Production of 5-ketogluconic acid using recombinant strains of *Gluconobacter oxydans*

Master's Thesis

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List of abbreviations

Abbreviation	**Description**
AI	Auto induction
BL21-P-XADH	BL21 (DE3) strain carrying pET30a vector integrated with XADH gene
DI	Deionized water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDP	Entner-Doudoroff pathway
E. coli	*Escherichia coli*
E. coli-P-XADH	Shuffle *E. coli* strain carrying pBBR1MCS-5 vector integrated with XADH gene
E. coli-P	Shuffle *E. coli* carrying pBBR1MCS-5 vector without integration of XADH gene
GA	Gluconic acid
G. oxydans	*Gluconobacter oxydans*
GDH	Glucose dehydrogenase
GADH	Gluconate dehydrogenase
GO49-P-XADH	Recombinant *G. oxydans* 50049 carrying pBBR1MCS-5 vector integrated with XADH gene
GO49-P	Control *G. oxydans* 50049 carrying pBBR1MCS-5 vector without integration of XADH gene
HPLC	High-performance liquid chromatography
HCl	Hydrochloric acid
Kb	Kilobase pairs
kDa	Kilodalton
LB	Luria-bertani
MPDH	Major polyol dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide

$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
OD	Optical density
PQQ	Pyrroloquinoline quinone
PPP	Pentose phosphate pathway
PCR	Polymerase chain reaction
RI	Refractive index
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAE	Tris base-acetic acid-ethylenediaminetetraacetic acid
TA	Tartaric acid
TB	Terrific broth
X. campestris	*Xanthomonas campestris*
XADH	Alcohol dehydrogenase from *Xanthomonas campestris*
YPS	Yeast extract-peptone-sorbitol
2-KGA	2-keto-D-gluconic acid
2-KGADH	2-keto-D-gluconate dehydrogenase
2-KGR	2-keto-D-reductase
2,5-DKGA	2,5-diketo-D-gluconic acid
5-KGA	5-keto-D-gluconic acid
5-KGR	5-keto-D-reductase

Abstract/Summary

5-Keto-D-gluconic acid (5-KGA) is a valuable compound that has a wide range of applications in the chemical and food industries and is commonly used as a precursor for manufacturing tartaric acid (TA). Industrial production of 5-KGA through chemical approaches was practiced in the early 1920's and was dropped immediately due to several drawbacks because of its complicated process that resulted in lower 5-KGA yield and higher accumulation of impure gluconic acid. As a promising solution for manufacturing 5-KGA, the biological approach through the mechanism of microbial fermentation was found to be cost-efficient with higher productivity of 5-KGA.

Gluconobacter oxydans (*G. oxydans*) belonging to the *Acetobacteraceae* family was commonly preferred due to their ability to perform incomplete oxidation suitable for industrial production. Compared to wild-type, over-expression of polyol dehydrogenase in recombinant strains of *G. oxydans* has proven to be successful in enhancing the production of 5-KGA and eliminating the co-production of other undesired products during fermentation.

The aim of this study was to increase the production of 5-KGA in *G. oxydans* strain DSM 50049 carrying plasmid pBBR1MCS-5 integrated with the gene encoding alcohol dehydrogenase (XADH) from *Xanthomonas campestris*. The recombinant *G. oxydans* strain was cultivated at different culture parameters for determining optimal conditions for maximal activity and expression of XADH.

During cultivation in glucose medium, the recombinant *G. oxydans* generated a maximum yield of ~ 0.7 g 5-KGA / g glucose at 25°C, pH-6.0 along with co-production of 2-KGA. The results obtained from SDS-PAGE, activity assay and resting cell reaction were found inconclusive to prove the expression of XADH using low-copy number plasmid, pBBR1MCS-5. Furthermore, the formation of target protein bands from the insoluble cell fractions of BL21 (DE3) strain carrying pET30a vector was found satisfying to continue further studies on the expression of XADH.

Results have shown that cultivation parameters affect the performance of the enzymes during oxidation which lends support the idea of developing different stages of pH during fermentation for increasing the productivity of 5-KGA. If the yields of 5-KGA were improved after optimizing the strain and culture conditions for maximal XADH activity, further studies will be focused on large-scale cultivation and developing framework for developing strategies to improve 5-KGA productivity.

Keywords: *Gluconobacter oxydans*; *Escherichia coli*; 5-Keto-D-gluconic acid; Alcohol dehydrogenase

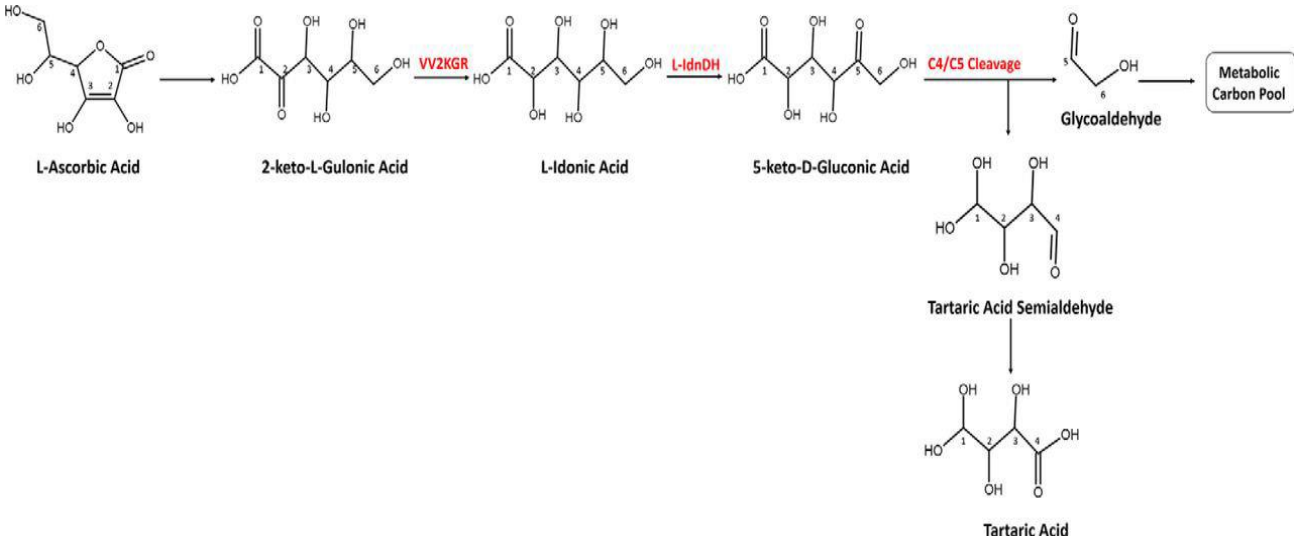
Introduction

5-Keto-D-gluconic acid (5-KGA) based products have a wide range of applications in food industries, e.g., acidulent and preservative in jams, jellies, lime-flavored beverages, and preparation of effervescent tablets in pharmaceutical industries [1]. Furthermore, it is used as a critical ingredient in gold and silver plating, cleaning and polishing metals, and manufacturing Rochelle salt, insecticides, wool dyes, etc. [2]. Of these products, 5-KGA is primarily used in the manufacturing of L-(+) tartaric acid (TA) or xylaric acid (Scheme 1-2) by oxidation of 5-KGA in the presence of oxygen, noble gases, vanadate, and air [3]. The process of manufacturing 5-KGA from glucose using chemicals was followed in the late 1920's which was not recommended due to the low yield of 5-KGA and higher accumulation of gluconic acid (GA) impurity. Harmful chemicals such as nitric acid, hypobromous acid and bromine were used to perform glucose oxidation, resulting in the generation of toxic waste hazardous to the environment [4-6].

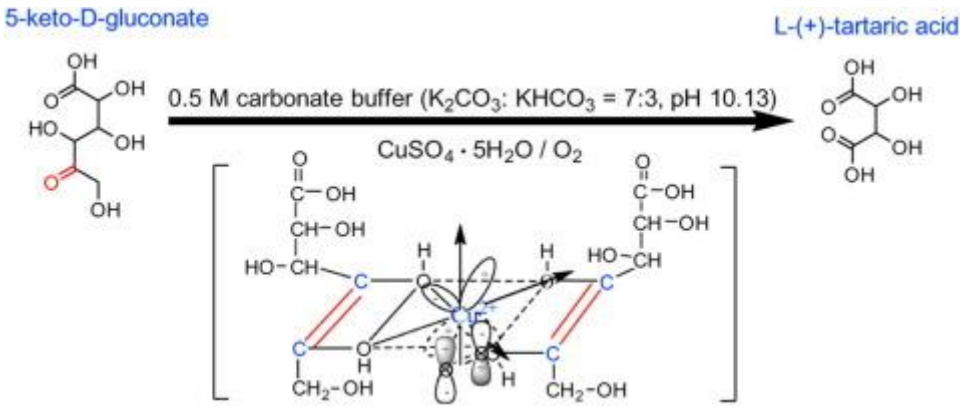
Biological routes for producing 5-KGA were achieved through microbial fermentation using *Acetobacter*, which was not efficient due to their difficulties in glucose fermentation and formation of oxalic acid [7]. From genus *Gluconobacter*, the *G. oxydans* was found to be ideal for performing glucose oxidation and formation of 5-KGA along with other essential by-products including 2-keto-D-gluconic acid (2-KGA), and 2,5-diketo-D-gluconic acid (2,5-DKGA) from d-glucose [8].

G. oxydans is a gram-negative, non-motile bacterium belonging to the family of *Acetobacteraceae*. It is either rod or oval-shaped with 0.5-0.8 µm × 0.9-4.2 µm in size. *G. oxydans* is commonly found in media with high sugar concentrations such as beer, fruits, wine, cider, garden soil, and soft drinks [9]. *G. oxydans* has an optimum growth temperature of 25 -30°C, with pH 5.5-6.0. They are obligate aerobes that requires oxygen to survive and produce energy by metabolizing substances such as fat or sugar. *G. oxydans* can cause incomplete oxidation of alcohols, sugars, and acids which results in the significant production of various oxidation products. This unique ability of *G. oxydans* makes it quite interesting for industrial applications [10].

The applications of *G. oxydans* (Appendix A, Figure 1) includes the production of GA, from D-glucose, L-sorbose from D-sorbitol, acetaldehyde from ethanol, and the formation of D-galactonic acid from D-galactose [9, 11]. Interestingly, 5-KGA is an attractive compound due to the possibility of converting it into L-ascorbic acid, 4-hydroxy-5-methyl-2, 3-dihydrofuranone, and xylaric acid [12-15].



Scheme 1. Tartaric acid biosynthetic pathway from the C4/C5 cleavage of ascorbic acid. [15]



Scheme 2. Selective oxidation of 5-keto-D-gluconate into L-(+) tartaric acid using alkaline carbonate buffer and transition metal $CuSO_4.5H_2O$. [16]

Oxidation of glucose by *G. oxydans* (refer to Appendix A, figure 1) occurs in both periplasm and cytoplasmic regions of the bacteria, where a major part of glucose is being oxidized by the membrane-bound enzymes present in the periplasmic region. The oxidation of remaining glucose occurs in the cytoplasmic region using metabolic pathways such as Entner-Doudoroff (EDP) and pentose phosphate pathway (PPP) [17].

Glucose oxidation inside the cytoplasm occurs through $NADP^+$ independent enzymes that convert GA into 2-KGA and 5-KGA using 2-keto-D-reductase (2-KGR) and 5-keto-D-reductase (5-KGR) respectively [18-19]. In periplasm, $NADP^+$ dependent membrane-bound glucose dehydrogenase (GDH) utilizes D-glucose to form GA, which was further used by gluconate dehydrogenase (GADH), 2-keto-D-gluconate dehydrogenase (2-KGADH), and major polyol dehydrogenase (MPDH) for producing 2-KGA, 2,5-DKGA and 5-KGA, respectively [20].

Even though 5-KGA were commonly produced as a part of *G. oxydans* fermentation, it will often lead to the co-production of 2-KGA and 2,5-DKGA during cellular metabolism, which causes significant drawbacks in manufacturing 5-KGA at an industrial scale [21]. Formation of undesired by-products can be avoided through gene technology which involves knocking out the genes responsible for the production of GA to 2-KGA and 2,5-DKGA [22]. For improving 5-KGA yield in *G. oxydans,* several studies were carried out using membrane-bound MPDH which are cloned and expressed in host organisms during transformation [23].

One such study involves utilizing membrane-bound pyrroloquinoline quinone (PQQ) dependent D-sorbitol dehydrogenase, by overexpressing *sldAB* gene comprising both *sldA* (UniProt ID-Q8KIL1) and *sldB* (UniProt ID-Q8L1D5) in *G. oxydans* [24]. The results from this study were promising and recorded maximum 5-KGA productivity of 2.53 g/L/h. Interestingly, the membrane-bound enzymes such as 5-KGR (UniProt ID-P0A9P9) from *E. coli* [25], gluconate 5-dehydrogenase (UniProt ID-A0A1R3D7W9) from *Salmonella enteritidis* and gluconate 5-dehydrogenase (UniProt ID-P50199) from *G. oxydans* 621H can cause reversible reactions between GA and 5-KGA [25-27]. Apart from improved production of 5-KGA, over-expression of gluconate-5-dehydrogenase (GA-5-DH) was found to be toxic to *E. coli* and had a relatively low activity with GA [28].

Most of the membrane-bound dehydrogenase enzymes are associated with PQQ co-factor oxidation, which occurs on the periplasmic membrane that is linked directly to the respiratory chain in *G. oxydans* quinoproteins which are essential for the formation of 5-KGA [21]. The oxidizing activities of PQQ co-factor in *G. oxydans* are not completely known and are still debated over their role in cell growth and metabolism.

Alcohol dehydrogenase enzymes from *X. campestris* were found to be ideal for enhancing 5-KGA production in *G. oxydans* based on their high efficiency in *E. coli* by achieving 75% conversion yield in a shorter time (16 hours), with a lower cell density (OD_{600}-2.0) [29]. Both the pH and temperature activity profile of the enzyme were found similar compared to the optimal culture conditions in *Gluconobacter* which makes it an ideal candidate for performing experiments on enhancing the yield of 5-KGA in *G. oxydans*.

Aim/Objective

The aim of this study was to increase the production of 5-KGA in *G. oxydans* expressing XADH enzyme from *X. campestris*. The recombinant *G. oxydans* DSM 50049 was cultivated under different parameters including temperature and pH to determine optimal conditions for expression of XADH.

Materials & methods

1. Materials

5-Keto-D-gluconic acid with potassium salt and D-mannitol were purchased from Sigma (St. Louis, MO), and D-glucose was obtained from VWR (Leuven, Belgium). Other chemicals used in the preparation of buffers and culture media were purchased from Merck (Darmstadt, Germany), Alfa Aesar (Kandel, Germany), Thermo Scientific (Kandel, Germany) and Acros Organics (Geel, Belgium).

2. Overview of plasmids and microorganisms

Plasmids used for cloning and expression of alcohol dehydrogenase encoding genes in *G. oxydans* DSM 50049 (*GO49*-P-XADH) and shuffle *E. coli* (*E. coli*-P-XADH), BL21 (BL21-P-XADH) were the pBBR1MCS-5 and pET30a vectors, respectively, provided by the supervisors. The control strains (*GO49*-P and *E. coli*-P) were prepared through the transformation of the pBBR1MCS-5 vector without the integration of gene expressing alcohol dehydrogenase. The genes were under the control of 'gHp0169 promoter' (derived from *G. oxydans* genome).

3. Preparation of control strains

Transformation of pBBR1MCS-5 control vector in *G. oxydans* was performed in electroporation cuvette (Gene Pulser, 0.2 cm gap) containing 50 µL of *G. oxydans* DSM 50049 and 2343 strains competent cells and 5 µL control plasmids. Electroporation was done using Genepulser II, Bio-Rad, USA at 2.5 KV, 25 µF for 6 seconds. After electroporation, the cells were transferred in a 50 mL falcon tube containing 5 mL of yeast extract-peptone-sorbitol (YPS) broth composed of 50 g/L D-sorbitol, 3 g/L yeast extract, 3 g/L peptone (pH 6) and the cells were incubated at 30°C, 200 rpm for 3 hours in a shaking incubator (Electron, Infors HT, UK).

After incubation, the cells were centrifuged (3-16PK, Sigma, Germany) at 4,500 rpm for 10 minutes followed by discarding part of the supernatant. The cell pellet was resuspended with the remaining supernatant and was spread across plates containing YPS agar comprising of 15 g/L agar and 10 µg/mL gentamicin. The transformed cells were incubated at 30°C for 3-4 days until the colonies were formed. Colonies were picked from the culture plate and transferred into 50 mL falcon tube containing 5 mL of mannitol medium composed of 25 g/L D-mannitol, 5 g/L yeast extract, 3 g/L peptone and 20 µg/mL gentamicin. Finally, the tubes were incubated

in a shaking incubator at 30°C for 18 hours, followed by cultivation in 100 mL for preparing glycerol stock using mannitol medium.

4. Glycerol stock preparation

Glycerol stocks of both *G. oxydans* DSM 50049 recombinant and control strains were prepared separately. Initially, mannitol medium used for cell cultivation containing 5 µg/mL gentamicin and *G. oxydans* cells were incubated at 30°C in a shaking incubator (Electron, Infors HT, UK) at 200 rpm. After performing pre-cultivation, the sub-cultivation of cells was performed with an initial optical density (OD_{600})-0.1 at wavelength of 600 nm using the spectrophotometer (LAMBDA Bio +, PerkinElmer, USA). During the main cultivation, with an initial OD_{600} of 0.05 the cells were incubated until the OD reaches 1.2. Thereafter, 20% glycerol stock was prepared through mixing of 500 µL of cell culture with 500 µL of 40% glycerol in sterile 1.5 ml centrifuge tubes. The glycerol stocks were stored at -80°C until further use.

For preparing glycerol stock for *E. coli* strains, 5 µL of *E. coli* cells was inoculated in 50 ml falcon tubes containing luria-bertani (LB) medium comprising 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride. The falcon tubes were further incubated in a shaking incubator (Electron, Infors HT, UK) at 37°C, 200 rpm overnight. The glycerol stock was prepared using 40% glycerol containing *E. coli* cells with OD_{600}-1.2 and was stored at -80°C.

5. Production of 5-KGA using *G. oxydans* expressing XADH

The glycerol stock (300 µL) from both recombinant and control DSM *GO*50049 strains was pipetted into 50 mL falcon tubes containing 5 mL mannitol medium for incubation at 30°C. After 18 hours, the OD of the cells was measured with a spectrophotometer at 600 nm using mannitol medium as blank. Based on OD from pre-cultivation, *G. oxydans* cells were sub-cultivated in 10 ml of mannitol media having an initial OD_{600}- 0.1. Main cultivation was performed using a glucose medium containing 5 g/L yeast extract, 3 g/L peptones, and 10 g/L D-glucose, with cells having an initial OD_{600}- 0.05. During cultivation, both mannitol and glucose media were supplemented with 10 µg/ml gentamicin. Both *GO49*-P-XADH and *GO49*-P were cultivated under similar conditions by adding 5 µg/ml gentamicin; *GO49*-P served as the negative control.

From the cultivation flasks, 1 ml of sample was drawn at time intervals of 0 hr., 8 hr., 16 hr., 24 hr., 36 hr., 48 hr., 72 hr., 96 hr., & 120 hr., for measuring the OD, pH and quantifying 5-KGA, glucose, and GA. During main cultivation, the pH of the culture was manually adjusted using sterile 1 M NaOH every 2-4 hours until the first 16 hours of cultivation followed by every sampling time point.

6. Cultivation of *E. coli* for analyzing XADH expression

For the pre-culture, 5 µL of *E. coli* cells from the glycerol stock were cultivated in 5 ml LB medium and incubated at 37°C overnight. This was followed by main cultivation in 50 mL of complex autoinduction (AI) media. The preparation and composition of the AI medium can be found in Appendix B.

The initial experiments were performed with *E. coli*-P-XADH strain cultivated in 250 ml culture flasks containing 50 mL AI media supplemented with 10 µg/mL of gentamicin, and BL21-P-XADH strains cultivated in terrific broth (TB) medium supplemented with 50 µg/mL kanamycin. The flasks were incubated at 37°C for 5 hours followed by secondary incubation at 16°C, 200 rpm for 48 hours in the cooling incubator (Electron, Infors HT, UK). During the cultivation, the OD and pH were constantly measured every 12 hours along with 4 ml sampling of cells which was used for performing resting cell reaction and activity assay.

Second experiment involved cultivation of *E. coli* strains using AI media at 30°C and 37°C for 72 hours. Every 12 hours, OD and pH were measured followed by cell sampling for performing SDS-PAGE and activity assay.

7. Restriction digestion and optimization of PCR annealing temperature

Extraction of plasmid from cells began by cultivating *GO49*-P-XADH and *GO49*-P strains in 50 ml mannitol medium, and once the culture reached OD_{600}-1.8, the cells were transferred to 15 mL falcon tubes and centrifuged (3-16PK, Sigma, Germany) at 4,500 rpm for 20 minutes. After discarding the supernatant, the plasmids were extracted from the cells using a plasmid extraction kit (GeneJET® Plasmid Miniprep kit, Thermo Scientific, Lithuania). The DNA concentration was measured using NanoDrop® ND-1000, Thermo Scientific.

PCR was performed with a reaction volume of 10-25 µl in 0.1 ml PCR tubes using the thermal cycler (Labcycler Gradient, SensoQuest, Germany) for 35 cycles using both Phusion™ High-Fidelity DNA polymerase and Taq DNA polymerase. The PCR composition and the temperature conditions for PCR conditions are described in Appendix C, Table 3-4.

Double digestion was performed in a reaction volume of 20 µl (refer to Table-5) in sterile 1.5 ml centrifuge tubes. The tubes were centrifuged (CT 15E, Himac, Japan) at 15,000 rpm for 30 seconds and were incubated at 37°C using a thermostat (BTD Dry Block Heater, Grant, Netherlands) for 30 minutes.

8. Protein extraction and activity

8.1. Extraction of soluble & insoluble cell fractions

Initially, falcon tubes containing the cell culture were centrifuged (3-16PK, Sigma, Germany) at 4,500 rpm for 40 minutes followed by discarding the supernatant. The wet weight of cells from the pellet was calculated in each tube. The cells resuspended in the buffer were sonicated using the sonicator (UP400S, Hielscher Ultrasonics, Germany). 4-9 ml of bacterial suspension was sonicated four to five times for one minute at 40-70% amplitude and 0.5 cycles. Samples were kept on ice while sonicated to prevent heating.
Further, the sonicated cells were transferred into 1.5 ml centrifuge tubes and were centrifuged at 14,000 rpm for 30 minutes using a cooling centrifuge (Biofuge 13, Heraeus Instruments, Germany). The supernatant (soluble fraction) was filtered using a 0.45 µm filter syringe and the cell pellets (insoluble fraction) were resuspended in 0.1 M potassium phosphate (pH-6.0) buffer with an equal volume to the soluble fraction. Both the soluble and insoluble fractions from the *G. oxydans* cell strains were stored at 4 °C until they were used for performing SDS-PAGE and activity assay.

8.2. Enzyme activity

The reaction volume of 1 ml containing 5 g/L GA was prepared by adding 46 µL of 0.5 M GA along with 954 µL of soluble or insoluble cell fraction in glass vials. The vials were incubated at 30°C, 500 rpm using a thermoshaker (MHR 13, Hettich Benelux, Netherlands). During the reaction, 200 µl of the sample from the reaction vials were drawn at time intervals of 0 hr., 6 hr. and 24 hr. for HPLC analysis.

8.3. Resting cell reaction

During the main cultivation, 4 ml of the cell culture collected at different time intervals was transferred to 15 ml falcon tube under sterile conditions. The tubes were centrifuged (3-16PK, Sigma, Germany) at 4,500 rpm for 30 minutes and after discarding the supernatant, the cells were washed twice with 4 ml of 0.1 M potassium phosphate buffer (pH-6.0). The cell pellets were resuspended in 1 ml of buffer containing 5 g/L GA and were transferred to 4 ml glass vials and incubated at 30°C, 500 rpm using the thermoshaker (MHR 13, Hettich Benelux, Netherlands). During the reaction, 100 µL of sample volume was collected from the reaction vials during the time intervals of 0 hr., 3 hr., 24 hr. and 48 hr. for HPLC analysis.

9. Analysis

9.1. Agarose gel electrophoresis

The samples obtained from PCR and double digestion were analyzed using 1.0 % of agarose gel. Before casting the gel was stained with 1 µl of GelRed™ (Thermo Fisher Scientific). Each lane in the gel was loaded with sample containing 1X loading dye; and ladder was loaded in one of the lanes (GeneRuler 1kb DNA ladder, ThermoFisher Scientific). The system was filled with 1X tris base-acetic acid-ethylenediaminetetraacetic acid (TAE) buffer until the gel was immersed and the current was set at 75 V for 40 minutes (until the bands were completely separated) and the obtained bands were visualized using the gel reader (GelDoc GO Imaging System, BioRad Laboratories).

9.2. SDS-PAGE

SDS-PAGE was performed using an electrophoresis system (Mini-PROTEAN® Tetra Handcast Systems, BioRad Laboratories). Samples were prepared by adding 20 µL soluble or insoluble cell fraction into sterile 1.5 ml centrifuge tubes containing 6 µL of SDS 4X dye. The tubes were mixed by vortexing, followed by centrifugation (CT 15E, Himac, Japan) at 15,000 rpm for 20 seconds, and incubation at 98°C using a thermostat (BTD Dry Block Heater, Grant, Netherlands) for 10 minutes. The samples were examined using Mini-PROTEAN TGX Stain-Free precast gels from BioRad Laboratories (8.6 × 6.7 cm, 7.5% polyacrylamide gel, 15-wells) with 10 µL of sample loaded in each lane along with 1.5 µL of protein ladder (Precision Plus Protein™ Standards, BioRad Laboratories). The electrophoresis was performed using 1X SDS buffer, run through the stacking gel at 80 V for 15 minutes followed by 100 V for 40 minutes. Bands were visualized in a gel reader (GelDoc GO Imaging System, BioRad Laboratories).

9.3. High-performance liquid chromatography

The quantification of glucose, GA and 5-KGA from the fermentation broth, activity assay and resting cell reaction was analyzed using high-performance liquid chromatography (HPLC) (LC-4000 Series, JASCO, USA) equipped with a refractive index (RI-4030) detector. An Aminex HPX-87C (1, 300 x 7.8 mm, 9 µm particle size) liquid chromatographic column was purchased from BioRad Laboratories. The elution was performed using the isocratic mobile phase which consisted of 0.5 mM sulphuric acid and Milli Q water (30:70) delivered at a flow rate of 0.4 mL/min with a total running time of 22.5 minutes. The column oven was maintained at 65°C, while the autosampler was kept at room temperature. The volumetric composition of HPLC sample vials was explained in table-1, appendix-D.

For obtaining their respective standard curves, glucose, GA and 5-KGA were weighed and mixed with 10% sulphuric acid to make up the following concentrations: 10, 7.5, 5, 3.5, 2.5, 1.5 and 0.8 g/L for determining concentration based on the area of chromatogram peak obtained during HPLC.

9.4. Purification of proteins using ÄKTA start

Immobilized metal affinity chromatography was carried out using ÄKTA start (Cytiva, United States), using Ni Sepharose™ prepacked into column HisTrap™ HP 1ml (Cytiva, United States) designed for purifying His-tagged recombinant proteins. Before sample loading, the columns were regenerated separately using elution buffer (50 mM potassium phosphate,

500 mM sodium chloride, 500 mM imidazole, pH 6.0) and binding buffer (50 mM potassium phosphate, 500 mM sodium chloride, 20 mM imidazole, pH 6.0). The column was loaded with a sample volume ranging from 3-6 ml and during the sample loading the filtered lysate was stored on ice. The flow-through, elution and wash fractions were collected at a constant volume of 1.5 mL and stored at 5°C.

9.5. Yield calculation

5-KGA yield (gram 5-KGA/ gram Glucose) was calculated using the following equation:

$$Y_{(5-KGA/Glucose)} = \frac{P - Po}{So - S}$$

where, P- Product formed (g/L)

P_o- Initial product formed (g/L)

S- Substrate consumed (g/L)

S_o- Initial substrate (g/L)

Results & Discussion

During the cultivation of recombinant DSM-50049 strain (*GO49*-P-XADH) under uncontrolled pH conditions, the pH of the media was dropped to 3.6 during glucose oxidation with GA levels being maintained stationary throughout the fermentation. Under manually controlled pH conditions, the *GO49*-P-XADH generated a maximum 5-KGA yield of 0.68 g 5-KGA / g glucose during cultivation at 25°C, pH 6.0. The recombinant *G. oxydans* cultivated under pH 6.0-6.5, accumulated a maximum concentration of GA during the fermentation and showed higher cell growth compared to the control strain (*GO49*-P). Co-production of 2-KGA was observed throughout the cultivation (refer to below fig. 2, 4, 6 and 9) from both the recombinant and control strains.

While performing resting cell reaction, *GO49*-P-XADH cells cultivated at pH-6.5 achieved a 5-KGA yield of 0.63 g 5-KGA/ g GA, also the results from activity assay indicates no formation of 5-KGA with analysis using the cell fractions (both soluble & insoluble) from *E. coli* and *G. oxydans* strains. During SDS-PAGE, the target XADH band having a molecular mass of 83 kDa was observed in both recombinant and control strains of *G. oxydans* and *E. coli* carrying pBBR1MCS-5 vector. This resulted in difficulties on validating the expression of XADH from the recombinant strains during SDS-PAGE and resting cell reaction. Interestingly, the insoluble cell fraction from strain BL21 (DE3) carrying pET30a vector showed expression of XADH.

During agarose gel electrophoresis, the presence of XADH gene was confirmed in *GO49*-P-XADH used for experimentation. Gradient PCR was performed using M13 primers to ensure target amplification and to increase the specificity of primer binding and based on the results the optimal annealing temperature of M13 primers was found to be 59°C.

1. Optimizing conditions for XADH activity based on pH

During the cultivation of *G. oxydans* in glucose medium, maintaining the pH during fermentation is essential for the activity of gluconate and ketogluconate forming dehydrogenases such as XADH and stimulating cell growth. For enhancing the productivity of 5-KGA in recombinant strain *GO49*-P-XADH, it was important to perform pH profiling to determine optimal pH conditions favouring the expression and activity of XADH.

1.1. Activity of XADH in uncontrolled pH conditions

As a part of pH profiling, the study was carried out for characterizing the activity of recombinant strain while performing batch cultivation without controlling the pH during fermentation. The results obtained during fermentation have shown that cultures from both *GO49*-P-XADH and *GO49*-P strains (refer to fig.1 & 2) had a significant dropping of pH to 3.5 during cultivation.

The formation and accumulation of GA in the fermentation broth has precipitated a marked drop in the culture pH as a result of glucose oxidation [30]. The growth of *G. oxydans* in the culture was adversely affected due to the immediate drop of pH below the optimal range of 5.5-6.0 [31]. In addition, the activity of pentose phosphate pathway responsible for the oxidation of GA was almost inhibited at pH below 4.0 which caused the accumulation of GA during the fermentation [32].

During experimentation, *GO49*-P achieved a maximum yield of 0.28 g 5-KGA/ g Glucose in comparison with *GO49*-P-XADH with a difference of 0.03 g 5-KGA/ g Glucose. Even though both the strains demonstrated their ability to produce 5-KGA under lower pH conditions, it was difficult to conclude the expression and activity of XADH in the recombinant strain. Possibilities are that under strong acidic conditions the expression of XADH in *G. oxydans* would have been inhibited completely or will have the least enzyme activity.

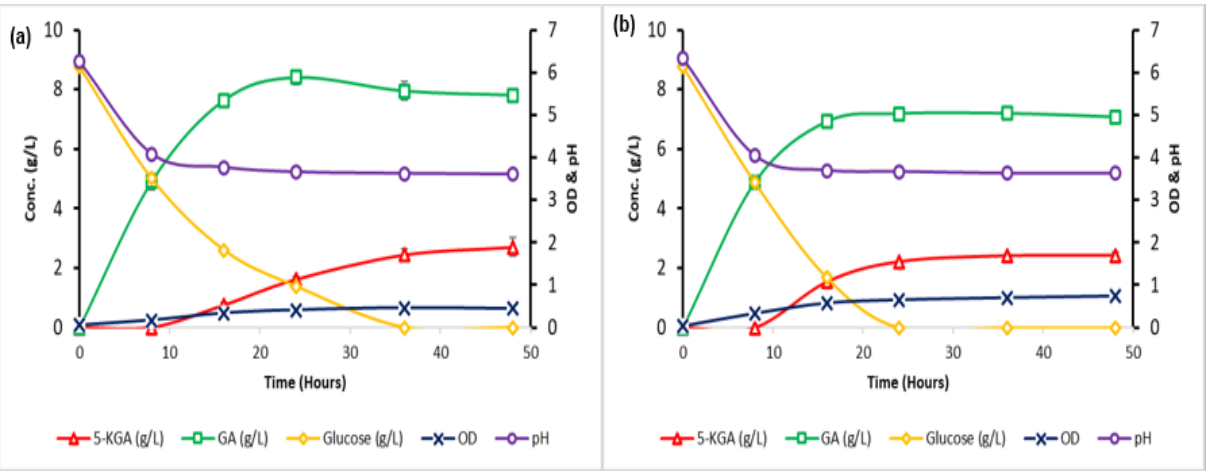


Figure 1. Production of 5-KGA via oxidative fermentation under uncontrolled pH conditions; a) *GO49*-P-XADH; b) *GO49*-P. Rate of glucose consumption in *GO49*-P-XADH was slower compared to *GO49*-P. Strains *GO49*-P-XADH and *GO49*-P recorded maximum OD_{600} of 0.442 and 0.736, respectively with pH of culture dropping from 6.3 to 3.63 by end of cultivation.

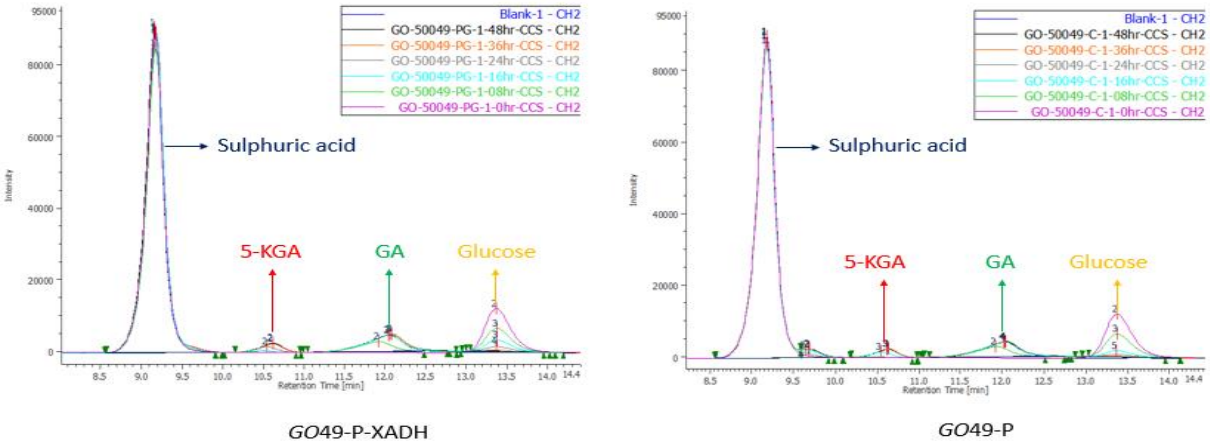


Figure 2. Chromatograms formed during HPLC illustrating the oxidation of glucose and GA during fermentation of *G. oxydans* under uncontrolled pH conditions.

1.2. Activity of XADH at pH 6.0-6.5

As the influence on culture pH is significant for expression and activity XADH in *G. oxydans*, it is important to have a data driven decision to finalize the ideal pH range for cultivation. Based on literature studies, the favourable pH for maximal activity of XADH was focused between the range of 6.0-6.5 [29].

Fig. 3a-b & 5a-b below indicates higher activity of glucose oxidizing enzymes such as glucose dehydrogenase (GDH) during cultivation at pH 6.0-6.5 depending on the rate of glucose consumption irrespective of cell mass. The recombinant strain shows favourable cell growth at pH 6.0 compared to cells cultivated at pH 6.2-6.5 (refer fig. 3 & 5).

The manual balancing of pH using strong base may have contributed to the rise of pH levels observed during cultivation, which could be avoided using an online system for constant monitoring and maintaining stable pH levels. During cultivation at pH ranging from 6.0-6.5, it was observed that the recombinant strains produced high concentration of 2-KGA in addition to 5-KGA (refer to fig. 4 & 6).

The dropping of 5-KGA concentration from the fermentation broth during cultivation observed from both the recombinant and control strains. This diminishing of 5-KGA from the culture could be caused due to the activity of non-specific membrane-bound enzymes such as 5-KGR and gluconate-5-dehydrogeanse (GA-5-DH), which causes reduction of 5-KGA to GA to be utilized for cell growth and metabolism [25-27, 33-34]. Depending on the substrate availability, these enzymes can be triggered by the cells to convert gluconates (2-KGA/5-KGA) back to GA, when the cells lack adequate substrate for performing EDP and PPP mechanisms [19].

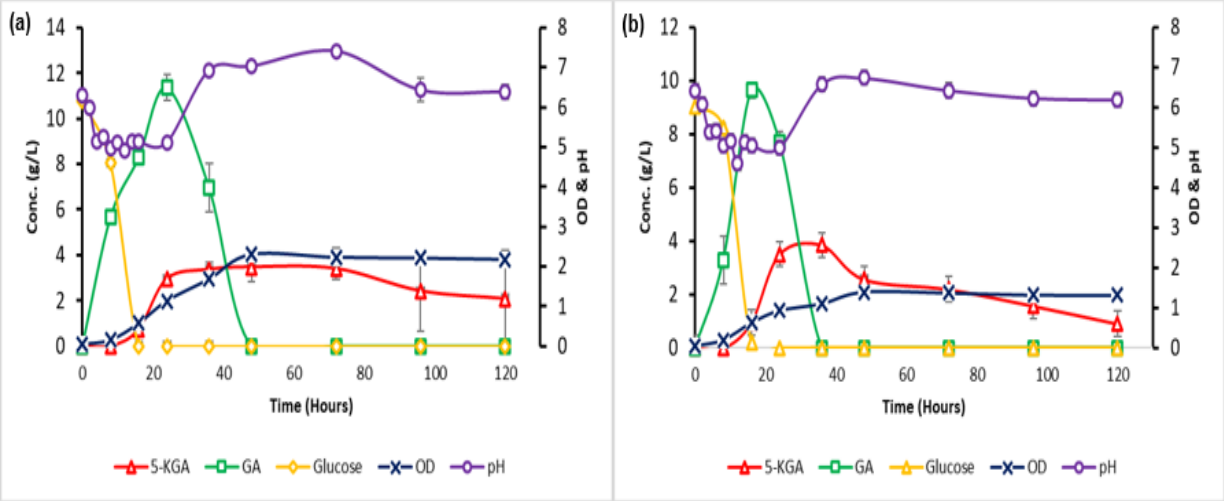


Figure 3. Production of 5-KGA from *G.* oxydans cultivated at 30°C, pH 6.2-6.5; (a) *GO49*-P-XADH; b) *GO49*-P. *GO49*-P-XADH produced maximum yield of 0.32 g 5-KGA/ g glucose, followed by 0.43 g 5-KGA/ g glucose, from *GO49*-P. *G.* oxydans cells achieved maximum OD_{600} of 2.186 and 1.397 with pH trends found almost identical in both strains.

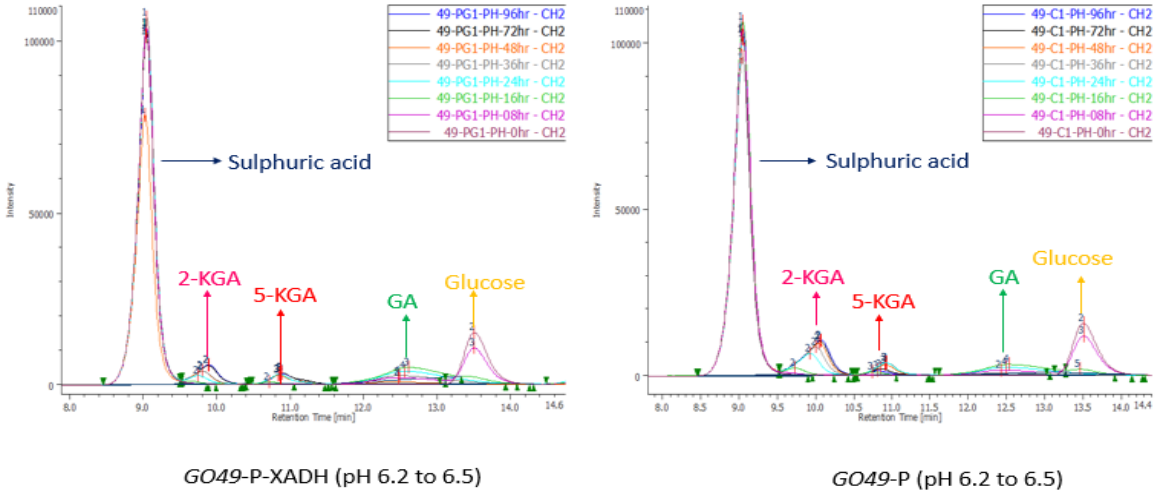


Figure 4. HPLC chromatogram obtained during the cultivation of *GO49*-P-XADH and *GO49*-P at pH 6.2 to 6.5.

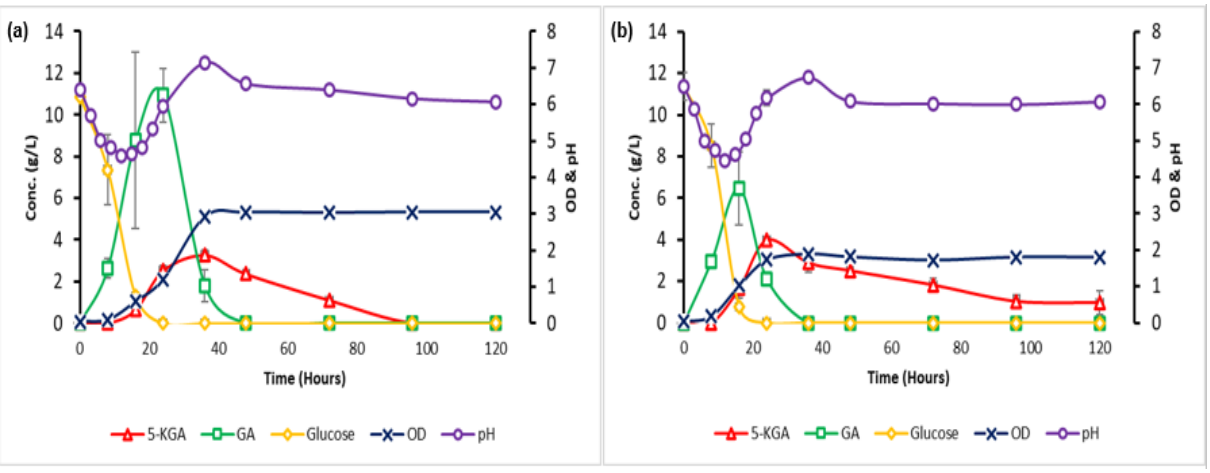


Figure 5. Production of 5-KGA via oxidative fermentation in *G. oxydans* at pH 6.0; a) *GO49*-P-XADH; b) *GO49*-P. Maximum OD_{600} of ~ 3.0 and ~ 1.9 was achieved by *GO49*-P-XADH and *GO49*-P respectively with pH trends from both strains reaching pH of ~ 6.0 by end of cultivation. Maximum yield of 0.35 g 5-KGA/ g glucose was obtained by *GO49*-P followed by 0.3 g 5-KGA/ g glucose by *GO49*-P-XADH. Transient accumulation of 5-KGA was observed in *GO49*-P-XADH.

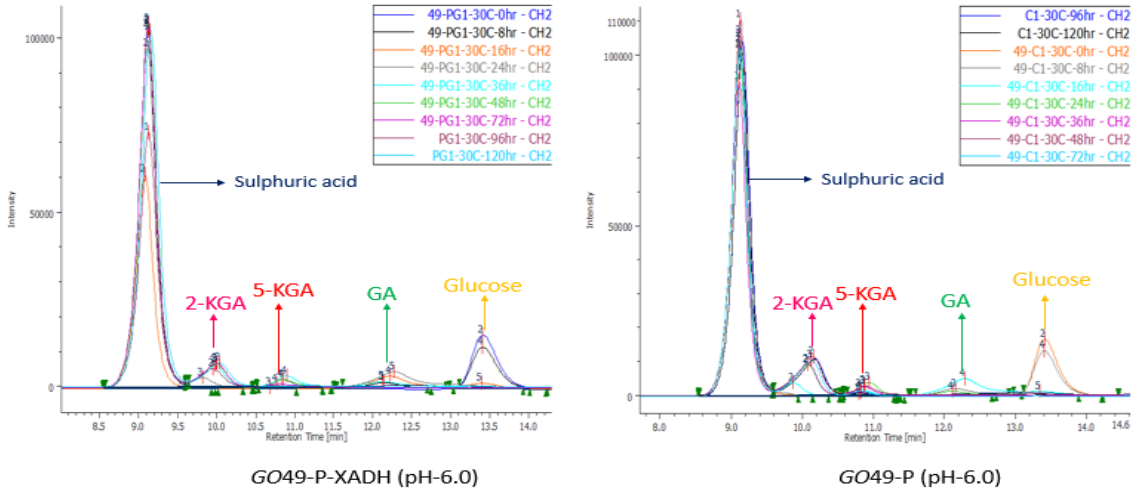


Figure 6. HPLC chromatogram representing glucose oxidation in *G. oxydans* cultivated at 30°C, pH-6.0 with formation of 2-KGA being detected during culture.

As an additional experimentation, the recombinant *G. oxydans* strain cultivated at pH-6.0 (refer to fig. 5a-b) was supplemented with 10 g/L GA into the fermentation broth after 120 hours of cultivation, to study if the cells have the potential to convert the added GA into 5-KGA in stationary phase. During sample analysis, the formation of 5-KGA was not detected from the culture which leads to hypothesize that genes responsible for the conversion of GA to 5-KGA, were expressed only during the exponential phase [35]. Another possibility includes the lower activity of 'gHp0169' promoter observed in *G. oxydans* when the cells reach the stationary phase during cultivation [36].

During the overall cultivation, *GO49*-P strain has a higher 5-KGA yield compared to *GO49*-P-XADH when cultivated at 30°C, pH 6.0-6.5 which suggests that the conditions were

not ideal for XADH activity. As a result, further studies involving screening for XADH proteins based on temperature was emphasized.

2. Optimizing conditions for XADH activity based on temperature

The following experiment was carried out to study the activity of XADH and yield of 5-KGA in *G. oxydans* during cultivation at 20°C and 25°C (refer to fig. 7 & 8). The pH of the culture during the cultivation was adjusted to 6.0, which was believed to be ideal for higher XADH activity based on studies performed with *X. campestris* [28-29].

When comparing the results, it was observed that both the recombinant and control strains from *G. oxydans* could produce 5-KGA during cultivation at 20°C and 25°C [37-38]. Also, it was noticed that *GO49*-P-XADH and *GO49*-P recorded an average maximum OD_{600} of 2.73 and 2.21, respectively, when cultivated at lower temperatures of 20°C and 25°C which leads us to a hypothesis that cultivating *G. oxydans* at optimal pH and temperature below 25°C could have supported the cell growth in the culture.

From fig. 7 & 8, we can see that rate of both glucose oxidation and GA oxidation was slower which indicates the lower activity of membrane-bound GDH and GADH which has an optimum working temperature of 30°C-50°C in certain bacterial species [39]. While analyzing 5-KGA yield at 20°C, it was found that *GO49*-P has higher yields compared to *GO49*-P-XADH. Interestingly at 25°C, *GO49*-P-XADH has better cell growth and yield of 0.68 g 5-KGA/ g glucose than 0.58 g 5-KGA/ g glucose in *GO49*-P. At temperatures below 30°C, certain membrane-bound enzymes from *G. oxydans* and *G. suboxydans* have been shown to have higher 5-KGA productivity, for example, GADH from *G. suboxydans* has documented maximum 5-KGA production at 15°C, pH-4.0 [40]. Formation of 2-KGA was observed at temperatures 20°C, 25°C (refer to fig. 9) and 30°C (from fig. 4 & 6), resulting in affecting the 5-KGA yield during fermentation.

As the 5-KGA yield in *GO49*-P-XADH at 25°C was higher compared to the control strain there are possibilities of XADH activity however, it is still difficult to prove that XADH was responsible for the enhancement of 5-KGA yield. As a result, it is important to study protein expression in recombinant strains to have a further understanding of this conclusion.

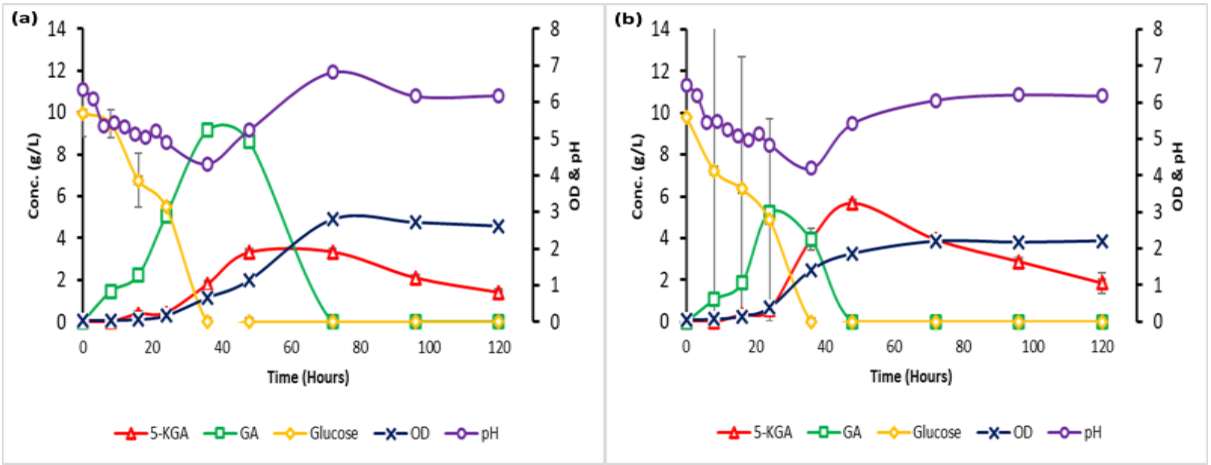


Figure 7. Production of 5-KGA by *G.* oxydans cultivated at 20°C, pH 6.0; (a) *GO49*-P-XADH; b) *GO49*-P. The cells from both strains have completely oxidized glucose within 36 hours of cultivation. By end of cultivation, OD from both strains was almost similar with a difference of 0.3 OD_{600}. 5-KGA

yield of 0.34 g 5-KGA/ g glucose and 0.58 g 5-KGA/ g glucose was obtained in *GO49*-P-XADH and *GO49*-P, respectively.

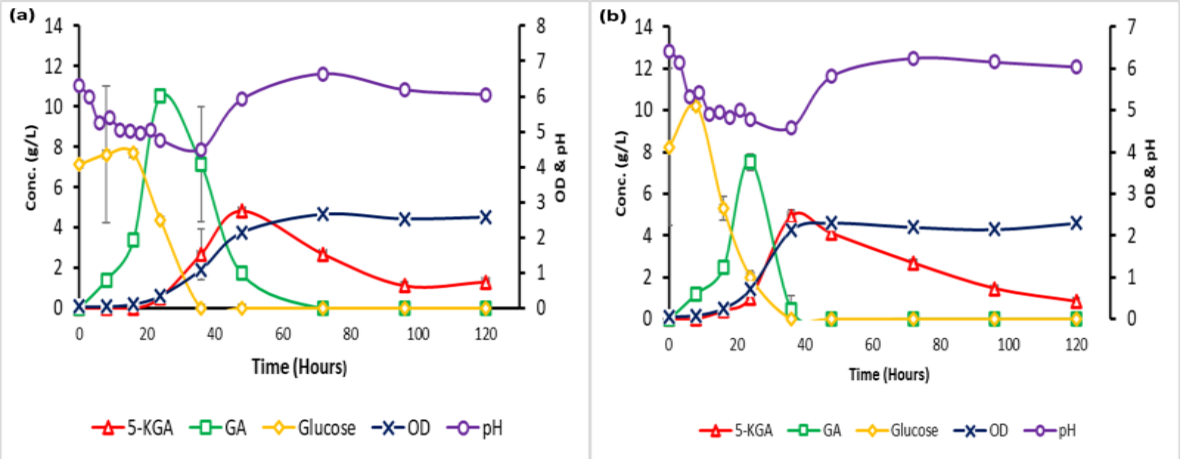


Figure 8. Production of 5-KGA from *G. oxydans* cultivated at 25°C; (a) *GO49*-P-XADH; b) *GO49*-P. The growth rate of *GO49*-P-XADH & *GO49*-P were tripled by end of cultivation with pH trends being identical similar to the trends observed at 20°C (refer to fig. 7a-b).

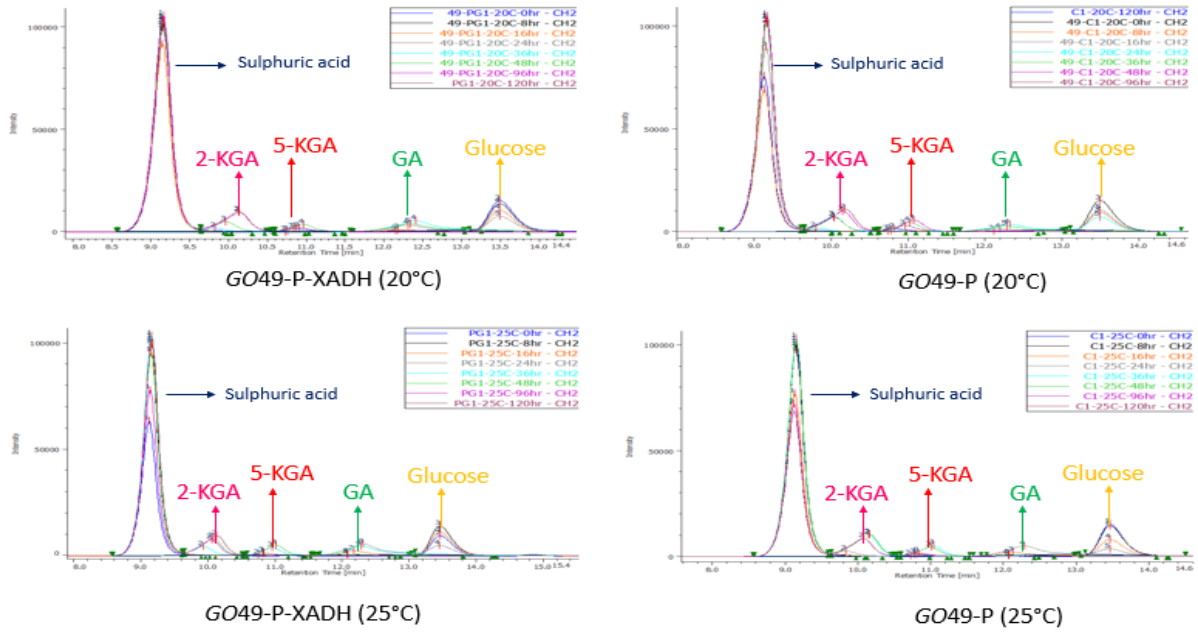


Figure 9. HPLC chromatogram illustrating glucose oxidation in *G. oxydans* cultivated at 20°C and 25°C.

3. Expression of XADH in *G. oxydans* and *E. coli* strains

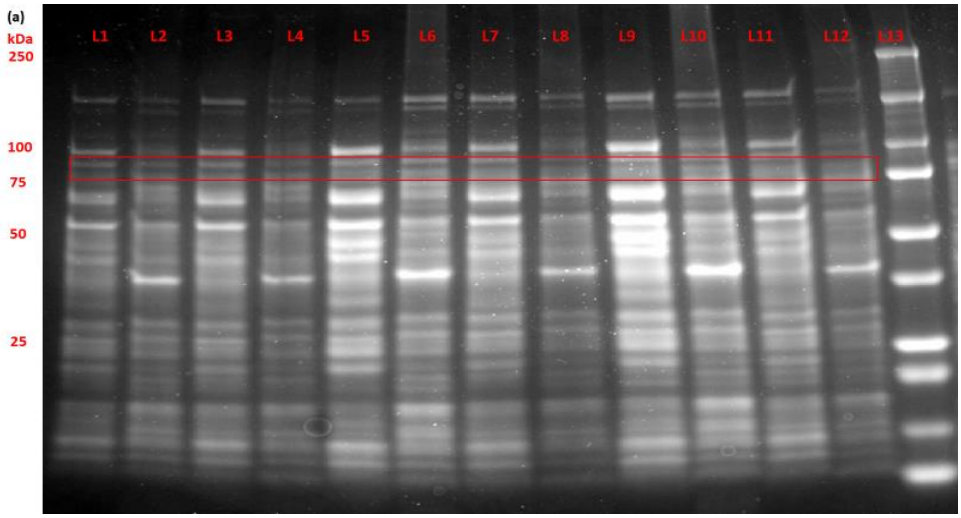
The following experiment was performed to analyze the expression of XADH in recombinant G. *oxydans* containing pBBR1MCS-5 along with *E. coli* strains transformed with pBBR1MCS-5 and pET30a vector.

While analyzing the cell fractions from recombinant *G. oxydans* using SDS-PAGE, it was observed that the expression of XADH was not detected during the analysis (refer to fig. 10a). From fig 10b, it was noticed that the target band in focus was present in both recombinant (*E. coli*-P-XADH) and control (*E. coli*-P) strains of *E. coli* which leads to difficulties in proving the expression of XADH. As a result, the SDS-PAGE was performed using purified protein fractions from ÄKTA start. From fig. 10c we can see that there was no band formed at 83 kDa from purified protein fractions, which could suggest that XADH was not expressed in shuffle *E. coli* strain containing the pBBR1MCS-5 vector. *E. coli*-P-XADH and BL21-P-XADH were analyzed for the presence of XADH, in which the former showed bands at 83 kDa in both soluble and insoluble fractions, whereas the latter showed band at expected molecular weight only in the insoluble fraction (refer to fig. 10d).

As an outcome of this study, the following suggestions could be helpful for providing better results before continuing with experiments further; 1) When performing protein purification using ÄKTA start, it is suggested to optimize the concentration of imidazole in the binding buffer to have a better binding of XADH protein to the His-Tag column. This is due to the reason that a higher concentration of imidazole often leads to lower protein yield along with improved purity, whereas imidazole at lower concentration could result in low purity during protein binding [41]. 2) Before sonication it is advisable to add protease or phosphatase inhibitors to cells as it can avoid possible denaturation of XADH proteins during sonication [42]. 3) During SDS-PAGE, it is important to prevent the samples from diffusing out of the well as it can lead to cross-contamination between samples, which can be avoided by running the gel immediately after loading the samples [43].

In conclusion, it was difficult to confirm the existence of XADH based on the protein bands obtained at 83 kDa, as the bands were observed in both the recombinant and control strains from *G. oxydans and E. coli* carrying pBBR1MCS-5.

This could suggest that XADH most likely has a lower gene expression rate in *E. coli a*nd *G. oxydans* containing the pBBR1MCS-5 plasmid. The factors responsible for lower protein expression are, 1) usage of low copy number plasmid pBBR1MCS-5 for gene expression, 2) lower promoter strength of the plasmid, 3) gene complexity, 4) poor efficiency during ribosome binding [44-45].



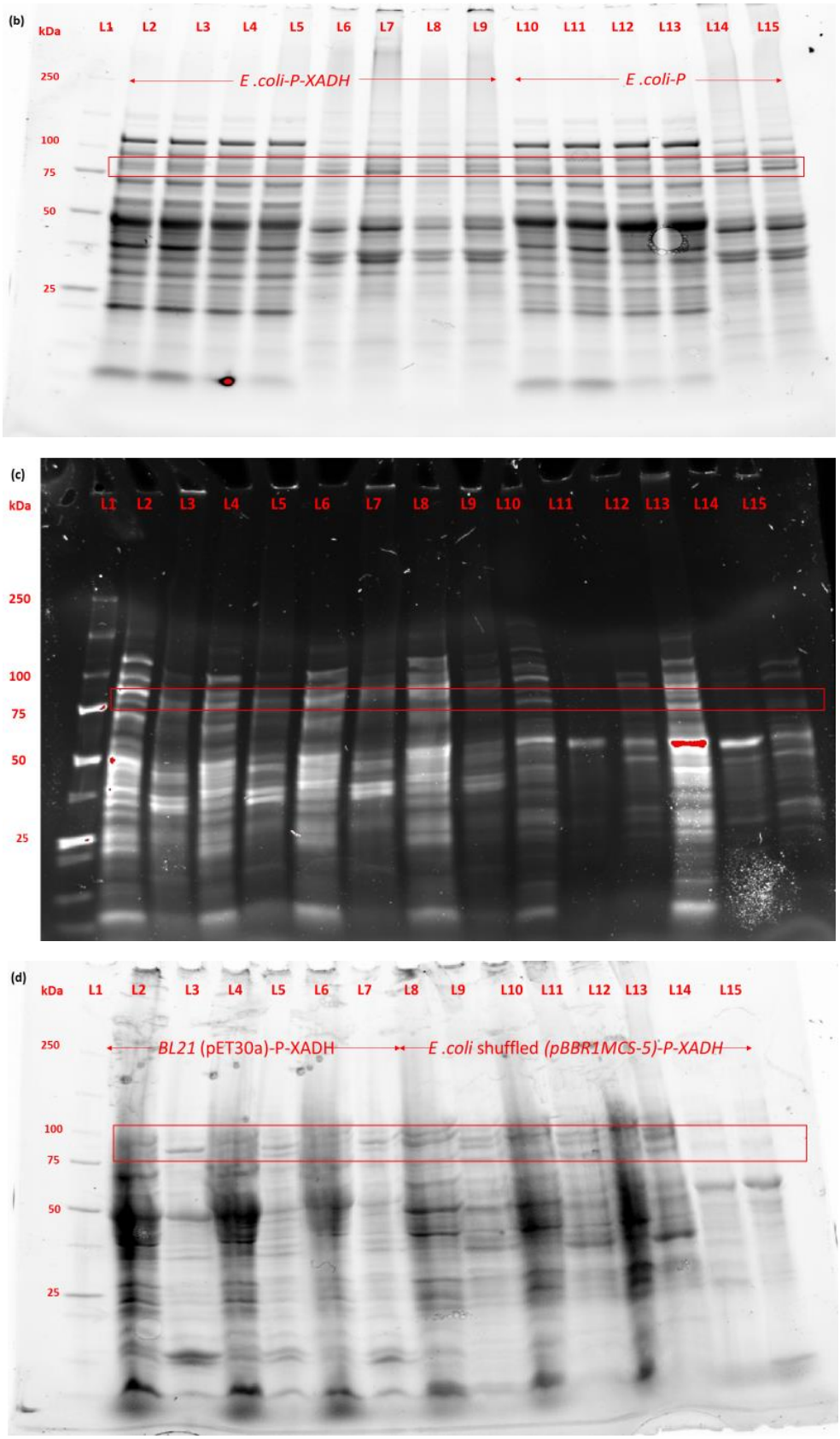


Figure 10. Screening for XADH protein having molecular weight 83 kDa in; (a) *GO49*-P-XADH and *GO49*-P; (b) *E. coli*-P-XADH and *E. coli*-P; (c) Purified protein fractions from *E. coli*-P-XADH and (d) BL21-P-XADH and *E. coli*-P-XADH.

4. Activity of XADH during activity assay and resting cell reaction

While performing an activity assay for studying the expression of XADH in recombinant *G. oxydans* and *E. coli* strains, during the reaction the formation of 5-KGA was not detected from both soluble and insoluble cell fractions (refer to fig. 11). During the resting cell reaction, it was observed that the resting cells from *GO49*-P-XADH cultivated at pH-6.5 (refer to fig. 12) achieved a 5-KGA yield of 0.63 g 5-KGA/ g GA.

The inability of the cell fractions to produce 5-KGA could be potentially due to the absence of PQQ co-factor and Ca^{2+} ions in the reaction mixture. Most quinoproteins including alcohol dehydrogenase require co-factors and metal ions for the reactivation of enzyme and to restore their activity lost during sonication [46-47]. Optimizing the conditions for activity assay should be emphasized as the information regarding the characterization of XADH and their ideal conditions for maximum activity is unknown.

The reason behind the formation of 5-KGA observed during the resting cell reaction is still unclear as it is difficult to predict the enzyme responsible for the formation of 5-KGA, since the expression of XADH was not proved during SDS-PAGE.

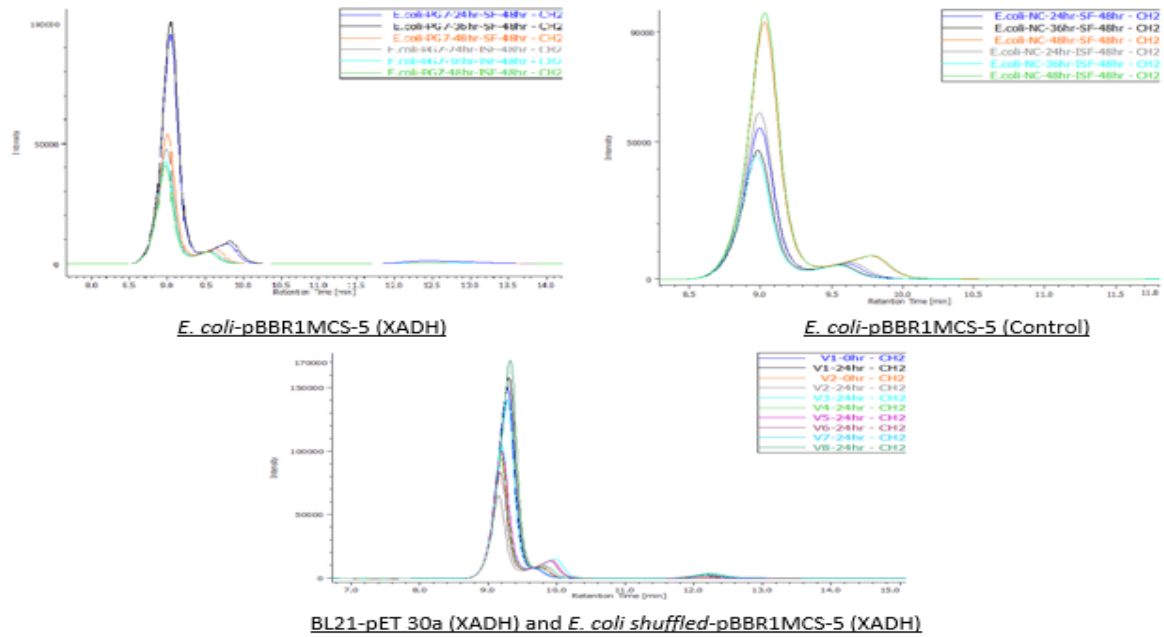


Figure 11. HPLC chromatogram obtained from activity assay performed using different *E. coli* strains and plasmids.

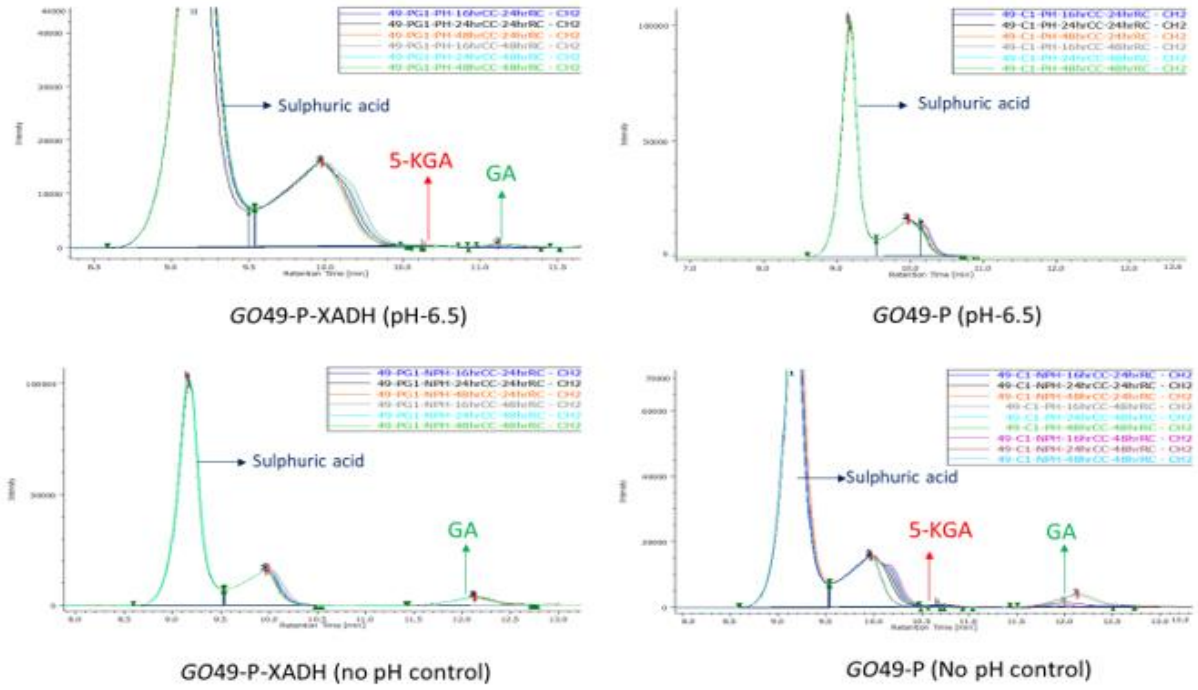


Figure 12. HPLC chromatograms obtained during resting cell reaction with *GO49*-P-XADH and *GO49*-P strains cultivated at pH-6.5 and uncontrol pH showing the formation of 5-KGA.

5. Confirmation of XADH gene in *G. oxydans* and determination of optimal annealing temperature for M13 primers

The main objective of this experiment was to verify the presence of recombinant XADH gene in *G. oxydans*. During agarose gel electrophoresis, the recombinant XADH gene has base pairs of 2,500 excluding from the pBBR1MCS-5 plasmid consisting of 4,500 base pairs. From fig. 13a, we can confirm the presence of XADH gene from the plasmid extracted from *GO49*-P-XADH.

While performing PCR with M13 primers (refer to Appendix C, Table 1) using pBBR1MCS-5 plasmid, it was observed that the XADH gene was not amplified during the reaction using PCR mix 1 (refer Appendix C, Table 2). After performing multiple attempts, the amplification of XADH gene was achieved using PCR mix 2 (refer to fig.13b-c and Appendix C, Table 2). Reasons behind the failure of gene amplification using PCR mix 1 could be due to 1) low efficiency of Phusion polymerase caused during longer storage, 2) lower concentration of M13 primers, and/or 3) longer annealing time [48].

Fig. 13b below illustrates the optimization of annealing temperature for M13 primers using gradient PCR performed using PCR mix 2. And fig. 13c shows the presence of XADH gene in the recombinant *G. oxydans* strain in comparison to the control strains.

From this experiment, we conclude that recombinant *G. oxydans* strain used for cultivation carries the XADH gene also, the optimal annealing temperature for performing PCR using M13 primers was determined as 59°C based on gradient PCR.

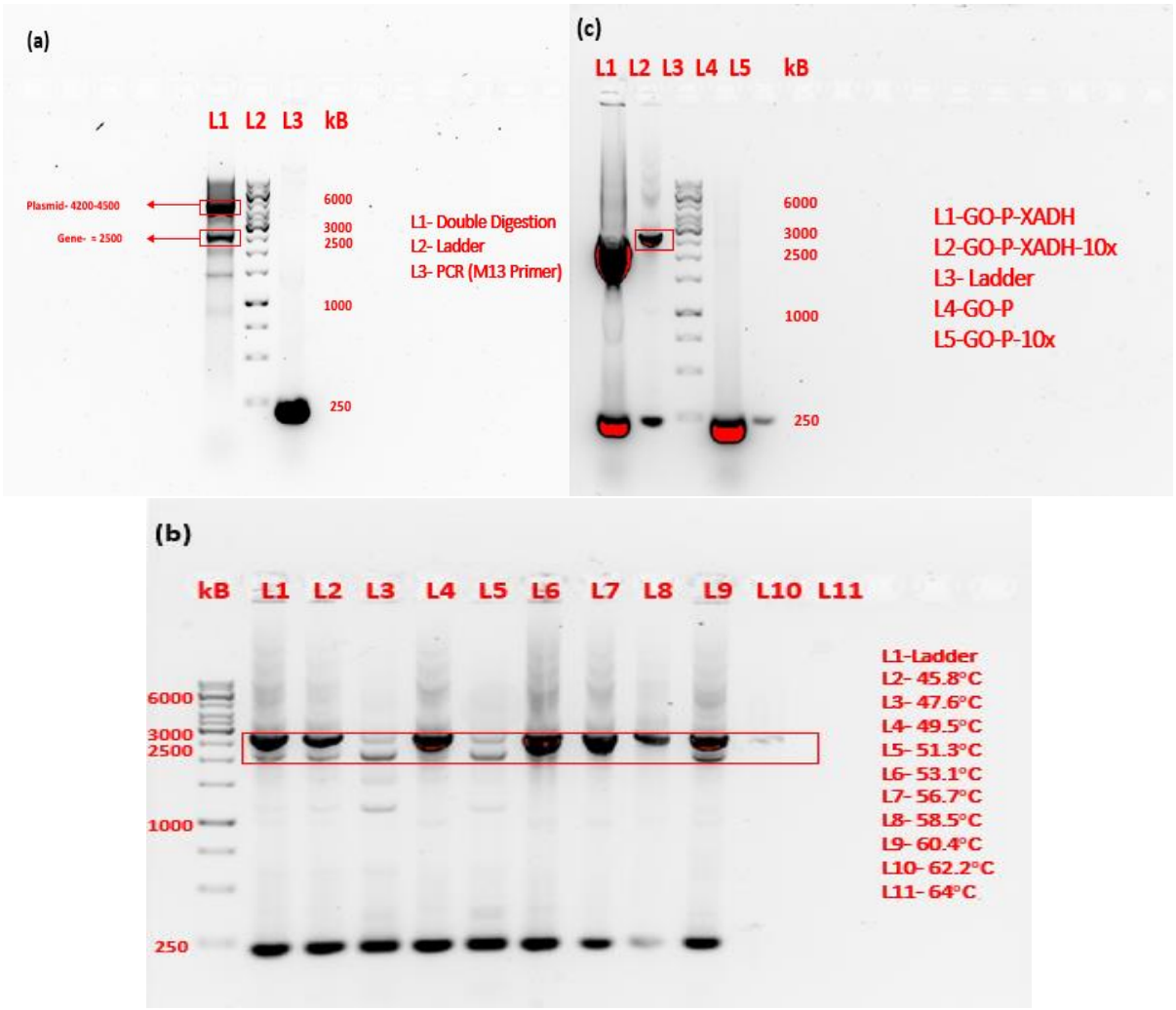


Figure 13. Agarose gel electrophoresis performed using *GO49*-P-XADH, (a) Double digestion and gene amplification using PCR mix-1, (b) Gradient PCR for optimization ideal annealing temperature using M13 primers and (c) Band comparison between *GO49*-P-XADH and *GO49*-P when performing PCR at 59°C.

Conclusion

During screening for optimal temperature and pH, the *G. oxydans* expressing XADH from *X. campestris*, generated a maximum 5-KGA yield of ~ 0.7 g 5-KGA/g glucose during cultivation in glucose media at 25°C, pH 6.0. Formation of 2-KGA and transient 5-KGA were identified as main problems affecting the yield during the cultivation of *GO49*-P-XADH. As results from SDS-PAGE and activity assay were found inconclusive to prove the expression and activity of XADH in recombinant *E. coli* strains containing pBBR1MCS-5 and pET30a plasmids. The minor accumulation of 5-KGA (0.43 g/L) documented from resting cells of *GO49*-P-XADH cultivated pH-6.5 were also considered indefinite to confirm the activity of XADH. Interestingly, the target band at 83 kDa was observed at the insoluble cell fractions of BL21-P-XADH which provides an opportunity to continue further working with pET30a vector for the expression of XADH.

Based on the constructive feedback obtained from our studies, it is imperative to carry out more experiments focusing on the characterization of XADH and enhancing the productivity of 5-KGA. As we know that each enzyme responsible for the biotransformation of D-glucose to 5-KGA has a specific optimum pH and hence during the course of fermentation it is important to develop different stages for pH favouring the oxidation for improving the productivity of 5-KGA [49]. During fermentation, constant monitoring of parameters including oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) helps in establishing a continuous dissolved oxygen (DO) control strategy which was proven to increase 5-KGA yield during cultivation [50].

Since most of the quinoproteins including secondary alcohol dehydrogenase from *X. campestris* are dependent on divalent cations Mg^{2+} and Ca^{2+} for their activity, the addition of $CaCl_2$ or $CaCO_3$ to the culture media helps in the activation of PQQ co-factor which significantly improves the production of 5-KGA [51]. Previous studies have credited the strategy of using combinatorial metabolic engineering in *G. oxydans* using the 'gHp0169' promoter for enhancing the accumulation of 5-KGA [24]. This strategy was used for the over-expression of recombinant proteins by combining two genes encoding the same enzymes with different subunits.

With constant experimentation and good mentoring, the alcohol dehydrogenase from *X. campestris* would prove to be a novel enzyme in the chemical industry for the manufacturing of compounds in an economical and sustainable way. The future scope for this study apart from optimizing the strain and fermentation process would be to perform larger-scale cultivations and designing of new strategies focusing on improving the productivity of 5-KGA suitable for industrial-scale production.

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Appendix A- Metabolic pathways of *G. oxydans*

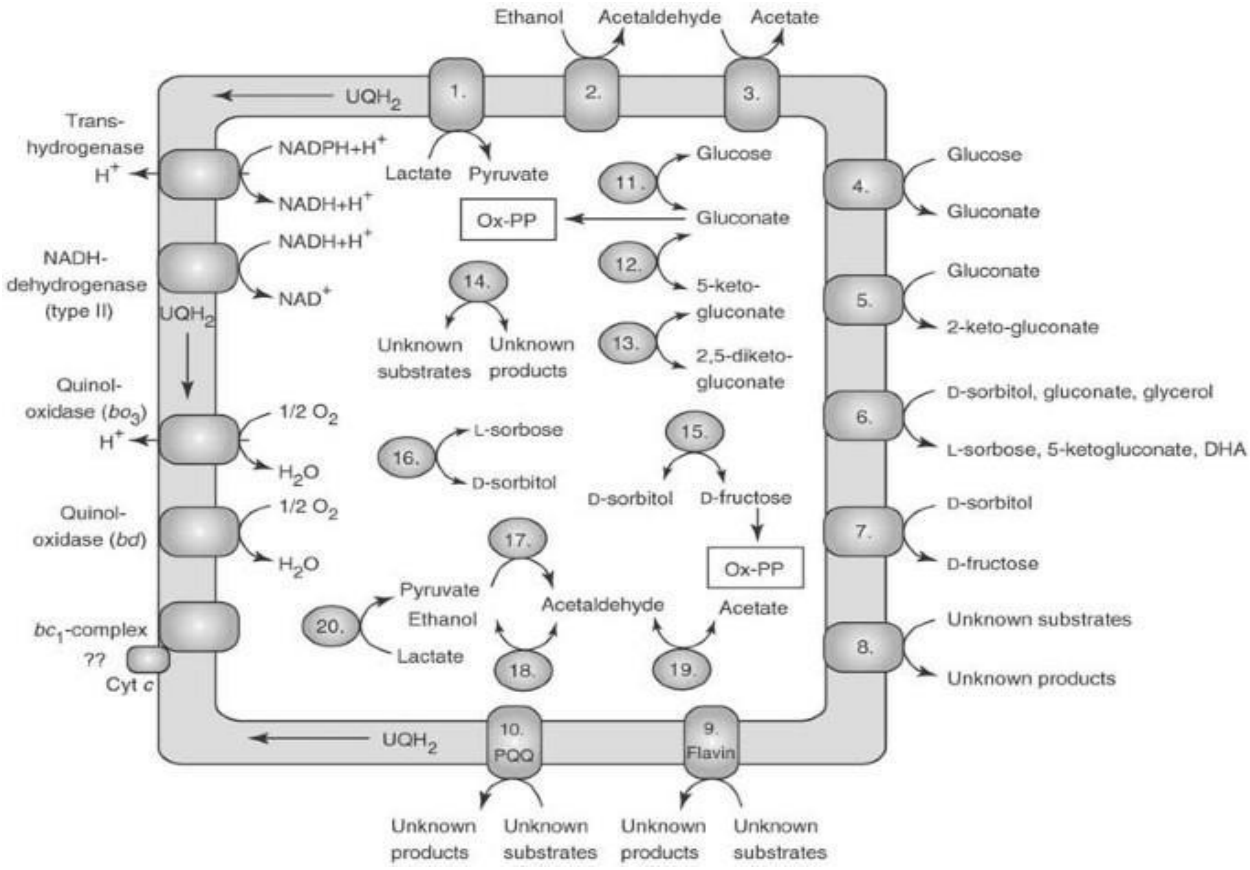

Figure 1. Respiratory chain and sugar/alcohol metabolism in *G. oxydans* 621H [52].

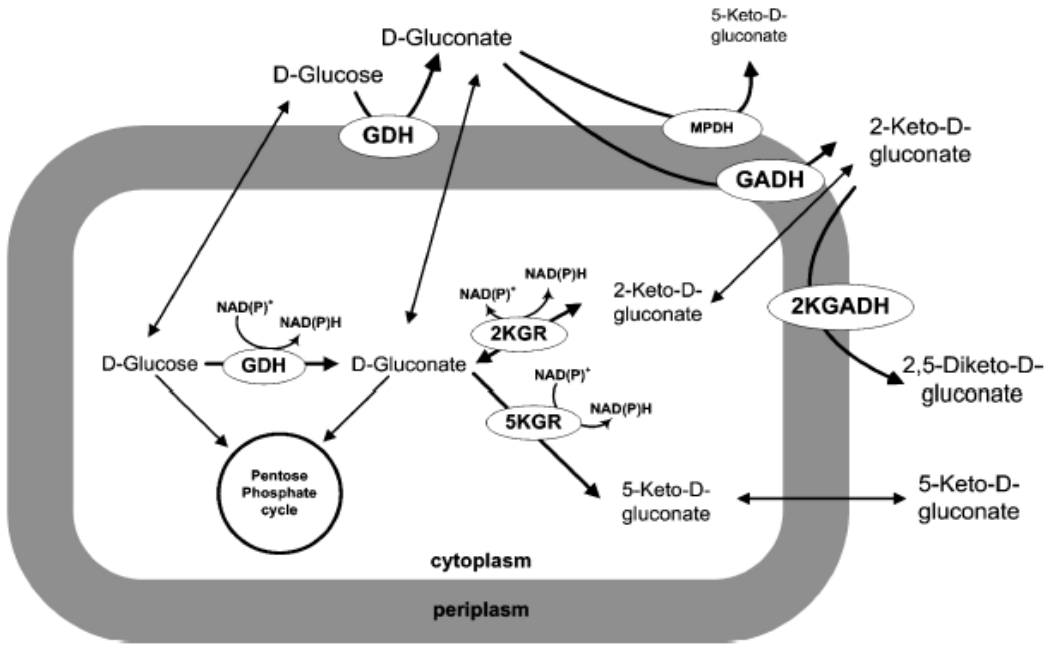

Figure 2. Glucose oxidation metabolism pathway in *G. oxydans species* [2].

Appendix B- AI media formulation

Table 1. Media components and composition for stock preparation.

Media Components	**Composition**
NPS 20x Stock	450 mL De-ionised (DI) water + 33 g $(NH_4)_2SO_4$ + 68 g KH_2PO_4 + 71 g Na_2HPO_4
5052 50x Stock	73 ml DI water + 25 g glycerol + 2.5 g glucose + 10 g α-lactose
ZY medium	925 ml DI water + 10 g tryptone + 5 g yeast extract
2M $MgSO_4$ stock	100 ml DI water + 49.3 g of $MgSO_4.7H_20$

Table 2. Volumetric composition for preparation of AI media.

ZY medium	**NPS 20X**	**5052 50X**	**2M $MgSO_4$**	**Final AI media volume**
46.4 ml	2.5 ml	1 ml	25 µl	50 ml
464 ml	25 ml	10 ml	250 µl	500 ml
928 ml	50 ml	20 ml	500 µl	1 L

Appendix C- PCR

Table 1. M13 primer sequence [53].

Primer	Sequence
M13 Forward	5'GTAAAACGACGGCCAGT3'
M13 Reverse	5'CAGGAAACAGCTATGAC3'

Table 2. Composition of PCR reaction mix with a reaction volume of 50 µl.

	PCR Mix-1		PCR Mix-2	
Cycle step	**Temperature (°C)**	**Time (mm: ss)**	**Temperature (°C)**	**Time (mm: ss)**
Initial Denaturation	98.0	3:00	95.0	5:00
Denaturation	98.0	0:30	95.0	0:30
Annealing	54.0	0:30	59.0	0:30
Extension	72.0	1:00	72.0	3:00
Final extension	72.0	10:00	72.0	10:00
Hold	4.0	∞	4.0	∞

Table 3. Composition of PCR reaction mix.

Mix-1		Mix -2	
Components	**Volume**	**Components**	**Volume**
Buffer	10 µl	Buffer	5 µl
M13 Forward primer (10 µM)	2.5 µl	M13 Forward primer (10 µM)	3 µl
M13 Reverse primer (10 µM)	2.5 µl	M13 Reverse primer (10 µM)	3 µl
Template	1 µl	Template	1 µl
dNTP's (10 µM)	1 µl	dNTP's (10 µM)	1 µl
Sterile Milli-Q water	32.5 µl	Sterile Milli-Q water	36.75 µl
Phusion polymerase	0.5 µl	Taq polymerase	0.25 µl

Table 4. Composition of double digestion mix.

Components	**Volume**
10X tango buffer	2 µl
SacI	1 µl
HindIII	1 µl
Whole plasmid	16 µl

Appendix D- HPLC

Table 1. Volumetric composition for HPLC vial preparation.

Experiment	Sample dilution factor	Volume of sample	Volume of Milli-Q water	Volume of 10% sulphuric acid	Total volume
Cell cultivation	10X	200 µL	1800 µL	40 µL	2040 µL
Activity assay	20X	50 µL	950 µL	20 µL	1020 µL
Resting cell reaction	10X	100 µL	900 µL	20 µL	1020 µL

Appendix E- ÄKTA start

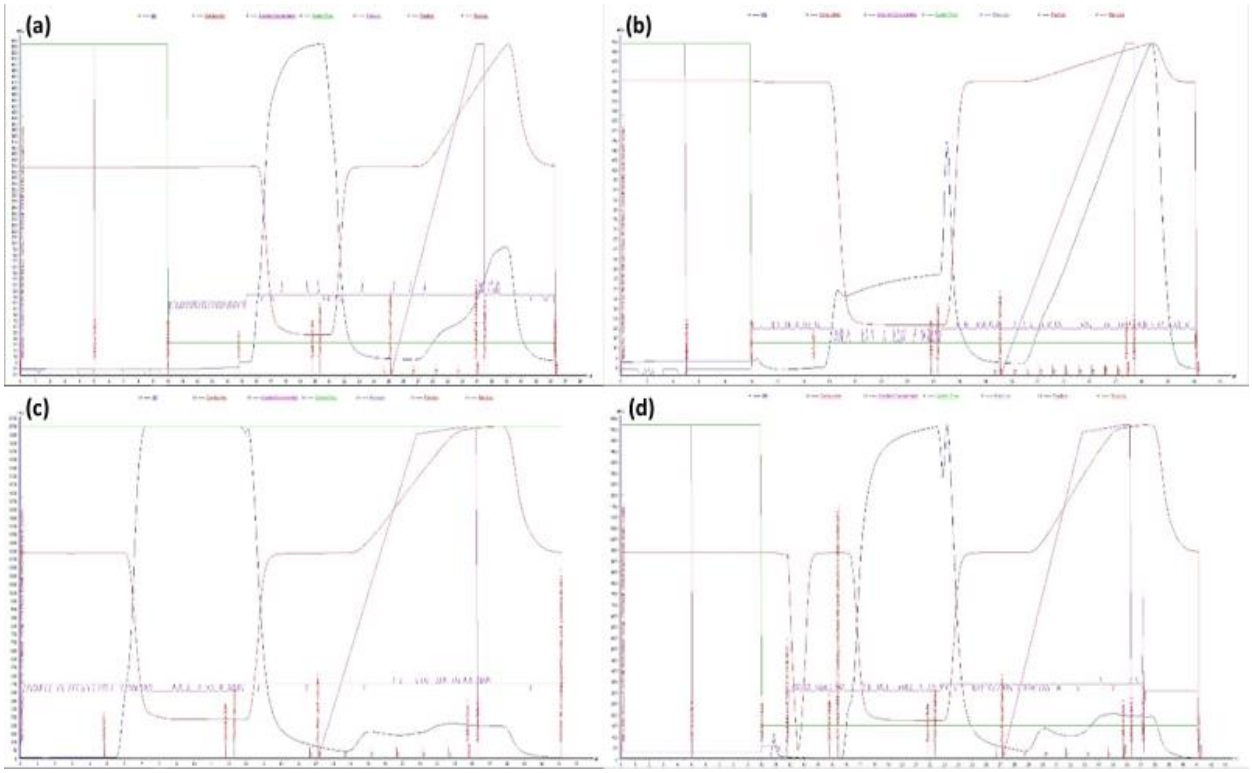

Figure 1. Schematic representation on conductivity signal obtained when performing IMAC using ÄKTA start from soluble cell fractions obtained from (a) *G. oxydans* DSM 50049 wild type and (b-d) *GO49*-P-XADH.