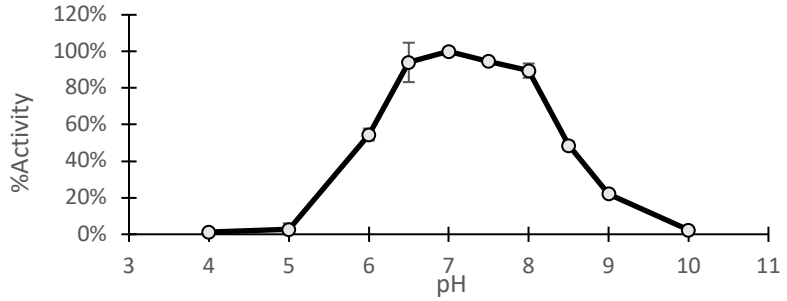

Figure 8. Effect of pH on PcAOX activity in its enzymatic activity assay. Enzymatic reaction conducted in a 1.5 mL cuvette comprising 4-aminopyridine (0.1 mM), DHBS (1 mM), ethanol (2 M), HRP (4 U · mL^{-1}), at 25ºC with different pH levels and their respective buffers depending in their pKa. pH 4, 5, 6, and 6.5: citrate buffer (50 mM). pH 7, 7.5, and 8: potassium phosphate buffer (50 mM). pH 8.5, 9 and 10: sodium bicarbonate buffer (50mM).

In comparison with ZpPDC, one of the other main enzymes in this system displays comparable optimal conditions, with pH ranges from 6 to 7 having high activities.[14] While pH 6 in a citrate buffer performed the reaction faster, pH 7 with tris-base buffer yields a high concentration of acetoin. Alptekin et at. found that free catalase shows maximum activity in pH 7 and 7.5.[22] The fact that all enzymes have similar optimal pH levels simplifies the decision-making process when it comes to selecting the preferred pH.

4.5.2. PcAOX activity towards temperature

The characterization of the optimal temperature revealed distinct behavior of PcAOX across different temperature ranges. As illustrated in Figure 9, the enzymatic activity exhibited a steady incline from 20ºC to 40ºC, reaching its maximum at 40ºC. Beyond this optimal temperature, a gradual decline in activity was observed, with a steady decrease from 40ºC - 60ºC, indicating thermal denaturation and subsequent loss of enzymatic function. These findings showcase the high thermostability of Nguyen et al.[14] found in their study, where the variation in temperatures had minimal impact on the relative PcAOX activity percentage, with values of 72% at 25ºC and 71% at 50ºC.

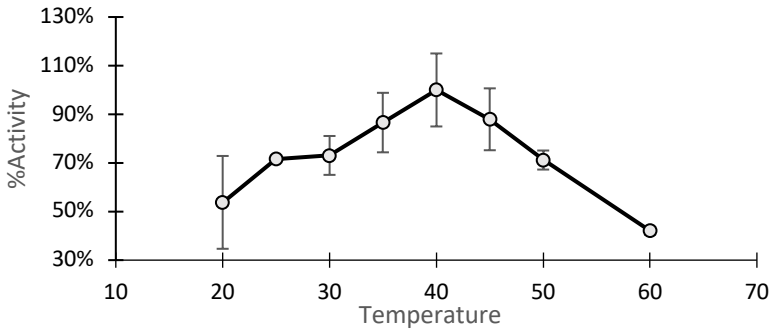

Figure 9. Effect of Temperature on relative PcAOX Activity percentage. Enzymatic reaction conducted in a 1.5 mL cuvette comprising 4-aminopyridine (0.1 mM), DHBS (1 mM), ethanol (2 M), HRP (4 U · mL^{-1}), and potassium phosphate buffer (42.7 mM), while maintaining a pH of 7.0. Temperatures ranged from 20, 25, 30, 35, 40, 45, 50, and 60ºC.

4.5.3. pH Stability of PcAOX

The pH stability assay of PcAOX revealed distinct patterns in the enzyme's resilience across a range of pH environments and its highly important feature for the implementation of the catalytic process. Figure 10 graphically represents the enzyme's behavior under different pH conditions. Notably, at pH 8, the enzyme displayed remarkable remaining activity levels surpassing 100%, peaking at 299% after 24 hours of incubation. Subsequently, a gradual decline was observed, stabilizing at a relatively consistent phase after 120 hours with a less pronounced decrease. This behavior has also been observed in the enzyme PDC, as reported in Alcover's thesis. After 8 hours of incubation, PDC showed an activity increase, maintaining remaining activities higher than 100% across all pH levels ranging from 5.5 to 8.[21]

At pH 9, the enzyme exhibited some hyperactivity of 257% after only 5 hours, followed by a steady decline until reaching 88% activity after 240 hours. At pH 7, the enzyme displayed exceptional stability, maintaining a somewhat consistent activity without

significant fluctuations over time, achieving 54% of remaining activity after 240 hours. In contrast, pH 4 exhibited near-zero activity from the onset, while pH 5 displayed a declining trend after 24 hours. Similarly, at pH 10, the enzyme's activity diminished to 0% after 100 hours, indicating limited stability in this alkaline environment. These findings underscore the enzyme's sensitivity to varied pH conditions and highlight the enzyme's susceptibility to extremely acidic and alkaline environments.

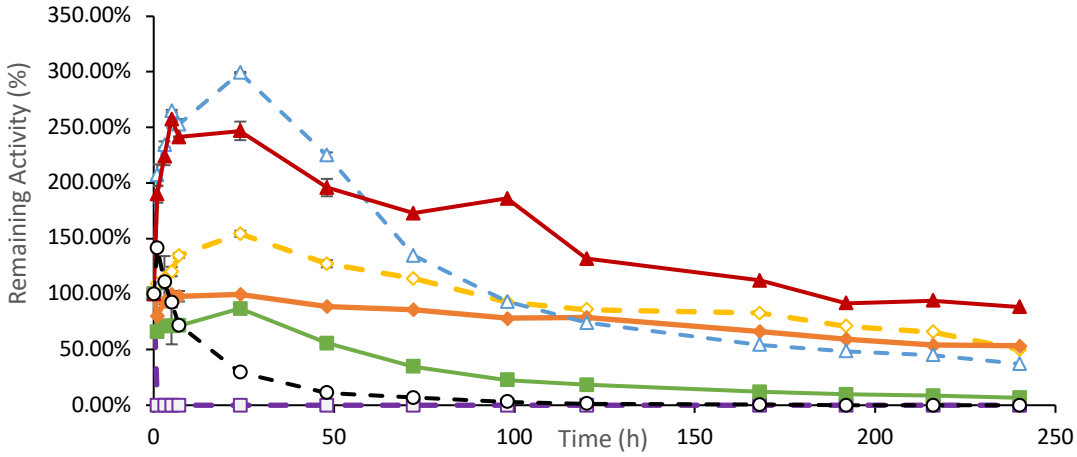

Figure 10. Profile of PcAOX stability at different pH conditions. Enzymatic reaction conducted in a 1.5 mL cuvette comprising 4-aminopyridine (0.1 mM), DHBS (1 mM), ethanol (2 M), HRP (4 U · mL^{-1}), and potassium phosphate buffer (42.7 mM), while maintaining a pH of 7.0 and 25°C. Markers represent distinct pH conditions: (□) pH 4, (■) pH 5, (◇) pH 6, (◆)pH 7, (△)pH 8, (▲)pH 9, and (○) pH 10. The graph illustrates the enzyme's varying stability responses in different acidic and alkaline environments in terms of the percentage of remaining activity over time.

4.5.4. Characterization of Optimal Ethanol Concentration

For further intensification studies, characterization of optimal ethanol concentration for the enzymatic reaction was done, involving the investigation of varying ethanol concentrations, as outlined in section 3.5.1. The highest enzymatic activity was attained at a concentration of 500 mM, reaching 27.46 U·mL^{-1}, a 21% increase compared to that obtained at 2 M.

Figure 11 illustrates a profile depicting enzyme activity toward ethanol concentration. As expected, activity is increased as substrate concentration increases. However, beyond 500 mM of ethanol enzymatic activity is reduced as ethanol concentration surpasses this value. The observed trend could be attributed that as the concentration surpasses the optima, it may start to exert inhibitory effects, leading to a decline in enzymatic activity.

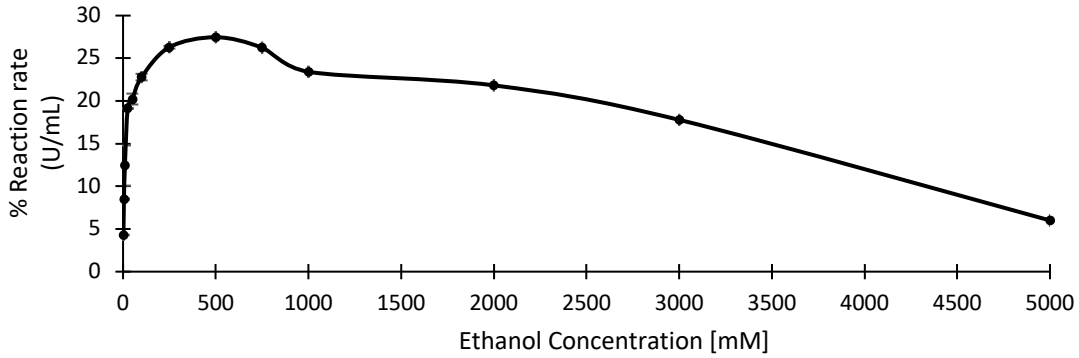

Figure 11. Optimization of Ethanol Concentration for Enzymatic Activity. Enzymatic reaction conducted in 1.5 mL cuvette with components including 4-aminopyrine (0.1mM), DHBS (1mM), HRP (4 U·mL^{-1}), and varying concentrations of ethanol (2mM, 5mM, 10mM, 25mM, 50mM, 100mM, 250mM, 500mM, 750mM, 1M, 2M, 3M, and 5M). The buffer volume was adjusted accordingly to maintain a total volume of 1.5 mL. The graph depicts the relationship between enzymatic activity and varying concentrations of ethanol.

This study revealed critical insights into the kinetics of the enzymatic system. Through data analysis, the Michaelis-Menten constant (K_M) and maximum velocity (V_{max}) were determined as 10.1 mM and 27.88 U·mL^{-1}, respectively. The K_M signifies the substrate concentration at which the enzyme achieves half of its maximum velocity, reflecting its binding affinity for ethanol. The lower KM compared to catalase implies a higher binding affinity for ethanol, suggesting a need for more catalase than PcAOX in the system. Furthermore, the Vmax value correlates with the maximum velocity observed at 500 mM of ethanol.

4.6. MULTI-ENZYMATIC SYSTEM USING PcAOX, ZpPDC, AND CATALASE

Having successfully produced, purified, and characterized the PcAOX enzyme, the integration of the enzyme into a multi-enzymatic system becomes feasible. This system composed of ZpPDC/PcAOX/Catalase, is central to the primary objective of this study, being the exploration of an alternative production of acetoin without the need for additional cofactors. Testing this reaction will determine its efficacy in acetoin production.

The enzyme activities selected for the reaction were determined based on specific criteria. 100 U·mL^{-1} was chosen as ZpPDC due to the optimal activity seen in prior studies involving the multi-enzymatic reaction system with ZpPDC, ScADH, and SpNOX.[12]. Catalase activity (200 U·mL^{-1}), was guided by the study realized by Garcia-Boffil, where the activity of the catalase was twice that of the primary enzyme in the system.[15] Additionally taking into consideration its higher K_M, (33.3 mM) it was deemed crucial to have an excess of catalase in the system. Finally, given the final volume of the reaction of

5 mL, the maximum activity that could be added to the reaction was 10 U·mL^{-1} of PcAOX, reflecting the initial utilization of PcAOX in this specific system without prior reference.

As depicted in Figure 12, the progression of the reaction indicates a near completion after 8 hours, with 99% of the ethanol consumed, resulting in a maximum of 22.6 mM of acetoin being reached. Notably, the observed dynamics involve the accumulation and subsequent consumption of acetaldehyde serving as an intermediate reagent within the reaction scheme. A residual 7.13 mM of acetaldehyde remains unconverted after 8 hours.

It is appropriate to mention that discrepancies in the mass balance have been noted, potentially suggesting an inconsistency in the chromatographic analysis or calibration standards. Further investigation into the calibration process may be warranted to reconcile the observed disparities and ensure the accuracy of the analytical measurements.

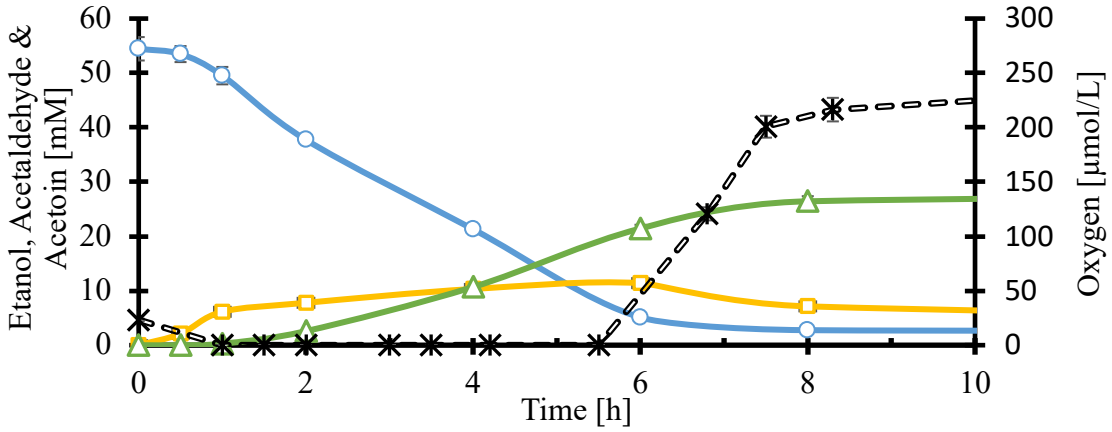

Figure 12. Profiles of ethanol(○), acetaldehyde (❑), acetoin (△), and oxygen concentrations (✱), throughout the reaction, Ethanol, acetaldehyde, and acetoin concentrations, the reaction was carried out at 25 ℃, pH 7.5, magnetic stirring, 250 mM Tris-Base Buffer, 50 mM Ethanol, 1 mM TPP, 1 mM MgCl2, 100 U·mL^{-1}, ZpPDC (purified), 200 U·mL^{-1} commercial catalase, and 10 U·mL^{-1} PcAOX.

Monitoring the oxygen levels confirmed that oxygen is a limiting factor to this reaction, within just 1 hour of reaction time, the oxygen concentration level dropped from 23.18 µmol/L to 0 µmol/L. To address this limitation, several strategies can be implemented. One viable approach involves the introduction of bubbling air or oxygen through a sparger into the reaction mixture to replenish oxygen levels. Additionally, optimizing the reaction setup by employing larger-scale equipment can enhance agitation efficiency, thereby augmenting oxygen transfer rates. After approximately 6 hours, the oxygen levels begin to rise again meaning the oxidation reaction is slowing down.

While the yield and conversion rates remain similar between the systems as depicted in Table 3, the difference lies in their initial velocities, influencing their productivities.

Specifically, after an 8-hour reaction, employing the ZpPDC/ScADH/SpNOX system yields a productivity of 2.9, whereas the ZpPDC/PcAOX/Cat system achieves a productivity of 3.5 $mM \cdot h^{-1}$, reflecting a notable 20.7% increase. Moreover, with the PcAOX system, there's a conservation of the cofactor, as no additional cofactor is required, unlike with the ScADH system, which necessitates the addition of a cofactor, NAD+/NADH, the market price of which ranges from €76 to €114 per gram.[23] Consequently, the PcAOX system presents a more economically viable solution over the long term.

The observed rate of acetoin production, being half of the ethanol's consumption rate, aligns with the stoichiometry of the reaction where 2 mol of ethanol yields 1 mol of acetoin.

Table 3. *Comparison of two multi-enzymatic systems for acetoin production. With the multi-enzymatic systems of ZpPDC/PcAOX/CAT carried out at 25˚C, pH 7.5, 250 mM Tris-Base Buffer, 50mM Ethanol, 1 mM TPP, 1 mM MgCl2, 100 $U \cdot mL^{-1}$ ZpPDC (purified), 10 $U \cdot mL^{-1}$ PcAOX, 200 $U \cdot mL^{-1}$ Catalase. The ZpPDC/ScADH/SpNOX system carried out at 25˚C, pH 7.5, 250 mM Tris-base buffer, 50mM Ethanol, 100 $U \cdot mL^{-1}$ ZpPDC (purified), 50 $U \cdot mL^{-1}$ ScADH, and 125 $U \cdot mL^{-1}$ of SpNOX.*

Multi-enzymatic system	Acetoin [mM]	Substrate Conversion [%]	Product Yield [%]	r_s [mM ethanol/h]	r_p [mM acetoin/h]	Productivity [mM/h]
ZpPDC/ScADH/SpNOX	22.9	97	100	4.4	3.3	2.9
ZpPDC/PcAOX/CAT	27.63	98	100	8.7	4.0	3.5

The ZpPDC/PcAOX/Catalase multi-enzymatic system demonstrates significant potential for acetoin production, yet the presence of substrate inhibition suggests the need for a more comprehensive investigation to optimize reaction conditions. Specifically, determining the maximum ethanol concentration that can be utilized without significantly slowing down the reaction is crucial. One potential solution is to explore the applicability of a fed-batch process to alleviate substrate inhibition. Addressing the limitation posed by oxygen, a proven limiting factor in the reaction requires careful consideration. Implementing strategies like the application of a sparger to bubble air or exploring alternative oxygenation methods may enhance productivity. Investigations into possible product inhibition and strategies like enzyme immobilization may enhance system efficiency and stability. Further research and optimization of reaction parameters are essential to maximize the system's potential for efficient acetoin production.

5. CONCLUSIONS

The PcAOX production in the bioreactor was carried out successfully obtaining enough enzyme to carry out the characterization.

The Ni^{+2}-Agarose affinity chromatography purification combined with a desalting step yielded promising results of 4.20 $mg \cdot mL^{-1}$ and 51.44 $U \cdot mL^{-1}$, maintaining a substantial portion of protein content and an increase in enzymatic activity respectively. Notably, the yield of enzymatic activity post-desalting reached 56.4%, with a purification level of 6.8.

Characterization studies delved into optimal conditions for PcAOX, revealing remarkable stability at pH 7.5, optimal activity at 40ºC, and a maximum enzymatic activity at 500 mM ethanol concentration, with inhibition observed beyond this point.

The coupled reaction of ZpPDC/PcAOX/CAT demonstrated it is possible to produce acetoin successfully without needing to add the cofactor $NADH/NAD^+$ as in the previous system where ZpPDC/ScADH/SpNOX[12] was used making it more cost-efficient, however, it depicts an inhibition by the substrate as well as the real-time monitoring of oxygen levels clearly indicating oxygen as a limiting factor in the reaction, therefore, more research is needed for further optimization.

Continued monitoring and optimization of the coupled reaction system could enhance the overall efficiency of the production of acetoin.

This study lays a solid foundation for the application of PcAOX in biocatalysis, offering insights into its purification and enzymatic behavior. Future research can build upon these findings to unlock the full potential of PcAOX in various industrial applications.

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