Zymobacter palmae pyruvate decarboxylase production process development: Cloning in *Escherichia coli*, fed-batch culture and purification

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List of abbreviations: PDC, Pyruvate decarboxylase; **DCW**, Dry cell weight, HCDC**,** High cell density culture; MANA-**agarose,** Amino functionalized agarose; **LB,** Luria-Bertrani medium; **DM** Defined medium; **TES**, Trace elements solution.

Practical Application

Pyruvate decarboxylase (PDC) is a very powerful tool in bioprocesses. PDC has an important role in the ethanol synthesis pathway; therefore its overexpression in target microorganisms is of interest for ethanol production. Moreover, PDC can be found in several multienzymatic systems of interest in biocatalysis such as the synthesis of chiral amines in combination with transaminases, where it drives the equilibrium towards formation of the target chiral amine by producing acetaldehyde and CO_2 from pyruvate. Despite its importance in biocatalysis, the use of PDC at large scale is hampered by the lack of an efficient production process. The present study develops a recombinant production and a purification process for PDC from *Zymobacter palmae* expressed in *Escherichia coli*, broadening its application of use at high scale. This is the first work reporting the evaluation of the production and downstream processing of PDC on a bioreactor scale.

Abstract

Pyruvate decarboxylase (PDC) is responsible for the decarboxylation of pyruvate, producing acetaldehyde and CO_2 and is of high interest for industrial applications. PDC is a very powerful tool in the enzymatic synthesis of chiral amines by combining it with transaminases when alanine is used as amine donor. However, one of the main drawbacks that hampers its use in biocatalysis is its production and the downstream processing on scale.

In this paper, a production process of PDC from *Zymobacter palmae* has been developed. The enzyme has been cloned and overexpressed in *Escherichia coli*. It is presented, for the first time, the evaluation of the production of recombinant PDC in a bench-scale bioreactor, applying a substrate-limiting fed-batch strategy which led to a volumetric productivity and a final PDC specific activity of 6942 U L^{-1} h^{-1} and 3677 U $gDCW^{-1}$. Finally, PDC was purified in FPLC equipment by ion exchange chromatography. The developed purification process resulted in 100% purification yield and a purification factor of 3.8.

1 Introduction

Pyruvate decarboxylase (PDC) (E.C. 4.1.1.1) is responsible for the decarboxylation of pyruvate producing acetaldehyde and CO_2 using Mg^{+2} and thiamine pyrophosphate (TPP) as cofactors [1]. This enzyme is a key biocatalyst in the fermentative ethanol pathways since the obtained acetaldehyde is subsequently reduced to ethanol by an alcohol dehydrogenase. PDCs are present in eukaryotes including plants, yeast and fungi. However, this enzyme has been found in a few prokaryotes: *Gluconobacter oxydans* [2], *Zymomonas mobilis* [3,4], *Sarcina ventriculi* [5], *Zymobacter palmae* [6,7] *, Acetobacter pasteurianus* [8] and *Gluconoacetobacter diazotrophicus* [9].

PDC is considered to be an enzyme of high interest for industrial applications. This biocatalyst has been widely studied for the production of bioethanol, especially from cellulosic biomass. In that sense, PDC has been cloned and overexpressed in several microorganisms, such as *Escherichia coli*, *Hansenula polymorpha*, *Bacillus sp* and *Klebsiella oxytoca,* to improve ethanol production [10–15]. Pyruvate decarboxylase has also been applied to the synthesis of pharmaceutical precursors [16,17], compounds for the cosmetic industry [18], or chiral amines and amino acids which play an important role in the pharmaceutical, agrochemical and chemical industries [19]. However, the use of PDC at industrial scale is still hampered. One of the main drawbacks that hinder the use of enzymes, including PDC, in large-scale industrial processes is the price of the biocatalyst. The production cost of enzymes, including their subsequent purification, is one of the main contributions to the final price of the enzyme [20–22].

Approximately 90% of all industrial enzymes are produced by recombinant microorganisms, especially for high-volume, low-value enzymes [21]. The use of *Escherichia coli* as a host for recombinant protein production is the most common strategy for process development [23–25]. Thus, Gram negative bacteria remain the preferred system for laboratory investigations and initial development in commercial activities and is a benchmark for comparison with other

88 expression platforms [25,26]. This is due to different factors such as ease of genetic
89 manipulation, high specific growth rates and availability of optimized expression plasmids. High
90 cell density culture (HCDC) techniques for culturing *E. coli* have been developed to improve
91 productivity, and also to provide advantages such as reduced culture volume, enhanced
92 downstream processing, reduced wastewater, lower production costs and reduced investment
93 in equipment [27]. High cell density cultures can be obtained by fed-batch operational
94 strategies, where the use of exponential feeding has been widely used as an efficient strategy
95 to maintain a desired specific growth rate before the induction of protein expression [28,29].

96 In the present paper the bacterial PDC from *Z. palmae* was cloned and expressed in *E. coli* under
97 the *lac* promoter, inducible by IPTG. The best-performing clone was selected according to results
98 obtained in Erlenmeyer-scale cultures using rich and defined medium. The selected clone was
99 then tested for the production of the enzyme at HCDC, applying a substrate-limiting fed-batch
100 strategy in a 2L-bioreactor. In addition, an efficient purification process was subsequently
101 studied and developed, aiming to obtain a process for PDC production including both, upstream
102 and downstream steps.

103 ## 2 Material and methods

104 ### 2.1 Reagents

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

107 Amino functionalized agarose (MANA-agarose) was purchased from Agarose Bead
108 Technologies® (ABT®, Madrid, Spain).

109 ### 2.2 Bacterial strains and plasmids

110 A pJAM3440 plasmid containing the *Zymobacter palmae* PDC gene was kindly donated by the
111 Microbiology and Cell Science Department of the University of Florida (Gaisnesville, FL, USA).

For protein overproduction, the gene was cloned into a pQE-40 vector and transformed into two *E. coli* strains, M15[pREP4] and SG13009[pREP4]. M15 and SG13009 strains were derived from *E. coli* K12 and have the phenotype NaIS, StrS, RifS, Thi–, Lac–, Ara+, Gal+, Mtl–, F–, RecA+, Uvr+, Lon+. Both pQE-40 and the strains were obtained from a QIAexpress type IV vector kit from Qiagen® (Venlo, Netherlands).

2.3 PDC cloning in *E. coli*

Plasmid pJAM3440 containing the *PDC* gene was isolated from the donated *E. coli* strain using a PureYield™ Plasmid Miniprep System from Promega® (Madison, WI, USA) according to the manufacturer's instructions. The *PDC* gene was then amplified by PCR using a Phusion High-Fidelity DNA Polymerase from Thermo Scientific® (Waltham, MA, USA) and two primers designed with the restriction sequences of *Bam*HI and *Sal*I inserted. Double digestion of the *PDC* gene and pQE-40 was performed with the mentioned restriction enzymes, followed by an overnight ligation at 16°C. After cleaning with Wizard® SV Gel and PCR Clean-Up System (Promega®), the new plasmid construction was transformed to M15[pREP4] and SG13009[pREP4] electrocompetent cells with a Gene Pulser II electroporator from Bio-Rad® (Hercules, CA, USA) (V= 2500 v; C= 25 μF; R= 200 Ω). Transformed clones were selected using LB-agar plates supplemented with ampicillin 100 mg L^{-1} and kanamycin 0.05 g L^{-1}. Transformations were confirmed by colony-PCR using GoTaq® Green Master Mix (Promega®).

2.4 Media composition

Luria-Bertrani (LB) medium, containing 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl, was used for pre-inoculum preparations and preliminary production studies in shake-flasks.

A defined medium (DM) with glucose as the main carbon source was used for shake flasks and bioreactor cultivations. For shake flask inoculums, the media contained 5 g L^{-1} glucose, 13.2 g L^{-1} K_2HPO_4, 2.6 g L^{-1} KH_2PO_4, 2 g L^{-1} NaCl, 4.1 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.026 g L^{-1} $FeCl_3$, 0.005 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.1 g L^{-1} thiamine, 0.033 g L^{-1} citrate, 0.1 g L^{-1} ampicillin, 0.05 g L^{-1} kanamycin and 3 mL L^{-1} of trace elements solution (TES) (TES: 0.04 g L^{-1} $AlCl_3$, 1.74 g L^{-1}

138 $ZnSO_4 \cdot 7H_2O$, 0.16 g L^{-1} $CoCl_2 \cdot 6H2O$, 1.55 g L^{-1} $CuSO_4 \cdot H_2O$, 0.01 g L^{-1} H_3BO_3, 1.42 g L^{-1} $MnCl_2 \cdot 4H_2O$,
139 0.01 g L^{-1} $NiCl_2 \cdot 6H_2O$, 0.02 g L^{-1} Na_2MoO_4). The same composition was used for the batch phase
140 cultivation in the bioreactor, but with an increased glucose concentration of 25 g L^{-1}.
141 Feeding medium for fed-batch phase contained 425 g L^{-1} glucose, 11.6 g L^{-1} $MgSO_4 \cdot 7H2O$, 0.6 g L^{-1}
142 $FeCl_3$, 0.35 g L^{-1} thiamine, 0.09 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.1 g L^{-1} ampicillin, 0.05 g L^{-1} kanamycin and
143 76.6 mL L^{-1} of TES.
144 Stock solutions of ampicillin and kanamycin were prepared in a concentration of 10 mg mL^{-1} and
145 50 mg mL^{-1} respectively and stored at -20ºC. IPTG stock was prepared at 100 mM concentration
146 and stored at -20ºC.
147 Vitamins, antibiotics, TES $FeCl_3$, $CaCl_2 \cdot 2H_2O$ and IPTG were sterilized by filtration (0.2 µm).
148 Glucose and saline solutions were separately sterilized by autoclaving at 121ºC for 30 minutes.
149 **2.5 Cultivation conditions**
150 *Pre-inoculum*
151 From cryostocks stored at -80ºC, pre-inoculums were prepared in 15 mL of LB medium
152 containing 0.1 mg L^{-1} ampicillin and 0.05 mg L^{-1} kanamycin. The growth was performed in Falcon
153 tubes overnight at 37ºC and 150 rpm.
154 *Shake flask cultures in LB and define medium (DM)*
155 Around 5 mL of pre-inoculum was transferred to shake flasks containing 100 mL of LB or defined
156 medium and 0.1 mg L^{-1} ampicillin and 0.05 mg L^{-1} kanamycin in order to obtain an initial optical
157 density OD_{600} of 0.1. Cultures were maintained at 37ºC and 150 rpm until a OD_{600} of 1 was
158 reached. Then, protein production was induced by the addition of IPTG to a final concentration
159 of 1 mM.
160 *Inoculum preparation for bioreactor cultures*
161 Inoculums were prepared following the same procedure as described for shake flasks cultures
162 in 100 mL defined medium using 10 g L^{-1} of glucose. The cultures were kept at 37ºC and 150 rpm
163 until an OD_{600} of around 1.2 was reached.

Fed-batch cultures in bioreactor

Substrate limiting fed-batch cultures were performed in an Applikon ez-Control (Applikon Biotechnology®, Delft, Netherlands) equipped with a 2 L vessel. The first phase of the batch was performed by transferring 80 mL of inoculum to 720 mL defined medium. The temperature was maintained at 37°C and the pH at 7.00 by the addition of 15% NH_4OH. Oxygen saturation was maintained at 60% by supplying air at a flow rate of 1.5 L min^{-1} and with a cascade agitation from 450 to 1150 rpm. When the cascade was not enough to maintain that pO_2 value, pure oxygen was added with a maximum flow rate of 0.5 L min^{-1}.

The substrate limiting fed-batch phase was started when glucose was totally exhausted. An exponential addition of feeding medium took place to maintain a specific growth rate of 0.225 h^{-1} using an automatic microburete of 2.5 mL capacity. Furthermore, every 30 OD increase, 5 mL of a concentrated phosphate solution (500 g L^{-1} K_2HPO_4 and 100 g L^{-1} KH_2PO_4) was injected into the bioreactor. When OD_{600nm} reached a value of approximately 70, IPTG was injected to a final concentration of 100 µM in order to induce protein production. Fermentation ended when a glucose accumulation was detected. Fed-batch cultures were carried out in duplicate. The calculated error indicates the confidence interval with a confidential level of 90%.

2.6 Analytical methods

Monitoring bacterial growth

Optical density measurements at 600 nm (OD_{600}) were performed to determine cell concentration using a HACH D3900 (Hach®, Loveland, CO, USA) spectrophotometer. Biomass concentration expressed as Dry Cell Weight (DCW) was calculated considering that 1 OD_{600} is equivalent to 0.3 gDCW L^{-1} [30]. Analyses were carried out in duplicate.

Glucose analysis

Biomass was removed from 1ml of broth sample by centrifugation and filtration (0.45 µm). The obtained supernatant was then used for the glucose analysis using an enzymatic analysis

189 performed on an YSI 20170 system (Yellow Spring System). Analyses were carried out in
190 duplicate.

191 *Total protein content*

192 Culture samples were adjusted to an OD_{600} of 4 and centrifuged at 10,000 *g* during 10 minutes
193 at 4°C. The obtained pellet was then re-suspended in lysis buffer (200 mM citrate buffer (pH
194 6.2), 1mM of $MgCl_2$ and 1mM TPP). Cell suspensions were kept on ice and sonicated using a
195 Vibracell ® model VC50 (Sonic & Materials, Newton, CT, USA) with four times 15 second pulses
196 and 2 min intervals on ice between each pulse. The lysate was then centrifuged at 10,000 *g*
197 during 10 minutes and the clear supernatant was used for protein content analysis. Total
198 intracellular protein content present in cell lysates was determined with the Bradford Method
199 using a Coomassie Protein Assay Reagent Kit (Thermo Scientific®) and Bovine Serum Albumin
200 (BSA) as standard. The assays were performed in 96-microwell plates and Thermo Scentific®
201 Multiskan FC equipment was used for the absorbance reading. Analyses were carried out in
202 duplicate.

203 *SDS-Page electrophoresis*

204 To determine the percentage of enzyme among the rest of intracellular soluble proteins present
205 in the lysates, a NuPAGE electrophoresis system (Invitrogen®, Carlsbad, CA, USA) was used.
206 Lysates were obtained as previously described. A volume of 10µL sample was mixed with 5µL
207 *NuPAGE™ LDS Sample Buffer* (4X), 2µL *NuPAGE™ Reducing Agent* (10X) and 3µL deionized
208 water. After 10 minutes incubation at 70°C samples were charged to NuPAGE 12% Bis-Tris
209 electrophoresis gel and run using MES-SDS as running buffer at 200 V during 40 minutes. The
210 resultant gel was fixed with a solution containing 40% v v^{-1} and 10% v v^{-1} acetic acid in water and
211 stained with *Bio-Safe™ Coomassie Stain* (BIO-RAD). Pictures were taken with a *Gel Doc EZ*
212 *Imaging System* (Bio-Rad®) and analyzed with *Image Lab™ 6.0 Software* (Bio-Rad®)

213 *PDC enzymatic activity assay*

PDC activity present in lysates was determined by coupling the pyruvate decarboxylation with alcohol dehydrogenase (ADH) and following NADH oxidation at 340 nm (ε_{NADPH} = 6.22 mM^{-1} cm^{-1}) and 25°C with a SPECORD® 200 PLUS (Analytik jena®, Jena, Germany) spectrophotometer. Lysates were obtained as described in the previous sections. The reaction mixture contained 33 mM sodium pyruvate, 0.11 mM NADH, 3.5 U mL^{-1} ADH from *Saccharomyces cerevisiae* (Sigma-Aldrich®), 0.1 mM TPP and 0.1 mM $MgCl_2$ in citrate buffer 200 mM and pH 6. One unit of enzyme activity corresponds to the amount of pyruvate decarboxylase that converts 1 μmole of pyruvate to acetaldehyde per minute. Analyses were carried out in duplicate.

2.7 Enzyme purification

Cell disruption

Biomass samples (3 g) were suspended in lysis buffer (15 mL): 200 mM citrate buffer (pH 6.2), 1mM of $MgCl_2$ and 1mM TPP. Cell suspensions were kept on ice and sonicated using Vibracell ® model VC50 (Sonic & Materials®, Newton, CT, USA) with four times 15 second pulses and 2 min intervals on ice between each pulse. The crude cell lysate was then centrifuge at 10,000 *g* during 10 minutes at 4°C and cell debris was rejected.

PDC purification by sequential precipitation with ammonium sulphate

Ammonium sulphate precipitation was carried out in two steps, using a solution of saturated $(NH_4)_2SO_4$ (4.1 M at 25°C). In the first step of purification, $(NH_4)_2SO_4$ was added into a cell lysate in order to promote protein precipitation while keeping the PDC soluble. For that purpose, different concentrations of salt were tested. The mixture was incubated for at least 30 minutes at 25°C or 5°C. The precipitated protein was separated from the supernatant by centrifugation at 10,000 *g* for 5 minutes. In the second step, additional $(NH_4)_2SO_4$ was added to the resulting supernatant in order to increase salt concentration that promotes pyruvate decarboxylase precipitation; the mixture was incubated for 30 minutes at 5°C. The precipitated enzyme was separated from the supernatant by centrifugation at 10,000 *g* for 5 minutes. All fractions were

239 sampled to analyse enzyme activity and protein concentration. Samples corresponding to
240 precipitates were re-suspended in lysis buffer before the analysis.

241 *Enzyme purification by ionic adsorption using MANA-agarose*

242 Ionic adsorption was carried out first in batch mode, where 1.07 grams of MANA-agarose
243 support was mixed with 1 mL of cell lysate and adjusted to 20 U mL of support^{-1} using 8 mL of
244 phosphate or acetate buffer (at pH 6.2 and 5, respectively) containing 1 mM $MgCl_2$ and 1 mM
245 TPP (Thiamine pyrophosphate). The suspension was incubated at 25^{o}C for 30 minutes with mild
246 agitation and 100 µL aliquots of the supernatant and the suspension were taken every 15
247 minutes. After incubation, the supernatant was separated from the support by filtration, the
248 support was washed with 9 mL of the buffer as described above and a sample was taken from
249 the combined supernatants and analysed as above. Finally, elution of the adsorbed proteins was
250 done by adding 9 mL of the buffer containing 1 M NaCl and incubating at 25^{o}C for 1 hour with
251 mild agitation. Supernatant was recovered by filtration and a sample of 100 µL was taken. In
252 parallel, a blank was performed under the same conditions, except for the MANA-agarose
253 support that was substituted by distilled water. All the samples were used to analyse enzyme
254 activity, protein concentration and PDC content by SDS-Page electrophoresis.
255 Using Fast Protein Liquid Chromatography (FPLC) performed in an ÄKTA Pure (GE Healthcare®,
256 Chicago, IL, USA), 0.5 mL of cell lysate was applied at 0.1 mL min^{-1} to a MANA-agarose column
257 (packed with 1.16 mL of support), equilibrated with buffer A (50mM acetate buffer at pH 5). The
258 column was washed at 0.8 mL min^{-1} with buffer A during 5-15 column volumes. In the elution
259 step, a linear gradient of buffer B (50 mM acetate buffer, 1 M NaCl, at pH 5) from 0 to 100%
260 during 10 column volumes or a step gradient (first step at 31% of buffer B and second step at
261 100% buffer B during 10 column volumes each step) was applied at 0.8 mL min^{-1}. Within each
262 chromatographic procedure, fractions of 1 mL were collected and analysed for enzyme activity,
263 protein concentration and SDS-Page electrophoresis.

Desalting

Purified PDC fractions, obtained after MANA-agarose chromatography with step gradient, were pooled and applied at a rate of 10 mL min^{-1} to a XK 26/20 (GE Healthcare®) column packed with 60 mL of Sephadex 10 and equilibrated with lysis buffer (200 mM citrate at pH 6.2 with 1 mM $MgCl_2$ and 1 mM TPP). After sample application, lysis buffer was applied to the column at a rate of 10 mL min^{-1} during 2 column volumes. Fractions of 10 mL each were obtained and analysed for enzyme activity and protein concentration.

3 Results and discussion

3.1 Shake-flask preliminary experiments

Firstly, clones of *E. coli* strains M15[pREP4] and SG13009[pREP4] expressing PDC were prepared following the procedure described in the Methods section. Several transformed clones were obtained and two of them, one from each *E. coli* strain, were selected. The colony PCR confirmed that both selected clones contained the *PDC* gene.

Aiming to compare the capability of both clones to grow and to produce PDC, preliminary cultures were performed in shake flasks with 100 mL LB medium supplemented with the corresponding antibiotics (Figure 1). Both in the case of M15[pREP4][pQE-PDC] and SG13009[pREP4][pQE-PDC], growth took place with a maximum specific growth rate of around 0.9 h^{-1} and a final biomass concentration of about 0.65 gDCW L^{-1} was achieved. During the exponential growth phase an early pulse of IPTG, to a final concentration of 1 mM, was applied at approximately 0.3 gDCW L^{-1} in order to induce PDC expression. In both cases, 2 hours after induction, a maximum activity of around 5000 U $gDCW^{-1}$ was achieved and the culture contained a total protein concentration of about 230 mg protein $gDCW^{-1}$. No PDC activity or PDC protein were detected in the samples taken before induction (Figure 2), thus indicating that the PDC promoter is strongly repressed by the pREP4 plasmid in both strains. Therefore, using LB rich

media no significant differences were detected between M15[pREP4][pQE-PDC] and SG13009[pREP4][pQE-PDC] during cell growth or PDC expression.

One of the main objectives of this work was to prove that PDC can be produced in *E. coli* in a bioreactor using a substrate-limiting fed-batch strategy. Therefore, the performance of each strain was also studied in a defined medium which allows economical process scale-up. Under these conditions, M15[pREP4][pQE-PDC] and SG13009[pREP4][pQE-PDC] showed similar maximum specific growth rate and final biomass concentration in shake flask cultures (Table 1). Nevertheless, PDC specific activity obtained with the SG13009[pREP4][pQE-PDC] strain was 1.7-fold higher compared to that of M15[pREP4][pQE-PDC] (Table 1). According to these results, SG13009[pREP4][pQE-PDC] was selected for further scale-up experiments.

3.2 PDC production in bioreactor cultures using a substrate limiting fed-batch strategy

The selected strain SG13009[pREP4][pQE-PDC] was used for PDC production using a substrate limiting fed-batch strategy (see Methods section). Figure 3 shows glucose, DCW, and specific PDC activity profile against time. Glucose was completely depleted during the batch phase after 11 hours of culture, reaching a final biomass concentration of 9.32 gDCW L^{-1}. During this batch phase the yield was 0.37 gDCW g glucose^{-1} and the maximum specific growth rate reached 0.51 h^{-1}. The substrate limiting fed-batch phase was then started to maintain a constant growth rate of 0.22 h^{-1}. Once biomass reached 20 g L^{-1} (3.5 hours after initiation of the fed-batch phase), induction was performed adding a pulse of IPTG to a final concentration in the bioreactor of 100 µM. The culture was stopped after 4 hours post-induction since the glucose started to accumulate in the media, probably due to cellular metabolic stress [31–34]. The maximum biomass concentration and PDC specific activity were 34 gDCW L^{-1} and 3677 U $gDCW^{-1}$, respectively. Enzyme volumetric productivity was also calculated, achieving 6942 U L^{-1} h^{-1}. Protein analysis revealed a total protein concentration and of 432 mg $gDCW^{-1}$ with 30% being PDC (128 mg PDC $gDCW^{-1}$) which means a quality of 29 U mg PDC^{-1}. Similar values have been obtained previously by the research group applying the substrate limiting fed-batch strategy

with IPTG pulse induction. The production of rhamnulose-1-phosphate alsolase (RhuA) expressed *in E. coli* M15ΔGlyA [pREP4] following the aforementioned strategy led to 160 mgRhuA $gDCW^{-1}$ and 25.5 gDCW L^{-1} [32].

3.3 PDC purification by *s*equential purification with ammonium sulphate

Fractional purification with ammonium sulphate is based on the different solubilities of the proteins present in the lysate [35,36]. The process is based on the salting out effect produced by the $(NH_4)_2SO_4$. The strategy is based on a two-stage process. In the first step, the salt concentration is increased up to a value that leads to the maximum precipitation of lysate proteins whilst maintaining PDC soluble. Thus, contaminant proteins can be removed by simple filtration or centrifugation. In the second stage, the salt concentration is increased until PDC precipitates.

Firstly, different preliminary experiments were carried out at two different temperatures (5ºC and 25ºC) to determine the best condition to carry out the purification. The results showed that enzymatic deactivation occurred under at temperatures. However, at 25ºC the total recovered activity (supernatant and precipitate) was 58%, whilst at 5ºC it was 15% higher (73 %). . Thus, 5ºC was selected as the temperature to perform the purification study.

Regarding the first precipitation step, saturated concentrations of 10, 20, 40 and 50% w/v of $(NH_4)_2SO_4$ were studied. The optimum in terms of final specific activity was obtained using 40% $(NH_4)_2SO_4$ concentration, containing 85% of the total final recovered activity (supernatant and precipitate) and representing a 61% of the total initial activity of the lysate. However, the specific activity was not increased compared to the lysate due to the activity loss suffered during the process. Aiming to reduce the enzyme deactivation during the first precipitation step at 40% w/v of $(NH_4)_2SO_4$ concentration, the incubation time was reduced from 2 h to 0.5 h. Results showed that the final specific activity reached 12.0 U mg $protein^{-1}$; corresponding to a purification factor of 1.5 (Table 2). The resultant supernatant contained 91% of the total final recovered activity

(supernatant and precipitate) and represented 82% of the total initial activity of the lysate (Table 2). Therefore, the obtained results demonstrate that the precipitation process is improved when incubation time is reduced from 2 to 0.5 hours due to a reduction in enzyme deactivation and treatment with a40% w/v of $(NH_4)_2SO_4$ concentration at 5^{o}C for 0.5 h was optimum for the first precipitation step.

Regarding the second purification step (Table 2), $(NH_4)_2SO_4$ concentrations of 65% w/v and 70% w/v were studied. The highest specific activity was obtained with 65% w/v of salt, reaching 15.7 U mg protein^{-1}, corresponding to a purification factor of 2. It represented a 97% of the total final recovered activity (supernatant and precipitate) and 79% of the total initial activity of the lysate. The use of 70% w/v salt concentration led to a 25% decrease in the purification factor. Therefore, 65% w/v salt concentration was selected as the best option for the second precipitation step.

In summary, sequential purification with the $(NH_4)_2SO_4$ strategy, applying a two-step process of 40% w/v and 65% w/v of $(NH_4)_2SO_4$ leads to a final purification factor of 2 and a purification yield of 79%.

3.4 PDC purification by ionic adsorption using MANA-agarose

Ion exchange purification is based on the interaction that takes place due to the difference in charge of protein and functional groups in the chromatography matrix. Ion exchange is one of the most used chromatographic techniques for the separation and purification of proteins, nucleic acids, polypeptides, polynucleotides, among others. The reasons for the success of ion exchange are its widespread applicability, its high resolving power, its high capacity, and the simplicity and controllability of the method [37].

Monoaminoethyl-N-aminoethyl agarose (MANA-agarose) is a widely used matrix for protein purification by ionic adsorption. This matrix is positively charged at acidic pH leading to an ionic adsorption of those proteins that are negatively charged under the process conditions. PDC purification was performed using MANA-agarose under two different pH's (6.2 and 5) in a batch

364 strategy. Even though at pH 6.2 the support is positively charged, according to the pKa of the
365 matrix (6.7) and the PDC is negatively charged, according to the pI (4.9), it was hypothesized that
366 a lower pH could increase the amount of positively charged proteins in the lysate, thus reducing
367 the competition among PDC and other contaminant proteins to be adsorbed on the support.
368 The results showed a higher final specific activity when the process was performed under the
369 most acidic pH, reaching 23.5 U mg protein^{-1}, corresponding to a purification factor of 4 which
370 represents a 56% increase, compared with the process performed at pH 6.2 (Table 3). It should
371 be mentioned that in both cases, 100% of total initial activity was recovered after desorption
372 treatment (1M NaCl), indicating that no enzyme deactivation had occurred during the processes,
373 giving a 100% purification yield.
374 The results obtained with the ion exchange strategy showed a 2-fold improvement in the
375 purification factor and a 21% improvement of the purification yield compared to the sequential
376 ammonium sulphate precipitation strategy. Therefore, it was decided to optimize and
377 automatize the process using an FPLC. The objective of this study was to find the optimum salt
378 concentration for the elution process. Firstly, the elution step (see Methods section) was
379 performed by using a linear gradient from 0 to 100% buffer B (NaCl 1 M) during 10 column
380 volumes and the results are shown in Figure 4. Two main protein fractions were eluted whose
381 maximum absorbance was detected at 31.03 mS cm^{-1} (33% buffer B; 0.33 M NaCl) and 57.8 mS
382 cm^{-1} (68% buffer B; 0.68 M NaCl) eluent conductivity. The samples recovered in the first peak
383 mainly contained contaminant proteins since the activity was only a 1.6 % of the total initial
384 loaded activity with a final specific activity of 0.94 U mg protein^{-1}. The activity assay of the sample
385 from the second peak revealed that almost 100% of the total initial loaded activity was present
386 with a specific activity of 39.22 U mg protein^{-1}. Protein electrophoresis also corroborated the
387 results obtained by the activity assays (Figure 5), thus indicating that PDC was eluted during the
388 second main fraction corresponding to an eluent conductivity of 57.8 mS cm^{-1} (68% buffer B;
389 0.68 M NaCl). Using this strategy, a purification factor of 3.4 and a purification yield of 100% was

obtained. The decrease in purification factor from 4 to 3.4 compared to the batch strategy could be due to a slight overlap between the two main fractions obtained in the elution step.

Aiming to improve the purification process by eliminating peak overlap, a new approach was applied based on a two-stage elution step. In the first elution stage, a 33% buffer B (0.33 M NaCl), corresponding to a conductivity value of 31.03 mS cm^{-1} was maintained constant until the contaminant proteins were eluted. In the second stage, once all contaminant proteins had been eluted, the composition of the eluent was changed to 68% of buffer B (0.68 M NaCl), corresponding to a conductivity value of 57.8 mS cm^{-1}, keeping it constant until all PDC was eluted. In figure 6 the enzymatic activity and protein concentrations achieved during the purification process are depicted. As can be seen, during the first stage of the elution (31.03 $mS \cdot cm^{-1}$), no PDC activity was detected, thus indicating that only contaminant proteins were eluted. In the second stage it can be seen that PDC activity was detected, corresponding to PDC elution. The obtained purified protein presented a specific activity of 42.95 U mg $protein^{-1}$, corresponding to a purification factor of 3.8 and a 100% yield during this ionic exchange purification. The purification factor obtained with the optimized FPLC strategy using a two-stage elution represented a 2-fold improvement compared to the sequential purification with $(NH_4)_2SO_4$. Other authors have also reported a purification factor of 4 for the *Z. palmae* PDC expressed in *E. coli* using a two- step downstream process based on ion exchange chromatography and size-exclusion chromatography [6]. Other bacterial PDCs expressed in *E. coli* have also been purified by other authors. PDCs from *Acetobacter pasteurianus*, *Zymomonas mobilis* and *Sarcina ventriculi* were purified by ion exchange and size-exclusion chromatography, reaching a purification factor of 14, 12 and 6, respectively [6]. Studies on native bacterial PDC purification have also been reported by other authors. Lowe et al. reached a purification factor of 8 for native PDC from *S. ventriculi* by applying a three step process based on $(NH_4)_2SO_4$ precipitation, ion exchange chromatography and size exclusion chromatography [5]. Native PDC from *Acetobacter pasteurianus* was purified up to 110-fold by ionic exchange and

hydroxyapatite chromatography while native PDC from *Z. mobilis* has also been purified up to 447-fold by dye-ligand interaction and ion exchange chromatography [6, 38].

3.5 PDC Purification: desalting step

In order to get a PDC preparation ready for storage before utilization, a last step of desalting was performed by using the FPLC. After the PDC purification by ionic exchange with MANA-agarose using a two-stage elution step process, the eluted peak 2 containing the PDC (52,3 U) was desalted in order to remove NaCl. After sample application to the column, packed with Sephadex 10, desalting was performed with lysis buffer (200 mM citrate, pH 6.2, containing 1 mM $MgCL_2$ and 1 mM TPP) and the eluted peak corresponding to desalted PDC contained 54 U of PDC activity. Thus, a 100% yield was obtained for the desalting step.

4 Concluding remarks

A production process for recombinant PDC from *Zymobacter palmae* expressed in *E. coli* has been developed. The PDC was cloned and overexpressed reaching up to 30% of the total protein. In this work it is presented for the first time the production of recombinant PDC in a bench-scale bioreactor applying a substrate-limited fed-batch strategy which lead to volumetric productivity and a final PDC specific activity of 6942 U L^{-1} h^{-1} and 3677 U $gDCW^{-1}$, respectively.

A purification process has also been developed by comparing two strategies: sequential purification by $(NH_4)_2SO_4$ and ion exchange purification using MANA-agarose. The last one resulted in the best purification factor and purification yield, reaching values of 3.8 and 100% respectively, after process optimization by FPLC. Finally, the eluted PDC from ion exchange chromatography was desalted in order to remove NaCl and a 100% yield was obtained.

Thus, a complete PDC production process, including cloning in *E. coli*, production in a bench-scale bioreactor (upstream) and purification by ion exchange and desalting (downstream), was developed to achieve a PDC preparation ready for storage.

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Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

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570 **Table 1.** Maximum specific growth rates (h^{-1}), specific PDC activities (U mg^{-1}) and final biomass
571 concentrations (gDCW L^{-1}) obtained in shake flask cultures of M15[pREP4][pQE-PDC] and
572 SG13009[pREP4][pQE-PDC] in defined medium. Culture was performed at 37°C and 150 rpm.

Strain	Maximum specific growth rate (h^{-1})	Specific activity (U mg^{-1})	Final biomass concentration (gDCW L^{-1})
M15[pREP4][pQE-PDC]	0.43	3.1	0.45
SG13009[pREP4][pQE-PDC]	0.41	5.2	0.41

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586 **Table 2.** PDC purification by sequential precipitation with ammonium sulphate. Enzymatic
587 activity obtained after the two-step sequential precipitation with 40% w/v of $(NH_4)_2SO_4$
588 saturation in the first step and different percentages of $(NH_4)_2SO_4$ in the second step. Incubations
589 with $(NH_4)_2SO_4$ were performed at 5°C during 0.5 h. The initial activity of the second step
590 corresponds to the activity of the supernatant after the first step (40% w/v $(NH_4)_2SO_4$).

$(NH_4)_2SO_4$ (% Saturation)	**Total initial activity (U)**	**Activity in the precipitate (U)**	**Activity in the supernatant (U)**	**Recovered activity (%)**	**Specific activity (U mg^{-1})**	**Purification factor**
Lysate	34.1	-	-	-	7.9	-
40 (1st step)		2.6	27.8	81.6[a]	12.0[a]	1.5
65 (2nd step)	27.8	27.1	0.8	79.3[b]	15.7[b]	2
70 (2nd step)		26.4	0.7	77[b]	11.8[b]	1.5

591 a) In the supernatant

592 b) In the precipitate

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600 **Table 3.** PDC purification with MANA-agarose by using a batch-strategy. Firstly, PDC (20 U mL
601 support^{-1}) was absorbed in MANA-agarose at 25^{o}C during 0.5 h at different pH 6.2 (phosphate
602 buffer 50 mM) and pH 5.0 (acetate buffer 50 mM). Finally PDC was desorbed from MANA-
603 agarose with 1 M NaCl.

pH	Total initial activity (U)	Total initial protein (mg)	Recovered activity after NaCl desorption (U)	Recovered protein after NaCl desorption (U)	Recovered activity (%)	Specific activity (U mg^{-1})	Purification factor
lysate	20	3.49	-	-	-	5.7	-
6.2			20.3	1.4	100	15.1	2.6
5			20.3	0.9	100	23.5	4.1

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615 **Figure legends**

616 **Figure 1.** Shake flask cultures of M15[pREP4][pQE-PDC] and SG13009[pREP4][pQE-PDC] grown
617 with LB-medium. Biomass (*filled diamonds*) and PDC activity (*filled circles*) for M15[pREP4][pQE-
618 PDC] strain; biomass (*empty diamonds*) and PDC activity (*empty circles*) for
619 SG13009[pREP4][pQE-PDC] strain. Cultures were performed at 37°C and 150 rpm. The arrow
620 indicates the IPTG pulse for the induction.

621 **Figure 2.** SDS-Page electrophoresis of M15[pREP4][pQE-PDC] and SG13009[pREP4][pQE-PDC]
622 samples from shake flask cultures in LB-medium. M: molecular weight marker. PI: pre-induction
623 sample. 1 h, 2 h, 4 h: time after induction with IPTG. Pyruvate decarboxylase subunits
624 correspond to the 60 kDa bands. Culture was performed at 37°C and 150 rpm.

625 **Figure 3.** PDC production in fed-batch culture in a 2L-bioreactor using the strain
626 SG13009[pREP4][pQE-PDC]. Biomass (*black diamonds*), glucose concentration (*white circles*)
627 and PDC activity (*black triangles*). The start of the fed-batch phase is represented with the
628 vertical dotted line. The arrow indicates the IPTG pulse for induction.

629 **Figure 4.** FPLC chromatogram profile of PDC purification using MANA-agarose and a linear
630 gradient elution. The wash step was performed using buffer A (50 mM acetate buffer pH 5) and
631 elution step was performed mixing buffer B (1 M NaCl, 50 mM acetate buffer pH 5) with buffer
632 A linearly increasing (0-100% buffer B) over 10 column volumes.

633 **Figure 5.** SDS-Page electrophoresis of samples obtained in the linear gradient elution
634 chromatography. Each line corresponds to a 1 mL fraction from the FPLC after the
635 chromatographic process.

636 **Figure 6.** Enzyme activity and protein concentration profiles with FPLC using a two-stage elution
637 step. The elution first step is performed at 33% of buffer B (during 10 column volumes). The
638 second step is performed at 100% of buffer B (during 10 column volumes).

639 **Figure 1**

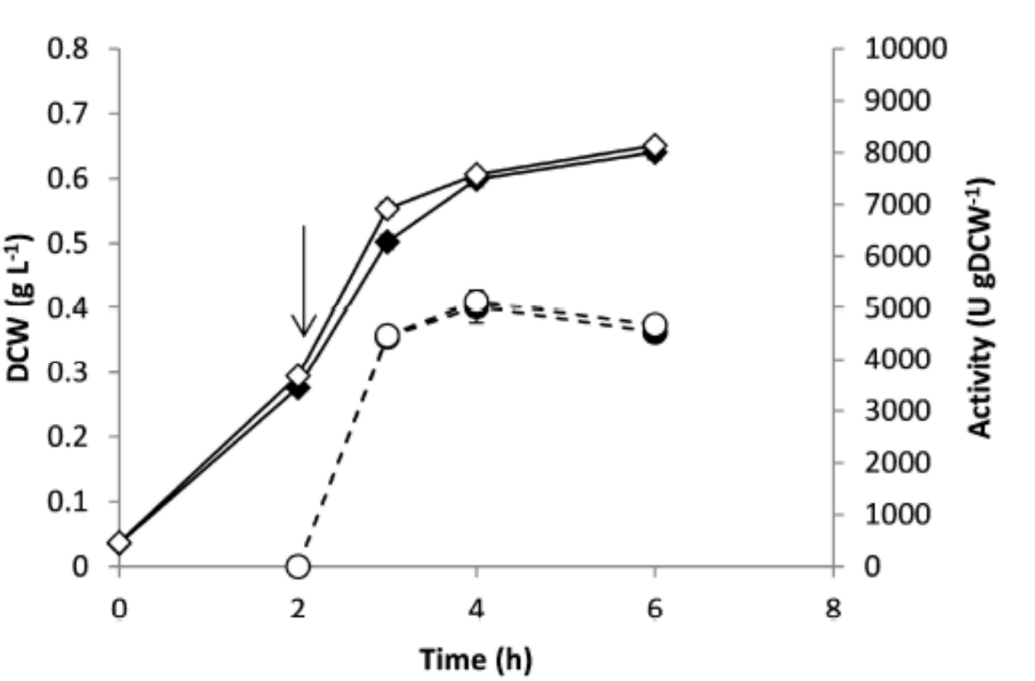

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Figure 2

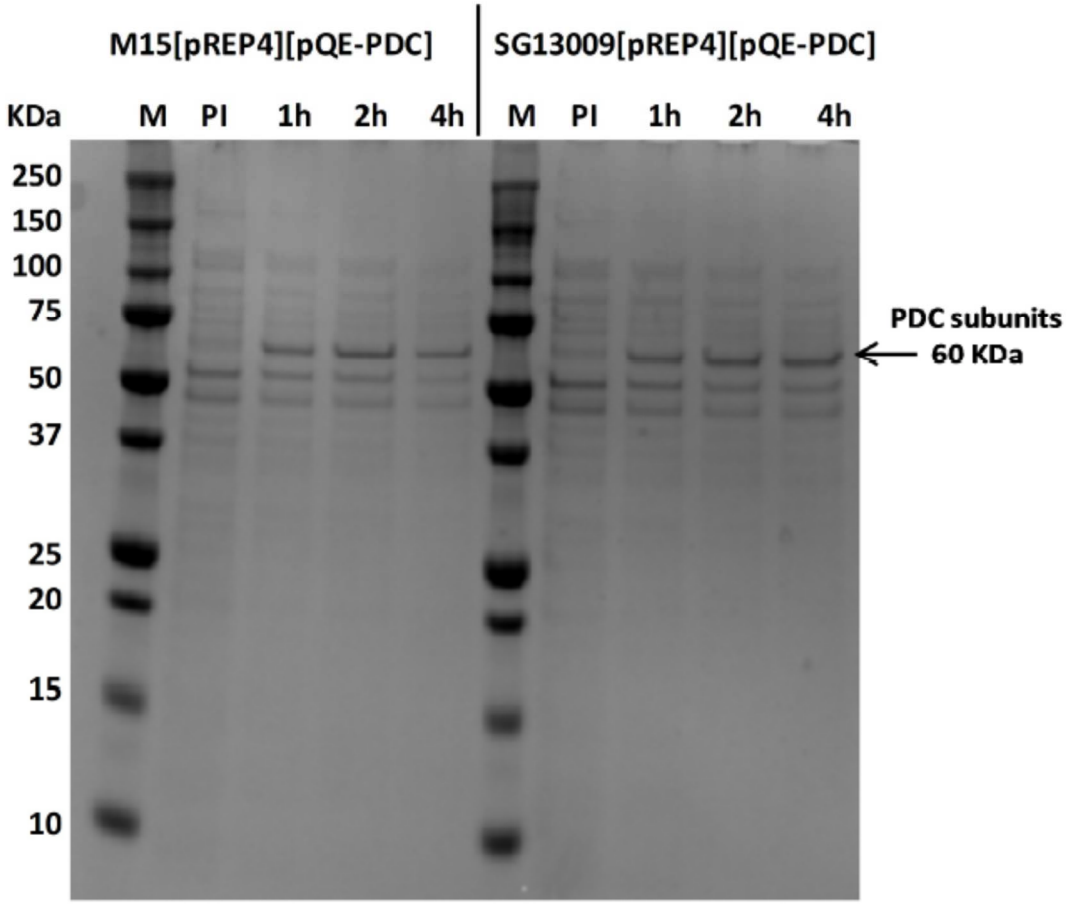
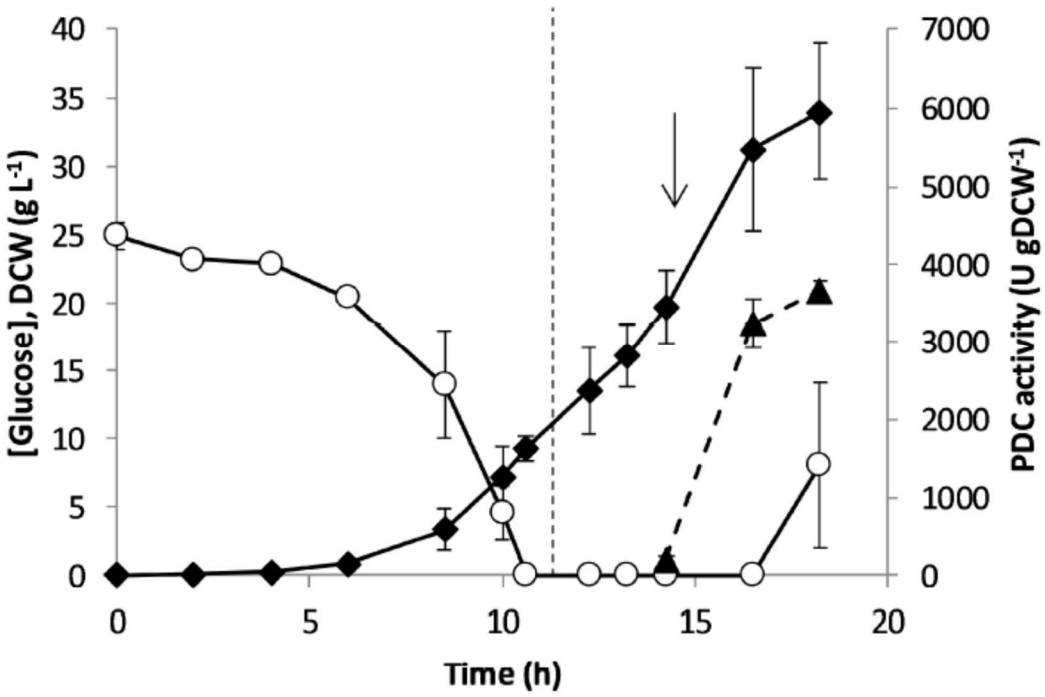

M15[pREP4][pQE-PDC]
SG13009[pREP4][pQE-PDC]
KDa
M
PI
1h
2h
4h
M
PI
1h
2h
4h
250
150
100
75
50
37
25
20
15
10
PDC subunits
60 KDa

Figure 3


[Glucose], DCW (g L-1)
PDC activity (U gDCW-1)
Time (h)
0
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672 **Figure 4**

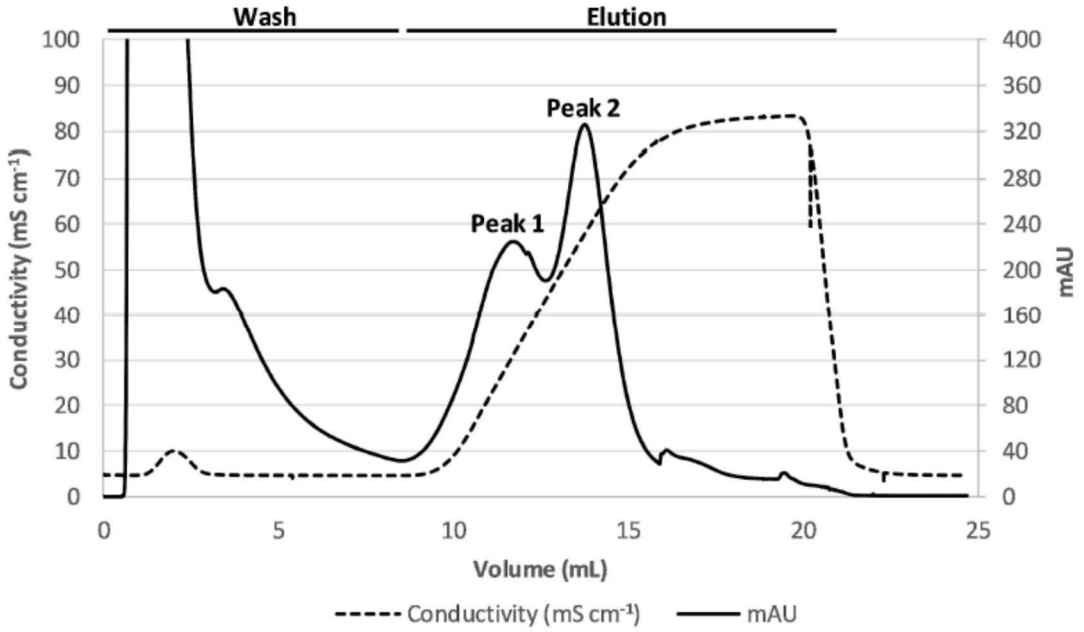

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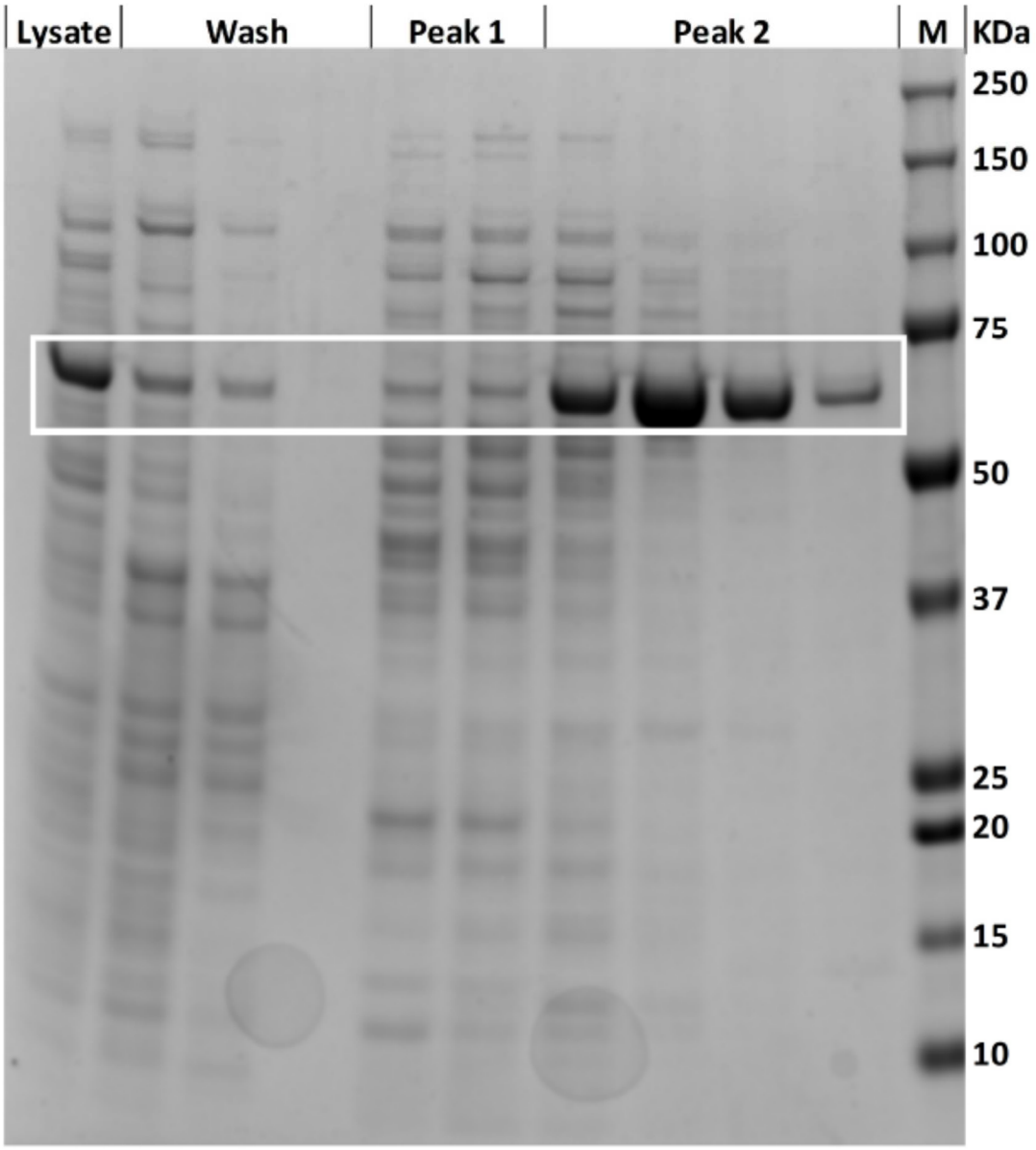
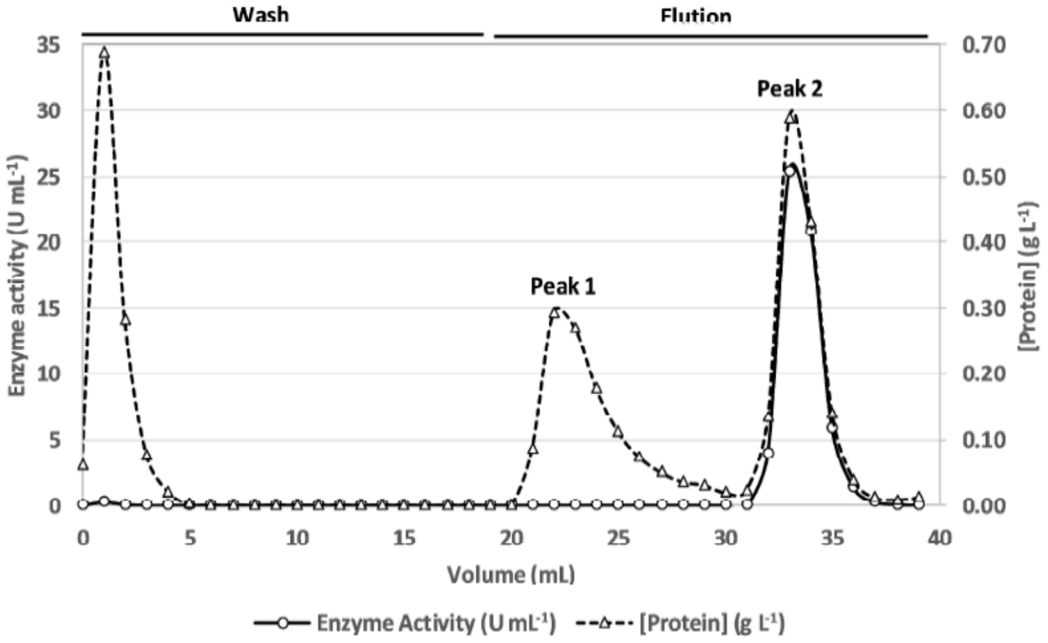
Figure 5



691 **Figure 6**



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