Synthetic Biology Approach to Construct an Efficient Production System of Itaconic Acid in Corynebacterium glutamicum

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(Degree)
博士（工学）

(Date of Degree)
2023-09-25

(Date of Publication)
2025-09-25

(Resource Type)
doctoral thesis

(Report Number)
甲第8742号

(URL)
https://hdl.handle.net/20.500.14094/0100485926

DOCTORAL DISSERATION

博士論文

Synthetic Biology Approach to Construct an Efficient Production System of Itaconic Acid in *Corynebacterium glutamicum*

(コリネバクテリウム グルタミカムにおけるイタコン酸の効率的な生産システムを構築する合成生物学的アプローチ)

July, 2023

2023 年 7 月

GRADUATE SCHOOL OF ENGINEERING

KOBE UNIVERSITY

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タグリード マグディ アブデルラゼク エルカサビー アタラー

PREFACE

This thesis is submitted by the author to Kobe University in partial fulfilment of the requirements for the degree of Doctor of Engineering. The studies were carried out between the period of 2020 and 2023 under supervision of Professor Chiaki Ogino in the Laboratory of Biochemical and Bioprocess Engineering, Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University.

First of all, the author wishes to express the extremely grateful to the Lord Almighty **Allah** for the blessing throughout my life and for giving me the patience and strength to continue throughout my research journey. The author would like to express her outmost sincere gratitude to her research adviser, Professor Chiaki Ogino for his continuous advice, guidance, support, encouragement and unconditional help throughout my research journey. The author would like to express her appreciation to Professor Akihiko Kondo, Associate Professor Hideo Kawaguchi, Associate Professor Prihardi Kahar and Masakazu Toyoshima, Ph.D. for their continual help, support and constructive criticisms throughout the research. By their knowledge, guidance and support the author could develop the research assembled in this thesis. The author appreciation goes also to, the lab technicians, staff and students of the Laboratory of Biochemical Engineering for their daily assistance.

The author would like to thank all colleagues; Katsumi Otta, Taiki Ono, Pamella Apriliana, Winda Tasia, Filemon Jalu, Radityo Pangestu, Tan Kar Ling, and Deddy Tri

Nugroho Adi for their encouragement, support, pleasant lab atmosphere, and unforgettable teamwork.

The author expresses warm thanks to her mother, her siblings, AbdEl-Razek Magdy, Mai Magdy, and Merna Magdy for their support, unconditional love and encouragement through this period of study.

The author would like to convey a special thanks from the heart to her daughter, Farida for being the source of love, kindness, and warmness throughout my life.

Last but not least, the author would also like to acknowledge the continuous support and valuable financial support from her sponsor, Egyptian Cultural Bureau in Japan on behalf of the Ministry of Higher Education and Mansoura University in her beloved country Egypt.

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CONTENTS

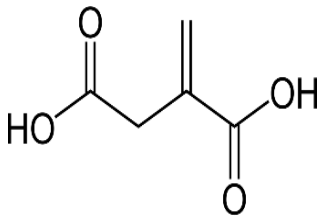
CHAPTER I

Introduction

I. 1. Itaconic acid

Itaconic acid (IA) present naturally as unsaturated carboxylic acid with five carbons and with other names of methylene succinic acid or methylene butanedioic acid (1). IA has unique properties (Table 1), where it has chemical formula of $C_5H_6O_4$ and molecular weight of 130.1 g/mol. It presents as a white to light beige crystals with a density of 1.573 g/ml at 25 °C, melting point of 165-168°C and a flash point of 268°C (1, 2). IA can dissolve in water up to 80.1 g/L at 20°C, which makes the purification process by polymerization is much easier. IA was found also to have the ability to dissolve in many alcohols such as methanol, 2-propanol and ethanol where the solubility increases by raising temperature (3, 4). The most important is that IA is biodegradable in nature. IA is a significant monomer due to its characteristic chemical properties where it contains methylene groups and two carboxylic acids groups which enable it to participate in many polymerization processes (5).

Table 1. Itaconic acid Characteristics



Chemical formula: $C_5H_6O_4$

Molecular weight: 130.1 g/mol

Appearance: white to light beige crystals

Density: 1.573 g/ml at 25 °C

Solubility: dissolve in water up to 80.1 g/l at 20°C

Melting point: 165-168°C

Flash point: 268°C

I. 2. Why the increasing need for IA?

IA can be used in various significant products as a monomer or substitute such as products from IA polymerization as polyIA, styrene butadiene rubber latex SBR which is the resulting polymer of IA, styrene and butadiene. Additionally, IA can be used as alternative to acrylic acid in the production of super absorbent polymers which are recently used in the production of unsaturated polyester resins (URP). IA can also replace sodium tripolyphosphate STPP in detergents industry (3). Furthermore, Mixtures of IA and acrylonitrile can give more dye than many other polymers. Carpets including IA are more resistant to abrasion. Moreover, IA can be used in the synthesis of emulsion paints to improve its adhering properties (6). In plastics and coatings, IA can be incorporated also by addition of 1-5% of IA to improve the final product by giving it lighter color, can be easily painted,

and separated, with antiseptic characteristics. Additionally, IA can be used in the medical industry as a solidifying agent in organosilaxans, which can be used in contact lenses (3)

I. 3. Metabolic Pathways involved in IA production

There are two known pathways for IA production in natural producers. First pathway occurs in the fungus *Aspergillus terreus* (Fig. 1) and involves transfer of metabolites between mitochondria and cytosol (13). IA is naturally produced during Krebs's cycle (TCA cycle) where citric acid is formed firstly by the aid of citrate synthase enzyme, then converted to *cis*-aconitate by aconitase. These first steps take place in mitochondria. After that, the transporter protein, mitochondrial *cis*-aconitic acid transporter protein (Mtta) which is a part of gene cluster including also *cis*-aconitase decarboxylase (*cadA*) gene which is the key gene in IA production transports *cis*-aconitic acid from mitochondria to cytosol where decarboxylation of *cis*-aconitate to IA occurs by *cadA*.

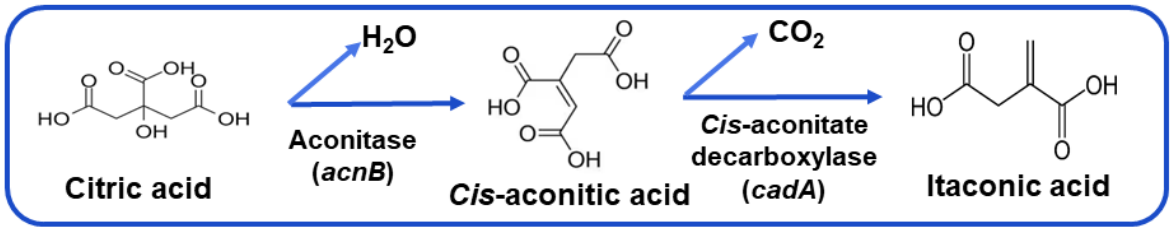


Fig.1 *Cis*-pathway depending on *cadA* gene for IA production in *A. terreus*.

IA can be produced also by the fungus *Ustilago maydis* (26), although it doesn't possess the *cadA* gene. It adopts another pathway for IA production. The production of IA from *U. maydis* includes two sets of enzymes, the first enzyme aconitase-delta-isomerase (*Adi1*) which catalyze the isomerization of *cis*-aconitate into *trans*-aconitate. After that, the second enzyme *trans*-aconitate decarboxylase (*Tad1*) catalyzes the decarboxylation of t*rans*-aconitate to IA (Fig. 2). Both enzymes are present in the cytosol, so transporter proteins mitochondrial tricarboxylate transporter (Mtt1) and itaconate transporter protein (Itp1), translocate *cis*-aconitate from mitochondria to cytosol where it is converted to IA.

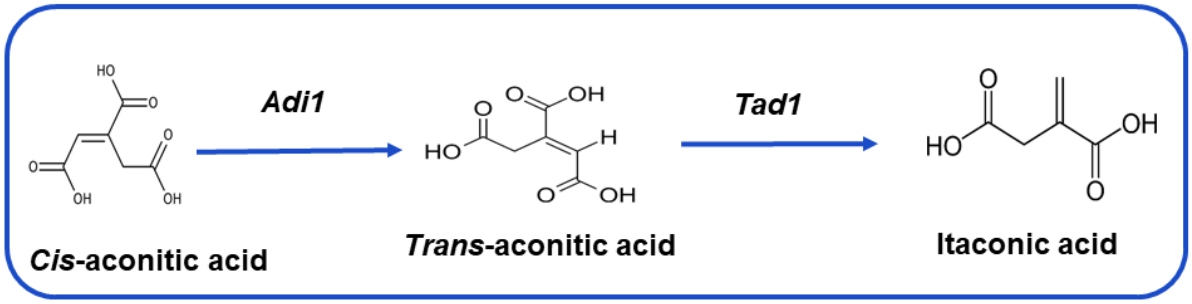


Fig. 2. *Tr*ans-pathway depending on *Adi1*/*Tad1* genes for IA production in *U. maydis*.

I. 4. Production of IA by natural producers

IA can be produced by many microorganisms naturally. The first report of production of IA by fungi using sugars was done by extraction of IA from the growth medium of an osmophilic fungus *Aspergillus itaconicus* (7). After that, *A. terreus* NRRL 1960 was isolated at the Northern Regional Research Laboratory (NRRL) of the U.S. Department of Agriculture in Peoria, Illinois and found to be more suitable for IA production

(8). Later, many fungal species other than *A. terreus* as *Ustilago zeae*, *U. maydis*, *Candida sp*., *Pseudozyma antarctica* and *Rhodotorula species* were reported to produce itaconic acid (9, 10, 11, 12). However, Up to now *A. terreus* is the most frequently used commercial producer of IA (13) (Table 2).

Not only *A. terreus* used as a natural producer of IA but also *U. maydis* (26) can be used as a native producer, and it has an advantage over *A. terreus* in its yeast-like growth. Also, wild *U. maydis* produced IA under nitrogen-limited conditions and with pH similar to *A. terreus*. Consequently, combination of metabolic, morphologic engineering by elimination of IA oxidase cyp3 and over expression of mitochondrial transporter (mttA) together with *insitu* crystallization of IA gave a high yield of IA from *U. maydis* (26).

Besides *A. terreus* and *U. maydis*, *U. cynodontis* also produces IA naturally and it is considered one of the most significant productive species in Ustilaginaceae family due to its tolerance to high pH compared to other smut fungi (27).

Table 2. Itaconic acid production from natural producers

Organism	Substrate	Titer (g/L)	Reference
A. terreus M-8	Starch hydrolysate	55	(14)
A. terreus IMI 282743	POME (palm oil mill effluent)	5.76	(15)
A. terreus SKR10	Sago starch	48.2	(16)
A. terreus	Jatropha seed cake	24.45	(17)
A. terreus	Jatropha seed cake	48.7	(18)
A. terreus CECT 20365	Olive & beet waste	44	(19)
A. terreus DSM23081	Hydrolysed wheat chaff	27.7	(20)
A. terreus CICC4040205	Hydrolysed wheat bran	49.65	(21)
A. terreus	Glucose	87.32	(22)
A. terreus M69	Corn stover hydrolysate activated with charcoal	33.6	(23)
A. terreus NRRL 1960	Glucose	38.89	(24)
A. terreus NRRL 1972	Glucose	41.0	(25)
A. terreus NRRL 1960	Glucose	26.3	(13)

I. 5. Production of IA in engineered bacteria

Not only fungi and yeast, but also IA can be produced by genetically engineered bacteria (Table 3). IA was produced many times by *Escherichia coli* due to many reasons

including, *E. coli* is a better choice for bioproduction because of its rapid growth in both aerobic and anaerobic conditions, its facultative anaerobic nature, commonly used in genetic engineering, and its nutritional needs are simple (28).

Consequently, many previous reports succeeded in the production of IA in engineered *E. coli*. IA was produced in recombinant *E. coli* by heterologous expression of α-amylase using cell surface display method and using soluble starch as the sole carbon source (29). IA was produced in with low yield in comparison, which may be due to many factors that needed to be controlled including, *E. coli* exhibits low *cadA* activity, low concentration of carbon source in the media, minimal medium can be used instead of LB medium, and the production can be further improved by applying some metabolic engineering (30).

Furthermore, in previous report IA was produced by recombinant *E. coli* by introducing citrate synthase and aconitase genes from *Corynebacterium glutamicum*, and by elimination of phosphate acetyltransferase and lactate dehydrogenase (29). IA was also produced under anaerobic conditions by expression of *cadA* with citrate synthase (*gltA*) and aconitase (*acnA*), and with using nitrogen-limited growth medium to decrease the competitive production of glutamate, as it depends on the presence of nitrogen in the medium (31). Additionally, *E. coli* produced IA by heterologous expression of immunoresponsive gene (*irg1*) with introduction of citrate synthase (*gltA*) and aconitase (*acn)* from *C. glutamicum* and deleting of phosphate acetyltransferase (*pta*) and lactate dehydrogenase (*IdhA*) pathways

with making comparison between codon-optimized and codon-harmonized *cadA* and *irg1*, where the codon-harmonized strain exhibited high enzymatic activity with no great effect on IA production (32).

Moreover, the yield of IA improved in *E.coli* by creation of library of synonymous codon variants (SCV) of *cadA* gene in the firstcodon except (ATG) start codon and optimizing culture conditions with glycerol feeding instead of glucose, and applying nitrogen-limited growth conditions (33). Recombinant *E. coli* also produced IA using various substrates by improving *cis*-aconitate flux and isocitrate pool by overexpression of aconitase, citrate synthase, PEP carboxylase and elimination of α-ketoglutarate formation by diminishing of isocitrate dehydrogenase (34).

Table 3. Attempts for IA production by engineered bacterial strains

Microbe	Substate	IA titer (g/L)	Reference
E. coli	starch	0.15	(50)
E. coli	glucose	0.69	(28)
E. coli	Glucose	0.377	(31)
E. coli	Glucose	0.56	(32)
E. coli	Glycerol	7.2	(25)
E. coli	Glycerol	3	(34)
E. coli	Glucose	4 47	(35)
E. coli	Acetate	3.57	(36)
C. glutamicum	Glucose	7.8	(49)
C. glutamicum	Sodium acetate	29.2	(51)

I. 6. Production of IA in engineered *Corynebacterium glutamicum*

Besides *E. coli, C. glutamicum* is considered as workhorse in the field of biotechnology, where it played an important role in the production of various bioproducts

starting from glutamate and recently used for production of other chemicals, fuels and polymers (37). Additionally, the complete genome sequence of *C. glutamicum* is now available (38, 39), which can allow researchers to understand more about this bacterium, easily engineer it and can also use a combination of Omics tools, synthetic biology tools and genetic engineering tools for successful metabolic engineering in *C. glutamicum*. *Corynebacterium* can be used also as synthetic biology platform chassis which can open new doors towards industrial microbial biotechnology other than classical ones. *C. glutamicum* is considered as GRAS generally recognized as safe host and safely used by human, has fast growth and high cell densities (40), lack recombinant repair system, which makes it genetically stable (41), has limited restriction modification system (42), has no autolysis and can maintain its metabolic activity under growth arrested conditions (43), exhibits limited protease activity, which makes it favorable host for production of recombinant protein (44), has metabolism plasticity and strong secondary metabolism characteristics (45), and can utilize wide range of carbon sources like pentoses and hexoses (46).

However, the wide range of bioproducts produced using *C. glutamicum*, till now there are few reports for production of IA from *C. glutamicum*. Therefore, additional research required to improve IA production using this multi-characteristic strain and this may require usage of more metabolic engineering, optimization of fermentation process, using synthetic biology in combination with Omics tools to enhance production in the future (47, 48).

In previous study, IA was produced in recombinant *C. glutamicum* by fusion of *cadA* with maltose-binding protein from *E. coli*, where the yield is further increased by applying nitrogen-limited growth conditions, and decreasing isocitrate dehydrogenase activity giving final titer of 7.8 g/L IA (49). IA was produced also by *C. glutamicum* using acetate as main carbon source reaching final IA titer of 29.2 g/L using fed-batch fermentation in 42 L bioreactor (51).

In our study we produced IA with a high final titer compared to previous studies (Fig. 3). In chapter II, engineered *C. glutamicum* strain expressing *cis*-aconitate decarboxylase gene from *A. terreus* (*cadA*) was constructed. The effect of different carbon sources on IA production in *C. glutamicum* was investigated. A mixture of substrates was also used to evaluate their effect on cell growth and IA production via *C. glutamicum*. The heterologous expression of *cadA* gene in the wild-type strain *C. glutamicum* ATCC 13032 resulted in a low IA production titer by using numerous carbon sources such as glucose, fructose, sucrose, maltose and mannose. Sodium acetate, however, was the carbon source that led to the highest IA production titer, while maltose led to optimal cell growth. The utilization of maltose and sodium acetate as the main carbon sources, resulted in an increase in IA production to a final titer of 12.63 g/L IA.

In chapter III, *C. glutamicum* was engineered by developing two distinct IA production pathways: *cis*-pathway that depends on *Irg1* gene from *M. musculus* and *trans*-

pathway (*Adi1*/*Tad1*) from *U. maydis*. The strain expressing *trans*-pathway (*Adi1*/*Tad1*) genes from *U. maydis* resulted in high IA titers by utilizing most carbon sources. Expression of the *trans*-pathway in *C. glutamicum* improved IA production than *Irg1* gene with a final titer of 12.25 g/L and molar yield of 0.22 mol/mol from glucose via fed-batch fermentation.

In chapter IV, three engineered *C. glutamicum* strains expressing the three different metabolic pathways (*cadA* gene, *irg1*gene and *Adi1*/*Tad1* genes) for IA production were used for IA production from plant-based biomass including; sweet sorghum juice and bagasse sugar lysate (BSL). Sorghum juice has a high concentration of soluble sugars (glucose and sucrose) with no chemical inhibitors. *C. glutamicum* strain expressing *Adi1*/*Tad1* genes from *U. maydis* which was used previously for IA from pure sugars, proved to be better also for IA production from sorghum juice and BSL which confirms that *trans*-pathway is better than *cis*-pathway in IA production by *C. glutamicum* not only from pure sugars but also from pant-based biomass.

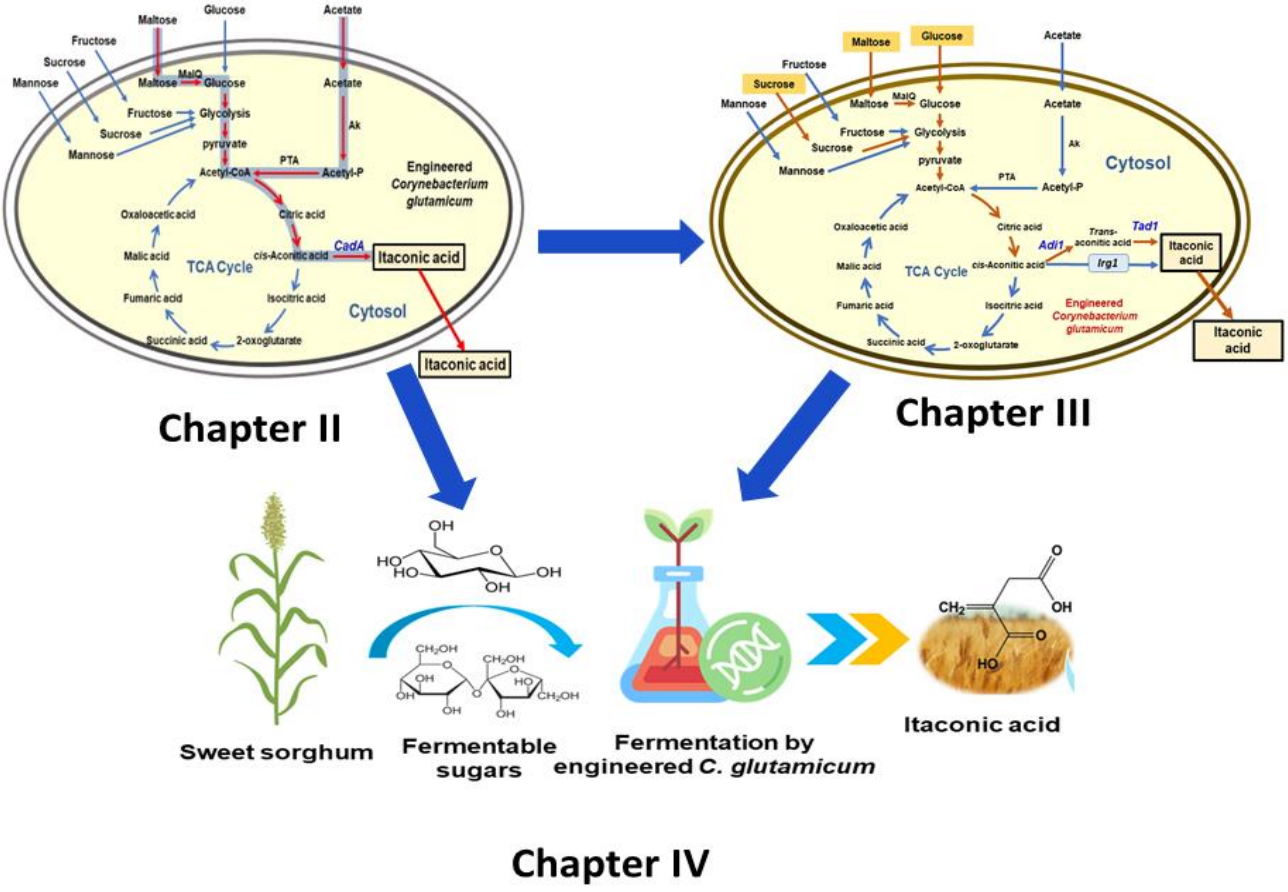


Fig. 3. Production of itaconic acid using three engineered *C. glutamicum* strains

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CHAPTER II

Co-utilization of maltose and sodium acetate via engineered *Corynebacterium glutamicum* for improved itaconic acid production

II.1. Introduction

A wide range of carbon and energy sources can be utilized by many microorganisms, and they adapt their metabolism and enzyme systems to the presence of a given carbon source or mixture of substrates. This adaptive process depends mainly on the substrate or on the repression of the genes of catabolism (1). Many organisms predominantly uptake only one substrate, and begin to utilize other available substrate(s) after the initial carbon source is completely consumed (2). This phenomenon is known as either biphasic growth or diauxic growth where the preferred substrate generally assists in a better growth rate. Optimal growth is a result of repression of the expression genes that are responsible for the enzymatic catabolism of a subordinate substrate as a result of the availability of the catabolites that result from the preferred carbon source, and this is referred to as carbon catabolite repression (1, 3, 4). Otherwise, a number of microorganisms simultaneously utilize more than one carbon source without possessing any catabolite repression in the case of the availability of diverse substrates (5, 6). *Corynebacterium glutamicum* is a gram-positive, non-sporulating and non-endotoxin bacteria that is considered a safe host (7), which was initially recognized

for its ability to produce many amino acids, and it is now widely used in the production of organic acids, diamines or alcohols (8). *C. glutamicum* also has the ability to harness various carbon sources to support the best growth and supply energy from sugars such as glucose, sucrose, fructose, mannose, maltose, and ribose.

Additionally, *C. glutamicum* has shown the ability to grow on either one carbon source or on a mixture of substrates (9). Microorganisms like *Escherichia coli* and *Bacillus subtilis* display diauxic or biphasic growth. The growth of *C. glutamicum* on substrate mixtures, however, demonstrates the ability to co-metabolize many carbon sources and display monophasic growth as shown in the mixtures of glucose and lactate, glucose and pyruvate, glucose and sodium acetate, and glucose and propionate (10, 11, 12, 13, 14, 15). Furthermore, the impact of maltose on sodium acetate and glucose consumption has been investigated for its role in enhancing the production of L-valine via *C. glutamicum* (9).

Itaconic acid (IA) is regarded as a promising bio-based product that could be used in polymer production as an alternative to acrylic and methacrylic fibers. Furthermore, it can replace many chemical intermediates such as styrene, 2-methyl-1,4-butanediol, and 3-methyl tetrahydrofuran (16, 17, 18).

However, many Microorganisms are involved in the production of IA, the highest concentration of IA (160 g/L) reached by using the natural producer filamentous fungus *Aspergillus terreus*, and now 400 t of IA are produced every year using glucose (19). There

are many obstacles in the production of IA by *A. terreus*. These include the high cost of production, sensitivity to shear stress, and changes in the oxygen supply where production can be stopped for many hours even if oxygen levels are depleted for a short duration (20). Moreover, *A. terreus* is considered a human pathogen that belongs to risk group 2 (21).

Consequently, recently many studies have focused on other possible microorganisms other than *A. terreus* for IA production. These alternatives have included fungi such as *U. maydis* (22, 23, 24) and bacteria such as *E. coli* (25, 26, 27) and *C. glutamicum* (28, 29). Thus far, *E. coli* has achieved the best IA titer among bacteria at final IA titer of 46.9 g/L using a temperature-controlled production strain (26). The highest IA concentration reached by *C. glutamicum* was 29.2 g/L by using sodium acetate as a sole carbon source via pH-controlled fed-batch fermentation in 42 L bioreactor in a process that combined pH and DO-coupled feeding of acetate (28).

In this study, the effect of different carbon sources on IA production in *C. glutamicum* was investigated. A mixture of substrates was also used to evaluate their effect on cell growth and IA production via *C. glutamicum*. The heterologous expression of a codon-optimized, *cis*-aconitate decarboxylase gene from *A. terreus* in the wild-type strain *C. glutamicum* ATCC 13032 resulted in a low IA production titer by using numerous carbon sources such as glucose, fructose, sucrose, maltose and mannose. Sodium acetate, however, was the carbon source that led to the highest IA production titer, while maltose led to optimal

cell growth. Higher concentrations of sodium acetate, however, led to a decrease in cell growth and a longer lag phase. Consequently, the combination of sodium acetate and maltose led to improved cell growth and IA production by the engineered strain *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$. The utilization of maltose and sodium acetate as the main carbon sources, resulted in an increase in IA production to a final titer of 12.63 g/L IA. This reflected the possibility of enhancing the cell growth and IA production in *C. glutamicum* by using mixture of carbon sources.

II.2. Materials and Methods:

II.2.1. Recombinant DNA work

The synthesis of all oligonucleotides used in this study was accomplished by Life Technologies Japan Ltd. Polymerase chain reaction (PCR) was carried out by following the standard protocols (30) with KOD one Start polymerase (Toyobo, Osaka, Japan). All the restriction enzymes used in the cloning were obtained from New England Biolabs (Hitchin, UK). Ligation was conducted using a Ligation high Kit (Toyobo) according to the manufacturer's instructions. Plasmid DNA was isolated using a DNA extraction kit (Cosmo Genetech, Seoul, Korea). Transformation of *C. glutamicum* was performed by electroporation as previously described (31) . The constructed plasmids were checked by colony PCR, then were confirmed via DNA sequencing using the 3500 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). DNA sequence data were then analyzed using the Genetyx program

(Software Development, Tokyo, Japan).

II.2.2. Construction of Bacterial strains and Plasmids

In this study, all bacterial strains and plasmids used or constructed are summarized in Table 1. BHIS medium, which is a mixture of 37 g/L of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI, USA) and 91 g/L sorbitol, was used for *C. glutamicum*, and Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was used for *E. coli*. The cultivation of *E. coli* strains was performed routinely by growing at 37 ºC for 16 h in LB medium containing 15 g/L agar and supplemented with kanamycin (50 µg/mL). *C. glutamicum* strains were cultured on 37 g/L BHI media with 15 g/L agar, supplemented with kanamycin (25 µg/mL), and incubated at 30 ºC for 24-48 h.

To construct the expression plasmids pCH-cad_{opt}, pCH-$cad_{opt}acnB_{opt}$, and pCH-$acnB_{opt}cad_{opt}$, the *cadA* gene from *A. terreus* with a codon-optimized sequence for *C. glutamicum,* and the fusion proteins $cadA_{opt}acnB_{opt}$ and $acnB_{opt}cadA_{opt}$ with the *cadA* gene from *A. terreus* fused with the aconitase gene (*acnB*) from *C. glutamicum* by $(GGGGS)_3$ linker protein were chemically synthesized by GeneScript Biotech (NJ, USA) and delivered as plasmids pMA-$cadA_{opt}$, pMK-$acnB_{opt}cadA_{opt}$, and pMK-$cadA_{opt}acnB_{opt}$. The pCH shuttle vector (*C. glutamicum-E. coli* shuttle vector, which is kanamycin resistant and contains a high expression constitutive promoter)(32) was used for expression of $cadA_{opt}$ *gene,* and the fusion proteins; $cadA_{opt}acnB_{opt}$ and $acnB_{opt}cadA_{opt}$ in the wild-type strain *C. glutamicum*

ATCC 13032. The codon-optimized *cadA* gene was amplified by p1-fw (5'-CTCTGGATCCATGACCAAGCAGTCCGCAGA-3') and p2-rv (5'-CTCTCTGCAGTTACACCAGTGGGGACTTCA-3') oligonucleotides using the plasmid pMA-$cadA_{opt}$ as a template. The PCR product was cut with BamHI and PstI, and cloned into the pCH shuttle vector, which was digested with the same restriction enzymes and yielding pCH-$cadA_{opt}$. In a similar way, in order to construct the expression plasmids pCH-$cad_{opt}acnB_{opt}$ and pCH-$acnB_{opt}cad_{opt}$, the codon-optimized fusion genes *cadAacnB* were amplified using p1-fw(5'-CTCTGGATCCATGACCAAGCAGTCCGCAGA-3') and p3-rv (5'-AAAACTGCAGTTACTTGGAGGAGGCTGCCAT-3') oligonucleotides, while the *acnBcadA* fusion genes were amplified with p4-fw (5'-AAAAGGATCCATGGAACTGACCGTGACCGAA-3') and p5-rv (5'-CTCTCTGCAGTTACACGAGTGGGGACTTCA-3'). Then, the resultant PCR products were digested with BamHI and PstI and cloned into pCH vector cut with the same enzymes resulting in pCH-$cadA_{opt}acnB_{opt}$ and pCH-$acnB_{opt}cadA_{opt}$ plasmids.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relative characteristics	Resource/ reference
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1,* Δ (*lac-proAB*)/*F'*[*tra*D36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Takara Bio
E. coli NovaBlue	*endA1, hsdR17* (*rK12*$^-$*mK12*$^+$), *supE44, thi-1 gyrA96*, *relA1, lacrecA1*/*F'*[*proAB*$^+$ *lacI*^{q} *Z1*M15 Tn*10* TetR]*;* used for gene cloning	Novagen
C. glutamicum ATCC 13032	Wild-type, biotin-auxotrophic	ATCC
C. glutamicum ATCC 21799	Wild-type, aminoethylcysteine-resistant, L-leucine auxotroph, lysine-producing strain	ATCC
C. glutamicum ATCC 13287	Wild-type, homoserine auxotrophic	ATCC
C. glutamicum ATCC 21253	Wild-type, homoserine and leucine auxotrophic	ATCC
C. glutamicum ATCC 13032 / pCH-*cadA*$_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon optimized *cadA* gene of *A. terreus*	This study
C. glutamicum ATCC 13032 pCH-*cadA*$_{opt}$*acnB*$_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA*	This study

	gene of *A. terreus* fused to the synthetic codon-optimized *acnB* gene of *C. glutamicum*	
C. glutamicum ATCC 13032 pCH-*acnB*$_{opt}$*cadA*$_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *acnB* of *C. glutamicum* fused to the synthetic codon optimized *cadA* gene of *A. terreus*	This study
C. glutamicum ATCC 21799 / pCH-*cadA*$_{opt}$	ATCC 21799 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA* gene of *A. terreus*	This study
C. glutamicum ATCC 21253 / pCH-*cadA*$_{opt}$	ATCC 21253 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA* gene of *A. terreus*	This study
C. glutamicum ATCC 13287 / pCH-*cadA*$_{opt}$	ATCC 13287 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA* gene of *A. terreus*	This study
pMA-*cadA*$_{opt}$	*AmpR*; *ColE1* origin, containing the synthetic *cadA* gene from *A. terreus*, codon-optimized for *C. glutamicum*	This study
pMK-*acnB*$_{opt}$*cadA*$_{opt}$	*KanR*; *ColE1* origin, containing the synthetic *cadA*	This study

	gene of *A. terreus* fused to the synthetic *acnB* gene of *C. glutamicum*, codon-optimized for *C. glutamicum*	
pMK-$cadA_{opt}acnB_{opt}$	*KanR*; *ColE1* origin, containing the synthetic *cadA* gene of *A. terreus* fused to the *acnB* gene of *C. glutamicum*, codon-optimized for *C. glutamicum*	This study
pCH	*KanR; E. coli-Corynebacterium* sp. shuttle vector	[1]
pCH-$cadA_{opt}$	*KanR*; containing the synthetic codon-optimized *cadA* gene of *A. terreus*	This study
pCH-$cadA_{opt}acnB_{opt}$	*KanR*; containing the synthetic codon-optimized *cadA* gene of *A. terreus* fused to the synthetic codon-optimized *acnB* gene of *C. glutamicum*	This study
pCH-$acnB_{opt}cadA_{opt}$	*KanR*; containing the synthetic codon-optimized *cadA* gene of *A. terreus* fused to the synthetic codon-optimized *acnB* of *C. glutamicum*	This study

II.2.3. Cultural media and conditions

The bacterial strain was preserved as 25% (v/v) glycerol stocks at - 80 ºC. For all IA production experiments, BHI was mainly used for the first preculture where 2 mL of BHI

liquid media with 4% (w/v) glucose was inoculated with a single colony of the desired *C. glutamicum* strain from a freshly streaked agar plate and incubated on a rotary shaker at 180 rpm for 8 h at 30 ºC. For both the second preculture and the main culture, modified CGXII (mCGXII) medium (1g/L urea, 1g/L KH_2PO_4, 1g/L K_2HPO_4, 42 g/L 3-morpholinopropanesulfonic acid (MOPS), 0.25 g/L $MgSO_4$.$7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4.7H_2O$, 0.1 mg/L $MnSO_4$ H_2O, 1 mg/L $ZnSO_4.7H_2O$, 0.2 mg/L $CuSO_4$ $5H_2O$, 20 mg/L $NiCl_2$. $6H_2O$, 0.2 mg/L biotin) with 4% (w/v) glucose as the carbon and energy source was supplemented with a limited amount of nitrogen as described previously (29) was used. The cells of the first preculture were used to inoculate 10 mL of the second preculture in a 50 mL test tube to a final optical density at 600 nm (OD_{600}) of approximately 0.2. After approximately 14 h of incubation at 30 ºC and 180 rpm the cells were then used to inoculate the main culture of 20 ml mCGXII in a100 ml baffled flask to an OD_{600} of approximately 0.2. Additionally, for investigation of different carbon sources for the production of IA, the same mCGXII media was used. Instead of glucose, however, different carbon sources (fructose, sucrose, maltose, mannose and sodium acetate) were tested with a final concentration of 4% (w/v). Moreover, mCGXII was also used with different concentrations of sodium acetate (50, 60, 70, 80, 90 and 100 g/L) as an alternative to glucose as the sole carbon source for the production of IA. Similarly, different concentrations of sodium acetate were combined with different concentrations of sugars like glucose, fructose, sucrose, and maltose, and were

tested as a replacement for glucose as the main carbon sources.

II.2.4. Experimental design

Response surface methodology (RSM) based on central composite design (CCD) was used for optimization of the concentration of the IA through the production process from *C. glutamicum* using two carbon sources as variables (maltose and acetate). A full factorial design with five levels for each variable, five center points and four-star points that resulted in a total of 13 runs was used to optimize the factors for IA production in a shake flask culture. Table 2 elucidates the investigated variables levels. Two coded variables were studied: high (coded value: +1.4142) and low (coded value: −1.4142). The real concentrations of variables were assessed with five levels for each: maltose concentrations (55.85 to 84.14 g/L) and sodium acetate concentrations (57.62 to 72.07).

Table 2. Coded and actual levels of IA production parameters.

Parameters	Unit	Levels				
		-α (-1.414)	-1	0	+1	+α (+1.414)
Maltose	g/L	55.85	60	70	80	84.14
Sodium acetate	g/L	57.62	60	65	70	72.07

II.2.5. Flux balance analysis

A genome-scale metabolic model of *C. glutamicum*, iCW773, was used in this study (33). To reveal the IA-biomass solution space, the maximum and minimum fluxes of IA production were calculated, using an objective function as the maximization or minimization of IA production, with each fixed growth rate from zero to the maximum value (34). All calculations were performed using MATLAB R2021a with COBRA Toolbox v3.0 (35, 36) and the opensource GLPK software (http://glpkmex.sourceforg e.net/), which is an application that solves linear programming. In this simulation, uptake rates of glucose, maltose, and sodium acetate were set to 0.3 mmol g^{-1} h^{-1}, 0.15 mmol g^{-1} h^{-1}, and 0.9 mmol g^{-1} h^{-1}, respectively, to ensure the same amount of carbon. In case of combined carbon sources (maltose with sodium acetate), uptake rates were set to 0.075 mmol g^{-1} h^{-1} for maltose and 0.45 mmol g^{-1} h^{-1} for sodium acetate, to ensure the same amount of carbon.

II.2.6. Statistical analysis

Each data is indicated by the average value with standard deviation as an error bar,

which were calculated from three biological replicate experiments. Data differences were compared using the paired Student's *t*-test with independent triplicated data. A *p*-value of < 0.05 was considered to be statistically significant. CCD design and Analysis of variance (ANOVA) were conducted using the Design Expert 13.0 statistical package (Stat-Ease, Inc., Minneapolis, MN, USA).

II.2.7. Analysis of organic acids and carbon sources.

High-performance liquid chromatography (HPLC) was used to quantify organic acids in the supernatants of the culture. Cultured broth samples were collected and centrifuged (15,000 rpm for 10 min at 4 °C). The concentration of IA in the resultant supernatant was measured via HPLC using a diode array detector SPD-20AV at UV210 nm (Shimadzu, Kyoto, Japan) equipped with a column (InertSustain 5 um, 150 mm x 4.6 mm internal diameter, GL Science, Tokyo, Japan). A mobile phase buffer of 50 mM NaH_2PO_4 with a pH of 2.1 and flow rate of 1.0 mL/min was used, and the temperature of the column oven was adjusted to 40 °C. The concentration of sodium acetate in the culture supernatant was also analyzed and measured using an HPLC fitted with a refractive index detector (RID-10A; Shimadzu). An ICSepICE-COREGEL-87H column (300 mm x 7.8 mm of internal diameter, Transgenomic Inc., New Haven, CT, USA) was used, and 5 mM H_2SO_4 buffer was used for elution at a flow rate of 0.6 mL/min at 80 ºC for 40 min. To evaluate the concentrations of sugars such as glucose, fructose, sucrose, mannose and maltose, a Shim-

Pack SPR-Pb column (250 mm x 7.8 mm of internal diameter, Shimadzu) was used at a flow rate of 0.6 mL/min, with MilliQ serving as the mobile phase at 80 ºC for 40 min for each sample.

II.3. Results and Discussion

II.3.1. IA production by engineered *C. glutamicum* strain

C. glutamicum is widely used to produce a variety of amino acids in the industrial sector. Recently, it has also been used to produce a variety of organic acids (37). In our experiment, we investigated the ability of the engineered *C. glutamicum* strain to produce IA. There are only two enzymes involved in the IA production pathway (38). The first key enzyme is aconitase (*acnB*), which is found in the tricarboxylic acid (TCA) cycle for the conversion of citric acid to *cis*-aconitic acid, then *cis*-aconitic acid is decarboxylated to IA by the *cadA* gene. In current study these two genes which are involved in IA production pathway were fused to enhance IA production. Three sets of genes were expressed by wild-type *C. glutamicum* ATCC 13032 using the expression vector pCH (with HCE-high constitutive promoter). These genes are *cadA* derived from *A. terreus* with a codon-optimized sequence, and two fused sets of genes, *cadAacnB* and *acnBcadA*, with codon optimized sequences giving the three plasmids pCH-$cadA_{opt}$, pCH-$cadA_{opt}acnB_{opt}$, and pCH-$acnB_{opt}cadA_{opt}$. These plasmids were transformed to wild-type *C. glutamicum* ATCC13032 giving the three strains of *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$, *C. glutamicum* ATCC 13032 pCH-

cadA$_{opt}$*acnB*$_{opt}$, and *C. glutamicum* ATCC 13032 pCH-*acnB*$_{opt}$*cadA*$_{opt}$.

The expression of the codon-optimized *cadA* and the two sets of fused genes, *cadAacnB* and *acnBcadA*, in the wild-type *C. glutamicum* ATCC 13032 led to IA production in the media with final titers of 0.242, 0.028, and 0.073 g/L, respectively (Fig. 1A). The glucose was completely consumed by all three constructed strains, and cell growth was the same for all of them **(**Figs. 1B and 1C). Consequently, it is obvious that the recombinant strain *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ was better than the two other constucted strains with the fused genes in the production of IA with a final titer of 0.242 g/L IA (Fig. 1A), and the fusion between the *cadA* gene from *A. terreus* and the *acnB* gene from *C. glutamicum* and their expression in *C. glutamicum* did not increase the concentration of IA in the media, while the expression of *cadA* gene only resulted in a better final concentration. Therefore, we suggest that the fusion strategy was not an appropriate strategy for expression of both genes and enhancement of IA production in *C. glutamicum*. Similarly, in a previous study using *E. coli* for the production of IA, fused *acnB* and *cadA* could not be expressed in soluble form. That result showed that the fusion strategy was not suitable for expressing the two fused enzymes in *E. coli*, which resulted in low levels of IA (0.117 g/L) after 48 h of fermentation (39). The self-assembly of *acnB* and *cadA*, however, improved the IA titer to 0.22 g/L, which was still low and not significant. In a similar manner, our results showed that the fusion between the two genes *acnB* and *cadA* was not suitable for the production of IA from *C.*

glutamicum giving low IA concentrations similar to that obtained by *E. coli*.

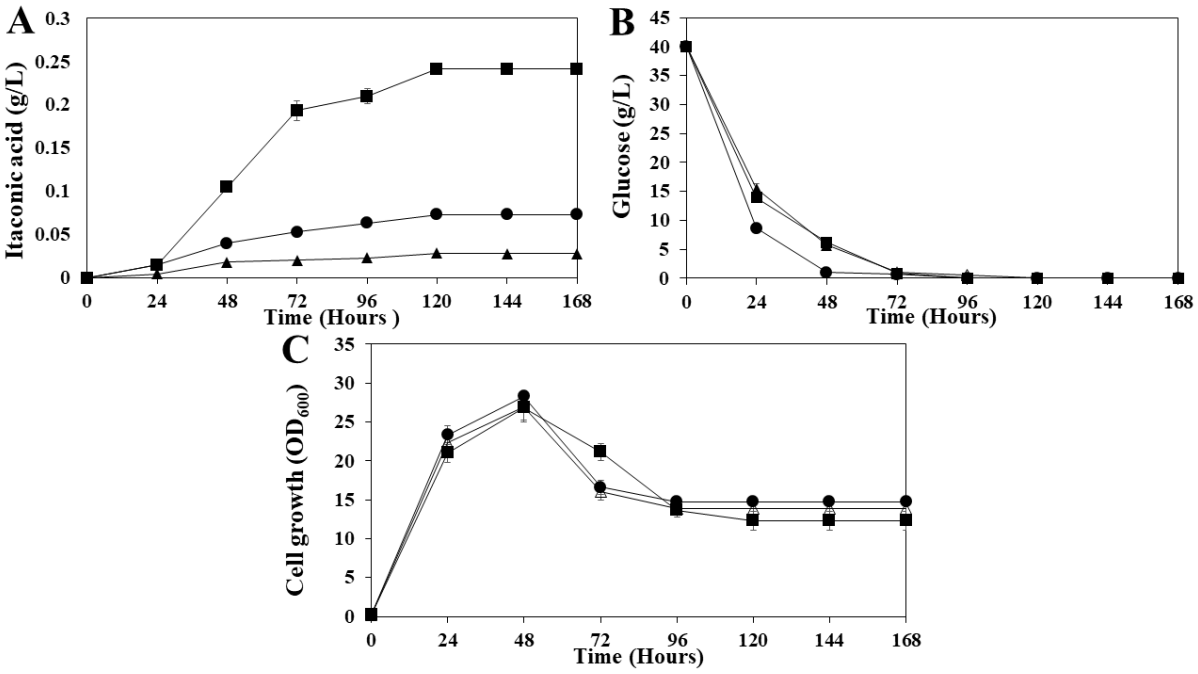

Fig. 1. Effect of expression combination on IA production. IA production (A), glucose consumption (B) and cell growth (OD_{600}) (C) of strains *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ (closed squares), *C. glutamicum* ATCC 13032 pCH-$acnB_{opt}cadA_{opt}$ (closed circles), and *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}acnB_{opt}$ (closed triangles), which were inoculated at initial OD_{600} of 0.2 in mCGXII media containing 40 g/L glucose and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

II.3.2. Comparison of different strains of *C. glutamicum* for production of IA

The present study is the first to evaluate the use of different strains of *C. glutamicum* to express the *cadA* gene for IA production. The plasmid pCH-$cadA_{opt}$ was chosen for

transformation to an additional three strains of *C. glutamicum*: ATCC 21799, ATCC 21253, and ATCC 13287 to examine the expression capability of *cadA* gene in other *C. glutamicum* wild-strains. This resulted in the following three strains: *C. glutamicum* ATCC 21799 pCH-$cadA_{opt}$, *C. glutamicum* ATCC 21253 pCH-$cadA_{opt}$, and *C. glutamicum* ATCC 13287 pCH-$cadA_{opt}$. These three strains were evaluated for production of IA from glucose under nitrogen-limited conditions. The results revealed that best strain for IA production was *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ with 0.242 g/L IA followed by *C. glutamicum* ATCC 21799 pCH-$cadA_{opt}$ with 0.15 g/L IA, *C. glutamicum* ATCC 21253 pCH-$cadA_{opt}$ with 0.045 g/L IA, and *C. glutamicum* ATCC 13287 pCH-$cadA_{opt}$ with 0.02 g/L (Fig. 2A**)**.

Additionally, the cell growth of *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ proved to be better than the other strains **(**Fig. 2C). Additionally, the glucose was completely consumed only by *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$. With the other strains, 3.53, 4.60, and 3.60 g/L glucose was not consumed by *C. glutamicum* ATCC 21799 pCH-$cadA_{opt}$, *C. glutamicum* ATCC 21253 pCH-$cadA_{opt}$, and *C. glutamicum* ATCC 13287 pCH-$cadA_{opt}$, respectively (Fig. 2B). Therefore, the wild-type *C. glutamicum* ATCC 13032 of *C. glutamicum* gave the highest IA production titer, and cell growth with complete glucose consumption. Using other strains resulted in lower IA production titer, which could be due to low *cadA* gene expression, slow growth and incomplete consumption of glucose. For this reason, *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ was chosen for further investigation.

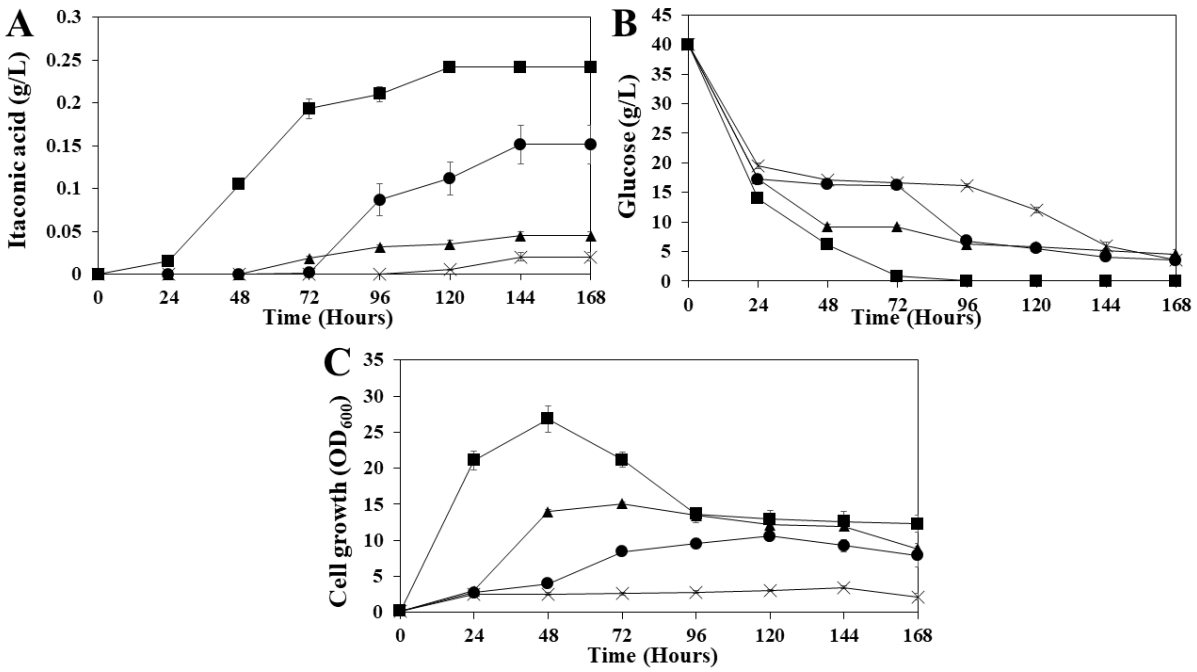

Fig. 2. Effect of a host strain of *C. glutamicum* for IA production. IA production (A), glucose consumption (B), and cell growth (OD_{600}) (C) were indicated. Strains used were as follows: *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ (closed squares), *C. glutamicum* ATCC 21799 pCH-$cadA_{opt}$ (closed circles), *C. glutamicum* ATCC 21253 pCH-$cadA_{opt}$ (closed triangles), and *C. glutamicum* ATCC 13287 pCH-$cadA_{opt}$ (multiplication symbols). These were tested for production of IA by cultivation at initial OD_{600} of 0.2 in mCGXII media containing 40 g/L glucose and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

II.3.3. Evaluation of different carbon sources for production of IA in *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$

There are various carbon sources that *C. glutamicum* uses for growth and energy

production including; monosaccharides like glucose, fructose, and mannose; disaccharides like sucrose and maltose; and, organic acids like acetic acid (8). In previous studies, IA was produced from metabolically engineered *C. glutamicum* ATCC 13032 with a final titer of 7.8 g/L from glucose (29) and 29.2 g/L from sodium acetate (28). Compared with previous studies, our study is the first to compare between different carbon sources for the production of IA from *C. glutamicum*. Six carbon sources (glucose, fructose, sucrose, maltose, mannose, and sodium acetate) were evaluated for production of IA from the selected strain *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ under nitrogen-limited conditions to improve the substrate availability for IA production. The results revealed that the best carbon source for IA production was sodium acetate with a final IA titer of 0.62 g/L, and the lowest carbon source was mannose with a final IA titer of 0.033 g/L **(**Fig. 3A). The IA titers from maltose, glucose, fructose, and sucrose were 0.293, 0.242, 0.223, and 0.13 g/L, respectively **(**Fig. 3A). It was found that sodium acetate, glucose, and maltose were completely consumed in the media, while fructose, sucrose, and mannose were not (Fig. 3B). Moreover, the best cell growth was reached when using maltose as a carbon source while the lowest was with mannose followed by sodium acetate (Fig. 3C).

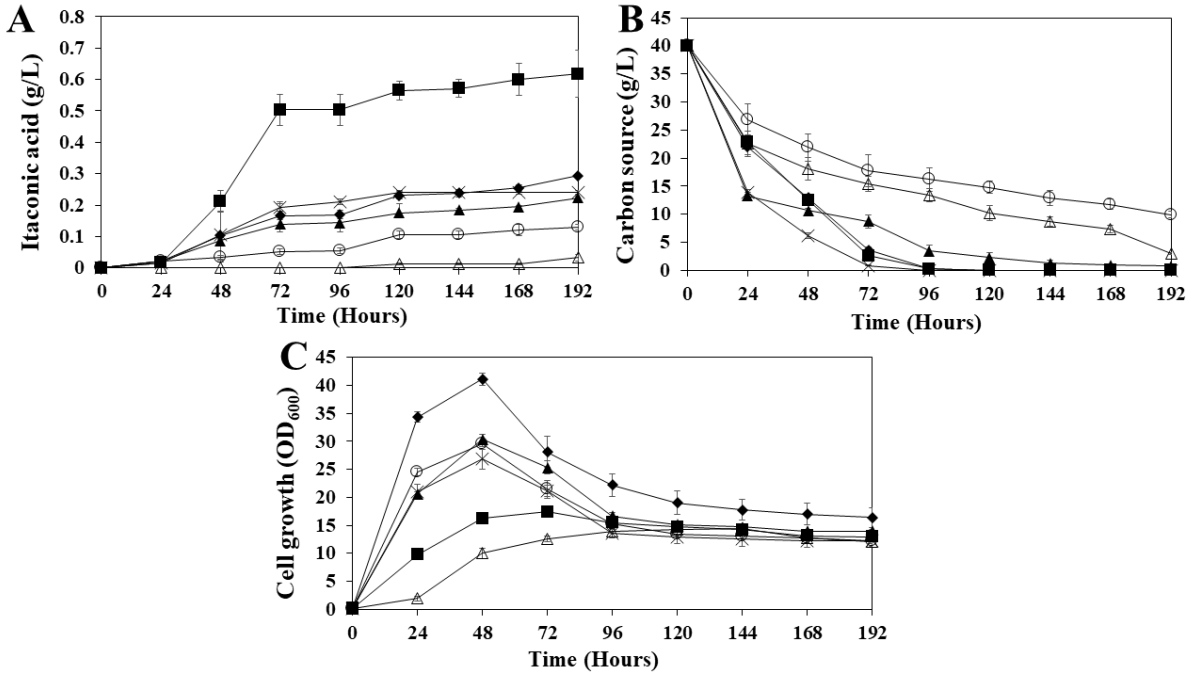

Fig. 3. IA production by *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ from different carbon sources. IA production (A), carbon source consumption (B), and cell growth (OD_{600}) (C) are indicated. *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different carbon sources including glucose (multiplication symbols), fructose (closed triangles), sucrose (open circles), maltose (closed diamonds), mannose (open triangles), and sodium acetate (closed squares) to obtain a final carbon source concentration of 40 g/L, under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

II.3.4. Usage of different sodium acetate concentrations for IA production

In a previous study, sodium acetate with different concentrations of 1, 5, 10, 15, 20, 25, and 30 g/L were used to determine its effect on bacterial growth. *C. glutamicum* was effective in growing on all concentrations with the full consumption of sodium acetate (40),

and has progressively shown that it was capable of growing on sodium acetate as the main carbon source at substrate concentration as high as 60 g/L.

In the current study, six different concentrations of sodium acetate (50, 60, 70, 80, 90, and 100 g/L) were investigated for IA production in *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ under nitrogen-limited conditions, resulting in final IA titer of 1.81, 2.79, 3.55, 2.74, 2.69, and 1.45 g/L using 50, 60, 70 ,80, 90, and 100 g/L of sodium acetate, respectively. By increasing sodium acetate concentration in the media, the final concentration of IA was improved **(**Fig. 4A), and the highest IA titer reached was 3.55 g/L using 70 g/L sodium acetate. However, the IA concentration began to decrease at concentration of sodium acetate higher than 70 g/L, cell growth was also decreased with the increase in the sodium acetate concentration in the media (Fig. 4C), showing that increasing the concentration of sodium acetate inhibited cell growth. In addition, *C. glutamicum* couldn't consume all the sodium acetate that was present in the media after 60 g/L (Fig. 4B). Consequently, evaluation of different concentrations of sodium acetate showed that increasing the sodium acetate concentration could increase the production of IA in the medium. Despite the increase in the final IA titer, increasing the sodium acetate in the medium to a concentration higher than 70 g/L inhibited cell growth (Fig. 4C), and the sodium acetate was not completely consumed in the medium (Fig. 4B), which resulted in decrease in the final IA titer (Fig. 4A). Similarly, in previous study, it has been shown that concentrations of sodium acetate in the media higher

than 50 g/L, however, did not lead to an obvious rise in biomass, although the nitrogen that exists in the medium should help in increasing the cell density (41). Also, it was found that there was an obvious expanding in the lag phase by increasing the concentration of sodium acetate to more than 10 g/L. This effect could have been a result of the specific inhibitory effects of sodium acetate such as an uncoupling of the trans-membrane pH gradient (42), and that could possibly influence the growth behavior once the crucial concentration is reached.

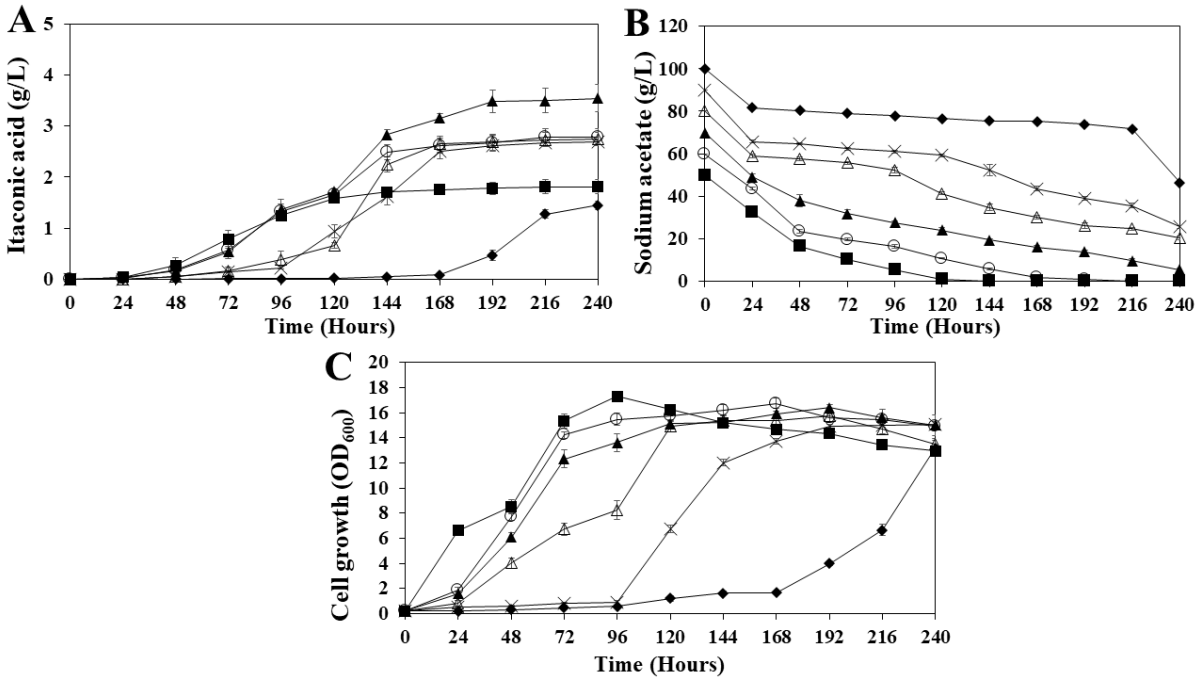

Fig. 4. Dose effect of sodium acetate on IA production. IA production (A), sodium acetate consumption (B) and cell growth (OD_{600}) (C) were indicated. *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media with different concentrations of sodium acetate: 50 g/L (closed squares), 60 g/L (open circles), 70 g/L (closed triangles), 80 g/L (open triangles), 90 g/L (multiplication symbols) and 100 g/L (closed diamonds) as the main carbon source under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

II.3.5. Combination between sodium acetate and different sugars for improvement of IA production

Most microorganisms can use more than one substrate for their growth and for the production of different metabolites and adapt their metabolism to the presence of certain substrates (1). Previous studies have shown that *C. glutamicum* has the ability to simultaneously use more than one carbon source (10, 12). Therefore, depending on this concept, mixtures of sodium acetate and other sugars were evaluated for enhancing cell growth and IA production in *C. glutamicum*.

In this study, it was found that sodium acetate is the best carbon source for the production of IA with a final IA titer of 0.62 g/L (Fig. 3A), however, sodium acetate resulted in a low cell growth compared to other sugars like glucose, fructose, sucrose and maltose (Fig. 3C). Consequently, the combination of sugars like glucose, fructose, sucrose and maltose with sodium acetate was evaluated with different three mixture concentrations: (20+20, 20+40 and 40+40) g/L respectively of glucose, fructose, sucrose and maltose with sodium acetate. The results showed that maltose-sodium acetate mixture was the best combination for production of IA with final IA titer of 4.96 g/L and a molar yield 0.2 mol/mol from a mixture of (40+40) g/L of maltose and sodium acetate (Fig. 5D), followed by glucose and fructose mixtures with sodium acetate with final IA titer of 3.43 g/L with a molar yield of 0.097 mol/mol from a mixture of (40+40) g/L of glucose and sodium acetate (Fig. 5A), and 2.17 g/L with a molar yield of 0.055 mol/mol from a mixture of (40+40) g/L of fructose and sodium acetate (Fig. 5B), while the lowest IA titer was from sucrose-sodium acetate mixture

with final IA titer of 0.76 g/L and a molar yield of 0.032 mol/mol from a mixture of (40+40) g/L of sucrose and sodium acetate (Fig. 5C) . Furthermore, both maltose and sodium acetate were completely consumed in case of all concentrations (Fig. 6). In the case of other sugars combinations with sodium acetate, the sodium acetate and fructose were completely consumed (Fig. 7). In case of combination between glucose or sucrose with sodium acetate, only by using (20+20) g/L combination, both sodium acetate and glucose or sucrose were consumed. In case of higher concentrations, the sodium acetate was consumed faster and both glucose and sucrose were not totally consumed (Figs. 8 and 9). However, the differences in consumption of carbon sources in different mixtures, all sugars combinations with sodium acetate were co-utilized by *C. glutamicum* and supported monophasic growth.

Additionally, the cell growth in the case of maltose-sodium acetate mixture was shown to be the highest compared to other combinations (Fig. 10D). It is also better than cell growth in case of using sodium acetate only as a main carbon source, even after increasing sodium acetate concentration in the medium (Figs. 3C and 3C). Moreover, in the case of other sugars combinations with sodium acetate, the cell growth was not high compared to using sodium acetate only as a sole carbon source (Figs. 10A, 10B and 10C). Consequently, it was found that maltose-sodium acetate mixture resulted in the highest final IA titer and cell growth with complete consumption of both maltose and sodium acetate.

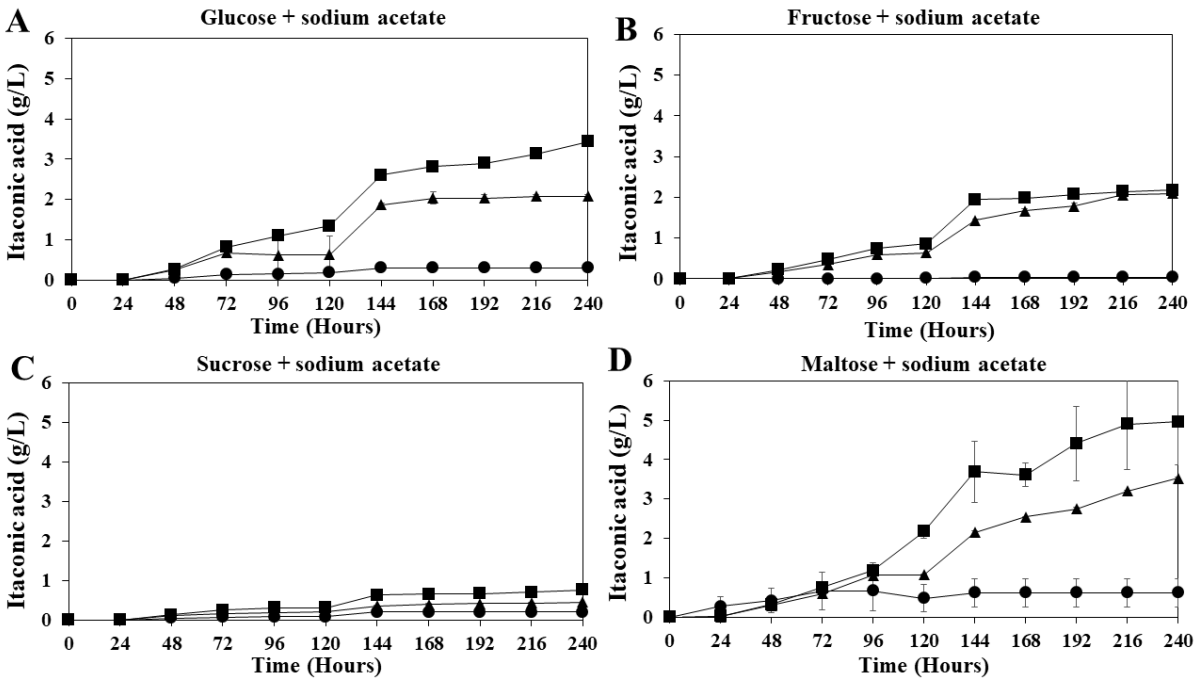

Fig. 5. The effect of combinations of sodium acetate and different sugars on IA production in *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain. IA production was indicated where *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media containing glucose and sodium acetate (A), fructose and sodium acetate (B), sucrose and sodium acetate (C), and maltose and sodium acetate (D) as main carbon sources and under nitrogen-limited conditions. Different combinations of glucose, fructose, sucrose and maltose with sodium acetate: 20+20 g/L (closed circles), 20+40 g/L (closed triangles), and 40+40 g/L (closed squares), respectively, were checked. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

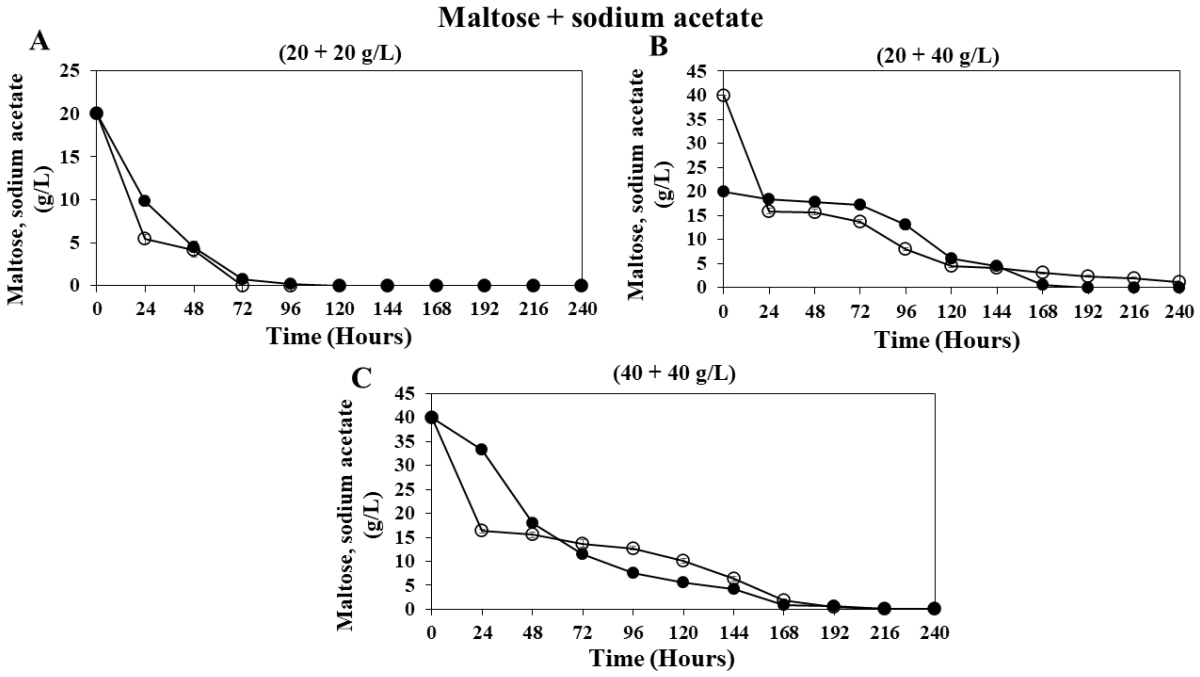

Fig. 6. Time course consumption of combined maltose and sodium acetate by *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$. Maltose (closed circles) and sodium acetate (open circles) consumption were indicated. *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different concentrations of maltose and sodium acetate: 20+20 g/L (A), 20+40 g/L (B) and 40+40 g/L (C), respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

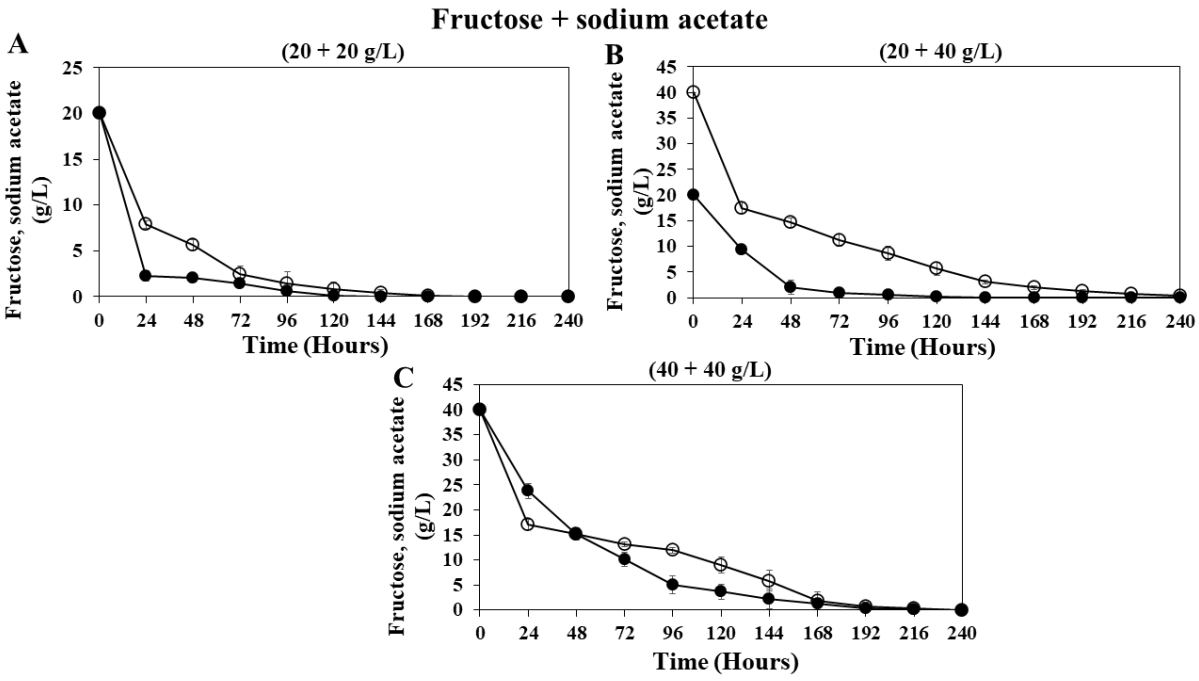

Fig. 7. Time course consumption of combined fructose and sodium acetate by *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$. Fructose (closed circles) and sodium acetate (open circles) consumption were indicated. *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different concentrations of fructose and sodium acetate: 20+20 g/L (A), 20+40 g/L (B) and 40+40 g/L (C), respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

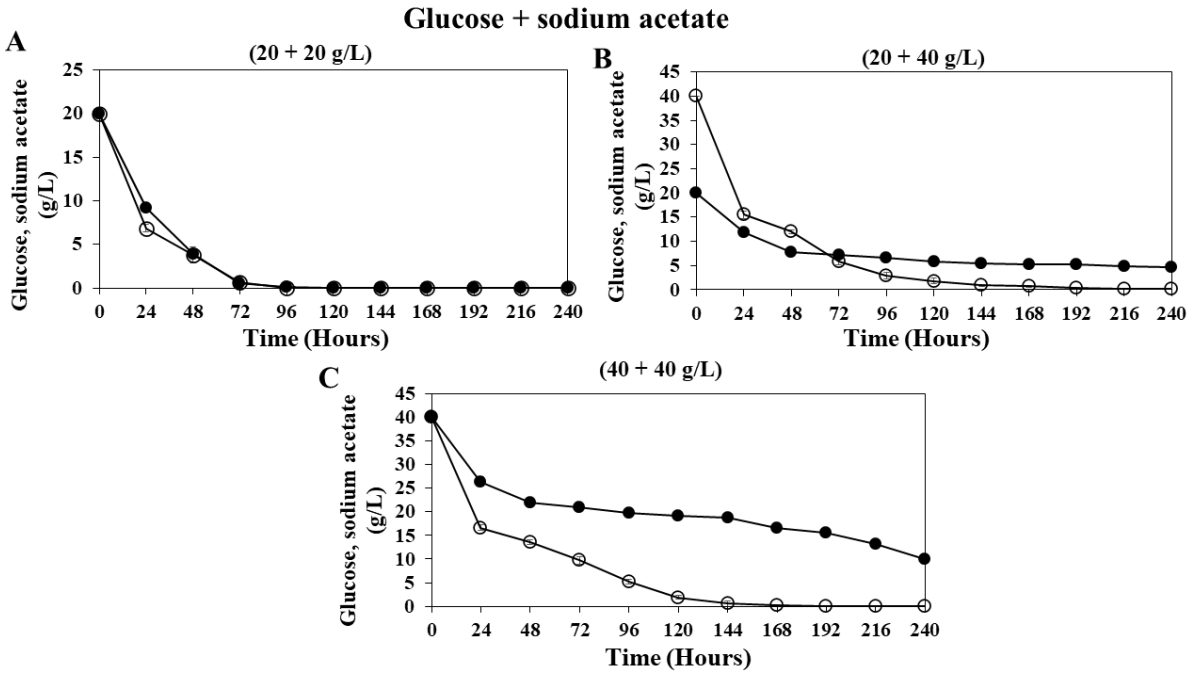

Fig. 8. Time course consumption of combined glucose and sodium acetate by *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$. Glucose (closed circles) and sodium acetate (open circles) consumption were indicated. *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different concentrations of glucose and sodium acetate: 20+20 g/L (A), 20+40 g/L (B) and 40+40 g/L (C), respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

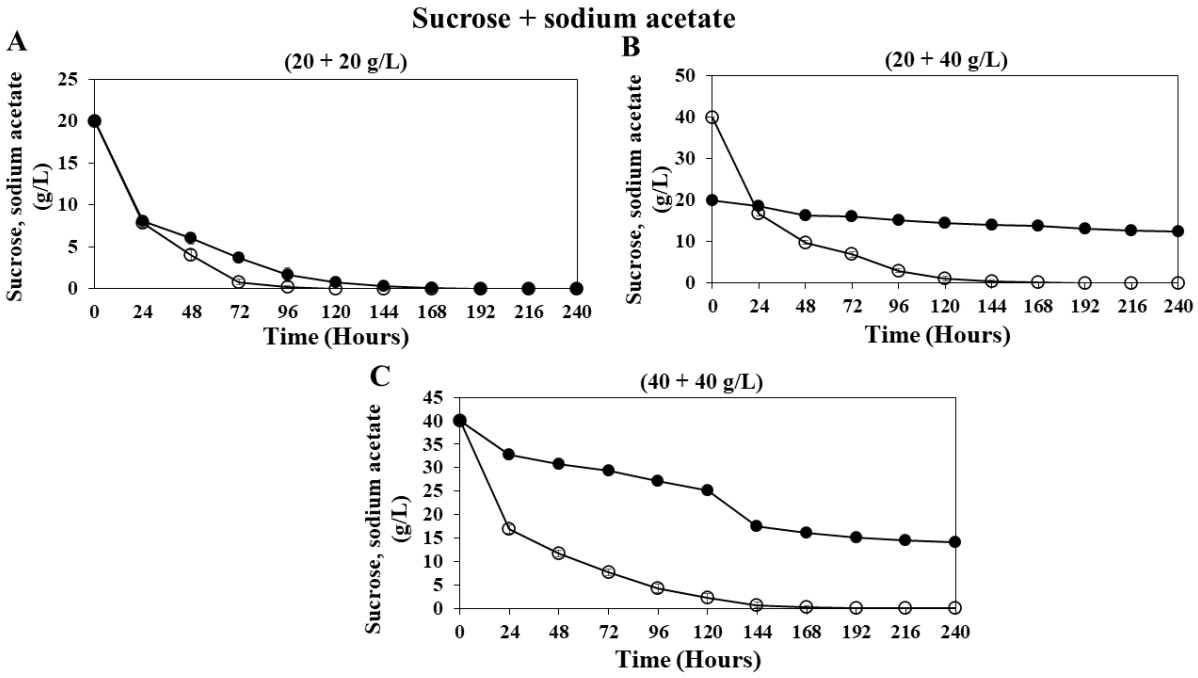

Fig. 9. Time course consumption of combined sucrose and sodium acetate by *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$. Sucrose (closed circles) and sodium acetate (open circles) consumption were indicated. *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different concentrations of sucrose and sodium acetate: 20+20 g/L (A), 20+40 g/L (B) and 40+40 g/L (C), respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

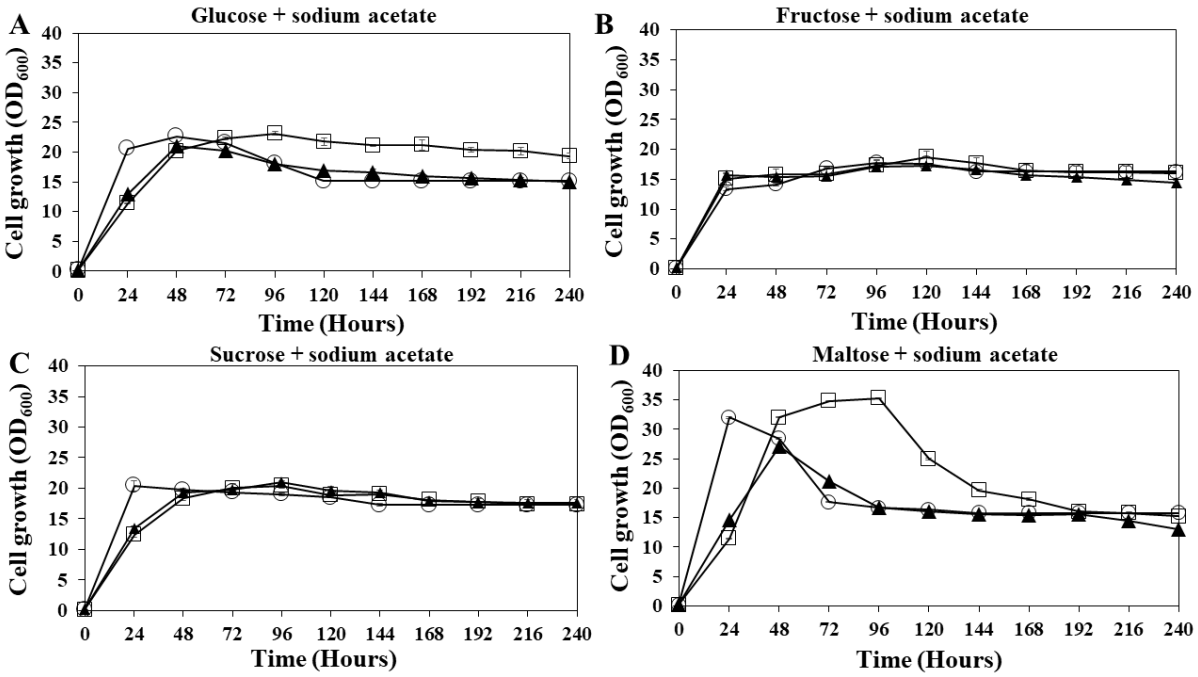

Fig. 10. The effect of combinations of sodium acetate and different sugars on cell growth by *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain. Cell growth (OD_{600}) was indicated, where *C. glutamicum* ATCC 13032 pCH-*cadA*$_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media containing glucose and sodium acetate (A), fructose and sodium acetate (B), sucrose and sodium acetate (C) and maltose and sodium acetate (D) as main carbon sources and under nitrogen-limited conditions. Different combinations of glucose, fructose, sucrose and maltose with sodium acetate: 20+20 g/L (open circles), 20+40 g/L (closed triangles), and 40+40 g/L (open squares), respectively, were checked. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

II.3.6. CCD experiment for the production of IA using a combination of maltose and sodium acetate.

In this study, the highest IA production titer was achieved by utilizing sodium acetate as a carbon source by *C. glutamicum* (Fig. 3A) while maltose recorded the highest cell growth (Fig. 3C). Also, the combination between maltose and sodium acetate resulted in the highest IA titer and the highest cell growth (Figs. 5 and 10). As a consequence of these results, RSM (Fig. 11) using CCD was used to study the effect that combining maltose and sodium acetate with different concentrations (Tables 2 and 3) would exert on IA production in *C. glutamicum*. A quadratic model was applied using Design Expert 13 software according to the coded values of the quadratic equation:

$$Itaconic\ acid = +9.03 - 2.22A + 0.3676B - 0.09AB - 0.2737A^2 - 1.94B^2$$

Furthermore, a Fisher test (F-test) was applied for evaluation of the statistical importance of the extracted model, where the analysis of variance (ANOVA) was calculated by dividing the mean squares by the model residual mean square (Table 4). The ANOVA results emphasized the possibility of applying the model. The resultant F-value for IA (17.92), also the achieved probability (*P*-value = 0.0007) with a low value, which confirmed the model significance. For IA, the A and B^2 terms were found to be significant while the B, AB and A^2 terms did not have a significant effect in the design space of the model. Consequently, only the terms A and B^2 were kept.

A lack-of-fit test was applied to compare the residual error with the pure error. The *p*-value for the lack-of-fit test showed a value greater than 0.05, as shown in (Table 4), which

reflects the insignificance of the lack-of-fit and reflected the model acceptance. Calculation of the IA equation for adequate precision or signal-to-noise value gave a value of 12.4506, which is higher than 4 and indicates the accuracy and significance of the model. In addition, the agreement between the predicted values and actual values was confirmed by using the predicted R^2 value.

Table 3. Values of variables (maltose and sodium acetate) in central composite design (real and coded values), and actual and predicted response (IA).

Run no.	Levels		Actual levels (g/L)		Response IA (g/L)	
	A	B	A: Maltose	B: Sodium acetate	Actual	Predicted
1	0	-1.414	70	57.62	4.74	4.62
2	0	0	70	65	9.08	9.03
3	1	1	80	70	4.34	4.87
4	1.414	0	84.14	65	5.44	5.35
5	1	-1	80	60	4.4	4.32
6	-1	-1	60	60	8	8.57
7	0	1.414	70	72.07	6.65	5.66
8	-1.414	0	55.85	65	12.63	11.62
9	0	0	70	65	9.73	9.03
10	-1	1	60	70	8.3	9.49
11	0	0	70	65	8.76	9.03
12	0	0	70	65	9.28	9.03

13	0	0	70	65	8.3	9.03

Table 4. ANOVA analysis on the IA (g/L) production.

Source	Sum of Squares	DF (degree of freedom)	Mean Square	*F*-value	*p*-value
Model	66.69	5	13.34	17.92	0.0007
Residual	5.21	7	0.7443		
Lack of fit	4.05	3	1.35	4.65	0.0858
Pure error	1.16	4	0.2902		
Total correlation	71.9	12			

R^2=0.9275, adjusted R^2 =0.8758, Predicted R^2=0.8758, Adequate Precision=12.4506

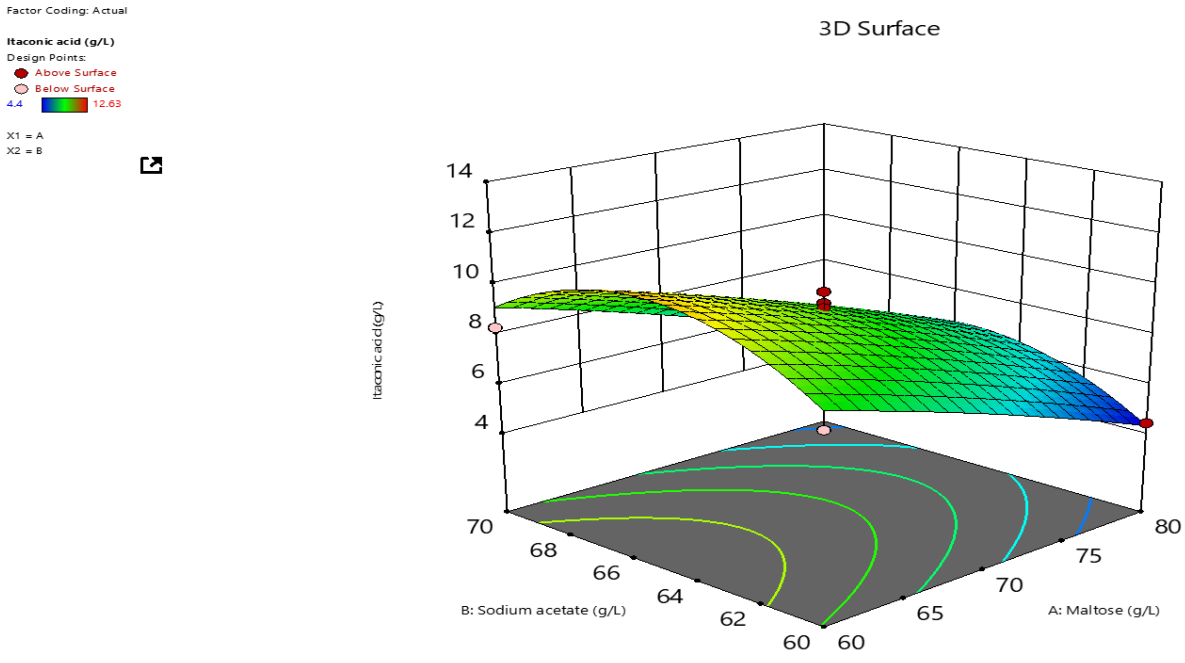

Fig. 11. Response surface of two factors maltose (A) and sodium acetate (B) on IA production by *C. glutamicum*.

II.3.7. Optimization of IA production parameters

Different combinations of maltose and acetate were examined to reach optimum concentrations. When combined, concentrations of 55.85 and 65 g/L of maltose and sodium acetate, respectively, resulted in the highest IA titer of 12.63 g/L (Fig. 12). Additionally, these results confirmed the nature of the monophasic growth of *C. glutamicum* where both acetate and maltose were simultaneously consumed, and cell growth was enhanced. Also, the reduction in the lag phase period is another improvement over using sodium acetate only that was characterized by a longer lag phase, particularly when using higher concentrations (Fig. 4C). Therefore**,** using CCD for optimization of IA production using maltose and sodium acetate as main carbon sources showed an enhancement of cell growth and IA production giving final IA titer of 12.63 g/L (Fig. 12) with a molar yield of 0.38 mol/mol, where *C. glutamicum* was able to simultaneously consume both carbon sources. Similarly, the effect of maltose on glucose and sodium acetate usage by *C. glutamicum* has also been investigated previously for improvement of L-valine productivity (9) where maltose was co-metabolized with both glucose and sodium acetate by *C. glutamicum*. Moreover, an enhancement in the productivity of L-valine has been investigated by co-metabolizing of acetate and maltose by *C. glutamicum*. Adding maltose also during the growth on a combination of glucose and sodium acetate has resulted in effective glucose consumption, as well as in improvement in L-

valine productivity. The results of these previous studies agree with our results.

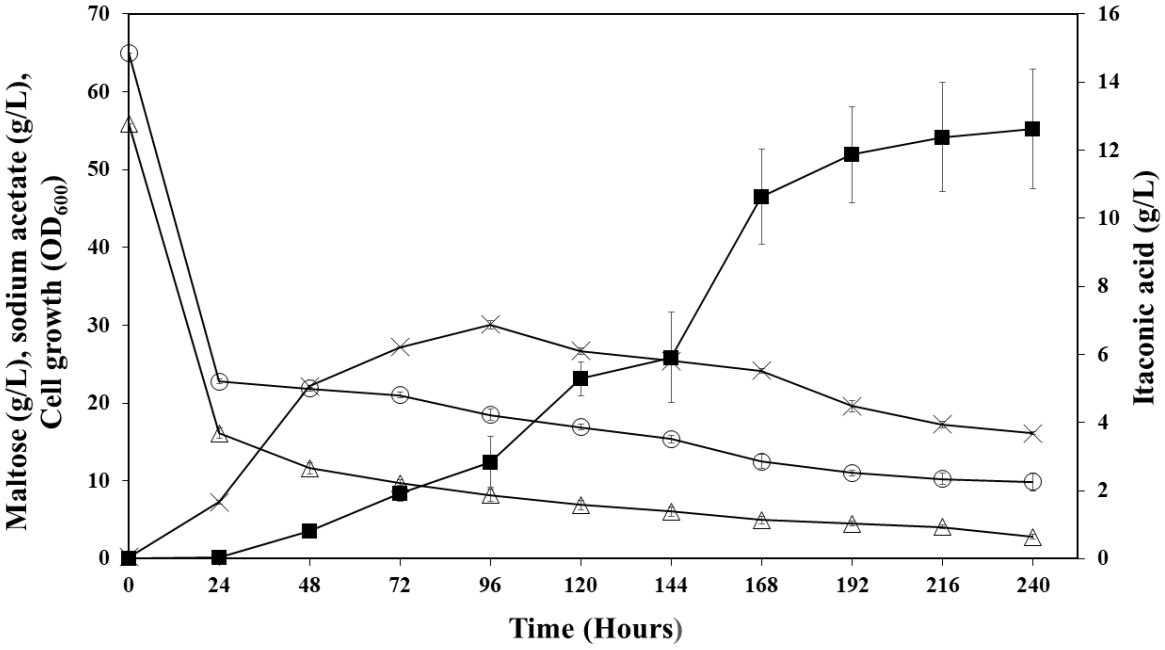

Fig. 12. Time course profile of IA production by the optimized mixture of maltose and sodium acetate. IA production (closed squares), maltose consumption (open triangles), sodium acetate consumption (open circles) and cell growth (OD_{600}) (multiplication symbols) were charted for the strain *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$. *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$ strain was inoculated at an initial OD_{600} of 0.2 in mCGXII media containing maltose and sodium acetate with final concentrations of 55.85 and 65 g/L, respectively, and under nitrogen-limited conditions. The data comprised average values, and standard deviations were calculated from three biological replicate experiments.

II.3.8. Flux balance analysis

The flux balance analysis (FBA) was calculated to reveal the IA-biomass solution

space, and the maximum and minimum fluxes of IA production were calculated. To equalize the number of carbon, uptake rates of glucose, maltose, and sodium acetate were set to 0.3 mmol g^{-1} h^{-1}, 0.15 mmol g^{-1} h^{-1}, and 0.9 mmol g^{-1} h^{-1}, respectively. In case of maltose with sodium acetate, uptake rates were set to 0.075 mmol g^{-1} h^{-1} for maltose and 0.45 mmol g^{-1} h^{-1} for sodium acetate, and O_2 uptake was set to 0.8 mmol g^{-1} h^{-1} which is a bit more anaerobic than the optimal value. FBA results also revealed that the biomass and IA production in the case of co-feeding of maltose and sodium acetate is better than sodium acetate (Figs. 13C and 13D). Consequently, FBA also supported our results including biomass and IA production, and it proved that the IA production could be better when using sodium acetate and maltose as a combined carbon sources than when maltose and sodium acetate were separately used. The tendency of cell growth also agreed with the results by FBA (Figs. 3 and 13).

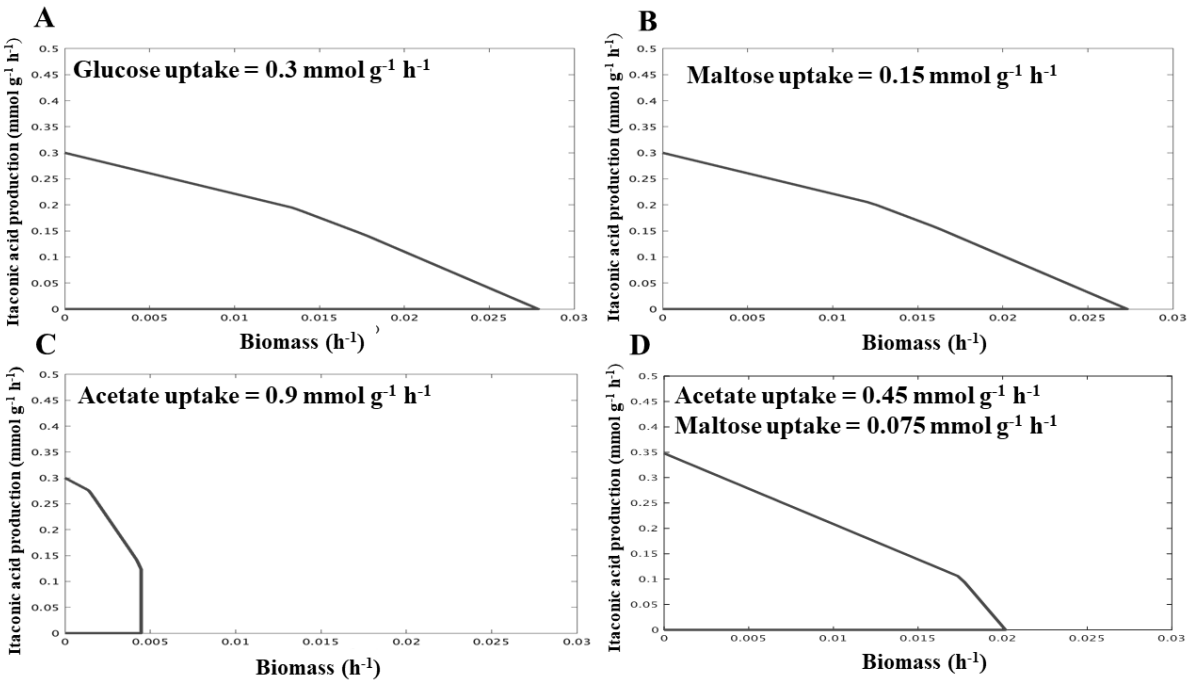

Fig. 13. Flux balance Analysis. (A): IA production in case of using glucose as a carbon source where glucose uptake was set to 0.3 mmol g^{-1} h^{-1}. (B): IA production in case of using maltose as a carbon source where glucose uptake was set to 0.15 mmol g^{-1} h^{-1}. (C): IA production in case of using sodium acetate as a carbon source where sodium acetate uptake was set to 0.9 mmol g^{-1} h^{-1}. (D): IA production in case of co-utilizing of maltose and sodium acetate as main carbon sources where maltose uptake was set to 0.075 mmol g^{-1} h^{-1} and acetate uptake was set to 0.45 mmol g^{-1} h^{-1}.

II.4. Conclusions

In this study, using maltose with sodium acetate as a combined carbon source mixture for the production of IA in *C. glutamicum* enhanced the cell growth, and decreased the lag phase giving a final IA titer of 12.63 g/L. This is the first study that has evaluated the

effect of combined substrate mixtures on the production of IA, and the results support the possibility of a simultaneous utilization of different carbon sources for IA production by *C. glutamicum*.

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CHAPTER III

Effect of different metabolic pathways on itaconic acid production in engineered *Corynebacterium glutamicum*

III.1. Introduction

Itaconic acid (IA) is an unsaturated dicarboxylic acid and a bio-derived potential platform chemical that is among the twelve most promising bio-based products and has gained considerable interest in recent years as its characteristics permit its usage in valuable applications (1, 2). There are several uses for it, including as a bio-plastics precursor (3), particularly when used as a bio-based replacement for acrylic acid, resins or coatings (4, 5). Furthermore, it can be used in the textile industry (6) and in medicine as either a drug carriers or confers antimicrobial properties (7). At the current selling price of 1,500-1,700 USD/ton, IA is already competing to replace fossil polyacrylic acid in the production of superabsorbent polymers (8).

There are many microorganisms that produce IA naturally, including *Aspergillus terreus* and *Ustilago maydis* (Fig. 1). IA production using *A. terreus* involves the transfer of metabolites between mitochondria and cytosol where IA is naturally produced during the Krebs cycle (TCA cycle) where citric acid is formed first by the aid of a citrate synthase enzyme and then is converted to *cis*-aconitate by aconitase. These first steps occur in the mitochondria. Next, mitochondrial *cis*-aconitic acid transporter protein (Mtta), which is a

part of gene cluster including also *cis*-aconitase decarboxylase (*cadA*) gene, which is the key gene in IA production, relocates *cis*-aconitic acid from the mitochondria to the cytosol where the decarboxylation of *cis*-aconitate to IA occurs by *cadA* (9).

IA also can be produced by *U. maydis*, which doesn't possess the *cadA* gene. In this process, another pathway (*trans*-pathway) is adopted, which includes two sets of enzymes; the first enzyme is aconitase-delta-isomerase (*Adi1*), which catalyzes the isomerization of *cis*-aconitate into the thermodynamically favorable isomer *trans*-aconitate, and the second enzyme is *trans*-aconitate decarboxylase (*Tad1*), which catalyzes the decarboxylation of *trans*-aconitate to IA, respectively. Both enzymes are localized in the cytosol, therefore, mitochondrial tricarboxylate transporter (Mtt1) translocate *cis*-aconitate from the mitochondria to the cytosol and itaconate transporter protein (Itp1) translocate IA from cytosol to extracellular space (10, 11).

Moreover, mammalian macrophages are known to possess the ability to produce IA during inflammation as an immune defense mechanism, which is linked to the *Irg1* gene from *Mus musculus*. *In vitro*, the mammalian immunoresponsive gene1 (*Irg1*) exhibits *cis*-aconitate decarboxylase activity, even though it possesses only 24% an amino acid sequence similarity with *A. terreus cadA* gene (12, 13).

At present, approximately 40,000 t of IA is produced every year via *A. terreus* and the process mainly uses glucose as a substrate. Although high titer of IA (more than 160 g/L)

could be produced using this microorganism (14), there are drawbacks that could result in increased production costs. These drawbacks involve sensitivity to shear stress and variations in the oxygen supply where the reduction of oxygen level for even limited-time, could stop the production for many hours (15). Moreover, controlling fungal morphology is difficult during the production process (16) due to the high sensitivity to the impurities that result from hydrolysis of biomass, and these impurities must be removed from the medium (17). Additionally, safety precautions should be taken when dealing with *A. terreus* because it is considered a risk group 2 an opportunistic human pathogen (8).

Consequently, besides *A. terreus,* different production hosts such as the fungus *U. maydis* have been used to produce IA (18, 19, 20). Other production hosts also include *Aspergillus niger* (21, 22, 23, 24, 25, 26), and bacteria such as *Escherichia coli* (27, 28, 29), and *Corynebacterium glutamicum* (2, 30). Thus far, the highest bacterial IA titer has been reached using *E. coli* (27) by developing a temperature-controlled strain that could produce IA at a final titer of 46.9 g/L. In case of *C. glutamicum,* the highest production titer achieved was 29.2 g/L via fed-batch fermentation with acetate as a carbon source (2).

C. glutamicum is considered as workhorse in the field of biotechnology where it played an important role in the production of various bioproducts starting from glutamate and recently has been used for production of other chemicals, fuels and polymers (31). Additionally, the complete genome sequence of *C. glutamicum* is now available (32, 33)

which can allow researchers to understand more about this bacterium, easily engineer it. Also, *C. glutamicum* is considered as GRAS generally recognized as safe host and safely used by human, rapidly grow with high cell densities (34), lack recombinant repair system which makes it genetically stable (35), limited restriction modification system (36), no autolysis and can maintain its metabolic activity under growth arrested conditions (37), it exhibits limited protease activity which makes it favorable host for production of recombinant protein (38), metabolism plasticity and strong secondary metabolism characteristics (39). Furthermore, *C. glutamicum* can utilize wide range of carbon sources like pentoses and hexoses (40). Consequently, *C. glutamicum* is an interesting host for production of IA.

Evaluation of *cadA* gene (*cis*-pathway) for production of IA by *C. glutamicum* and other engineered microorganisms was well explained using glucose and other carbon sources like sodium acetate (2, 30). The usage of other pathways including *trans*-pathway for production of IA from *C. glutamicum* was not well studied, so this study concentrates on the evaluation of the possibility for production of IA using other pathways other than *cis*-pathway (*cadA* gene).

In this study, *C. glutamicum* was engineered by developing two distinct IA production pathways: *cis*-pathway that depends on *Irg1* gene from *M. musculus* and *trans*-pathway (*Adi1*/*Tad1*) from *U. maydis,* which were then used for IA production at high titers using a wide range of carbon sources. The results confirmed the potential of *C. glutamicum* for IA

production using pathways other than the common *A. terreus cis*-pathway that depends mainly on the *cadA* gene. We also found that the strain expressing *trans*-pathway (*Adi1*/*Tad1*) genes from *U. maydis* resulted in high IA titers by utilizing most carbon sources. On the other side, expressing codon optimized *Irg1* gene from *M. musculus* in *C. glutamicum* resulted in a low IA titers, which reflects the great effect of different metabolic pathways in IA production by *C. glutamicum.*

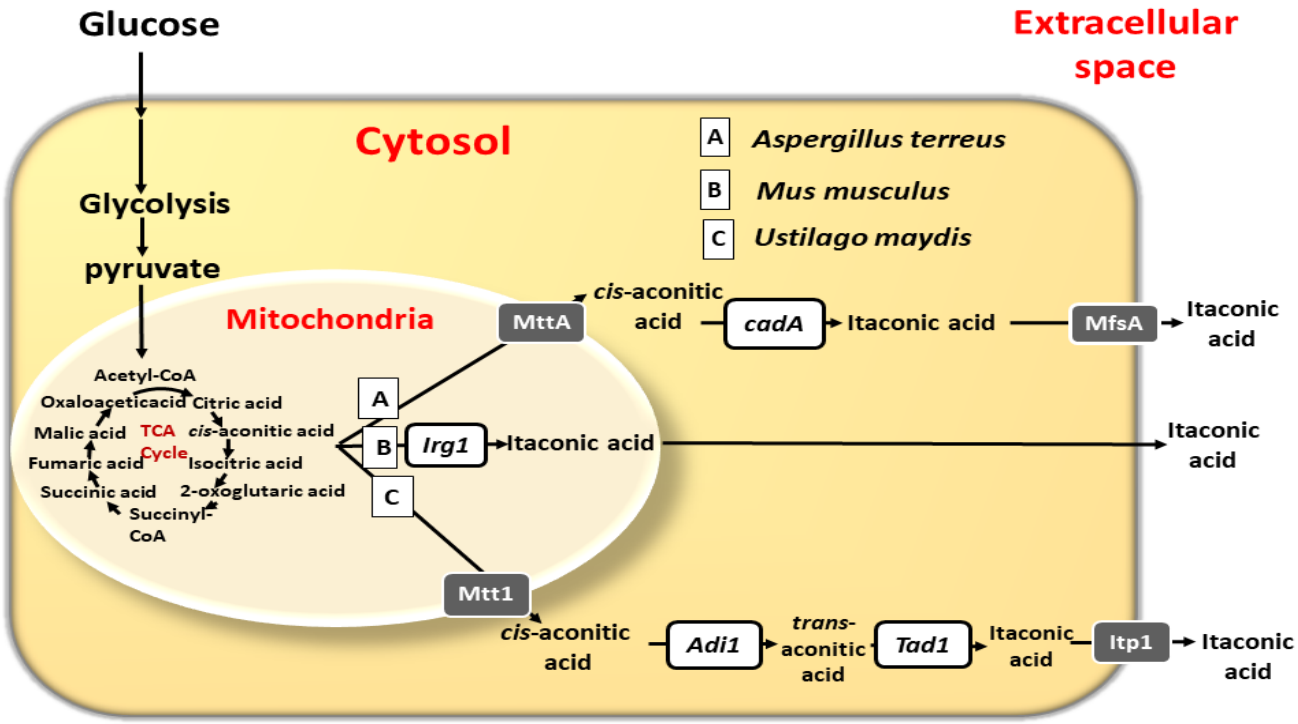

Fig. 1. Different metabolic pathways involved in itaconic acid (IA) production: A, metabolic pathway used by *A. terreus*, which involves conversion of *cis*-aconitate into IA by *cadA* gene; B, pathway used by mammalian cells where IA is formed via the action of *Irg1* gene; and, C, pathway adopted by *U. maydis*, which involves conversion of *cis*-aconitate into IA through (*Adi1*/*Tad1*) genes.

III.2. Materials and Methods

III.2.1. Recombinant DNA work

The synthesis of all oligonucleotides used in this study were accomplished by Life Technologies Japan, Ltd. PCR was carried out by following the standard protocols (41) with KOD One start polymerase (Toyobo Co., Osaka, Japan). All the restriction enzymes used in the cloning were brought from New England Biolabs (Frankfurt am Main, Germany). Infusion was conducted via the use of the HiFi DNA assembly Master Mix from New England Biolabs (Frankfurt am Main, Germany) according to the manufacturer's instructions. Plasmid DNA was isolated using a DNA extraction kit (Cosmo Genetech, Inc.). Transformation of *C. glutamicum* was performed by electroporation as previously described (36). The constructed plasmids were checked by colony PCR, then confirmed via DNA sequencing using the 3500 Genetic Analyzer (Applied Biosystem, Foster, USA).

III.2.2. Plasmid and *Corynebacterium* strain construction

The bacterial strains and plasmids used or constructed in this study are summarized in **Table 1**. For strain construction, BHIS medium, which is a mixture of 37 g/L of brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI, USA) and 91 g/L sorbitol, was used for *C. glutamicum*, and Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) was used for *E. coli* where agar plates contained 15 g/L of agar. The cultivation of *E. coli* strains was performed routinely by growing at 37 ºC for 16 h in LB medium

containing 15 g/L agar and supplemented with kanamycin (50 µg/mL). *C. glutamicum* strains were cultured on 37 g/L BHI media with 15 g/L agar, supplemented with kanamycin (25 µg/mL), and incubated at 30 ºC for 24-48 h.

To construct the expression plasmids pCH-Irg_{opt} and pCH-$Tad1_{opt}Adi1_{opt}$, *Irg1* gene from *M. musculus* and (*Adi*/*Tad1*) genes from *U. maydis* with a codon-optimized sequence for *C. glutamicum* were chemically synthesized by ThermoFisher Scientific and delivered as plasmids pMA-T-$Irg1_{opt}$, pMK-RQ-$Adi1_{opt}$, and pMA-T-$Tad1_{opt}$. The pCH shuttle vector (*C. glutamicum-E. coli* shuttle vector, which is kanamycin resistant and contains a high expression constitutive promoter) (42) was used for expression of *Irg1* gene and *Adi1*/*Tad1* genes in the wild type strain *C. glutamicum* ATCC13032.

The codon-optimized *Irg1* gene was amplified by p1-fw (5'GGAGATCTGATGGGATCCATGGGCCTGAAGTCCGTGAC3') and p2-rv (5'CCATGGGTCGACGAATTCTTAGATGTTGGTGAAGCGTGG3') oligonucleotides using the plasmid pMK-T-$Irg1_{opt}$ as a template. The resultant PCR product was cloned into a digested pCH shuttle vector with *Bam*HI and *Eco*RI enzymes via infusion. For construction of the expression plasmids pCH-$Tad1_{opt}Adi1_{opt}$, the codon-optimized *Tad1* gene was amplified using p3-fw (5'AAAGGAGATCTGATGGGATCCATGGCACCAGCACTGAACGCAAAC 3') and p4-rv (5'AGCCCATGGGTCGACGAATTCTTATGCGGAGGATGGACGGG 3') oligonucleotides,

and the resultant PCR product was cloned into a digested pCH shuttle vector with *Bam*HI and *Eco*RI enzymes via infusion. Then, *Adi* gene was amplified with p5-fw (5'GAGATCTGATGGGATCCATGCTGCACCCAATCGATACC 3') and p6-rv (5' ATGGGTCGACGAATTCTTAGGACAGGGAGCGATCGGAG 3') and used for PCR. The PCR product was then cloned into pCH-$Tad1_{opt}$ digested with *avr*II and *Mfe*I restriction enzymes via infusion, which resulted in pCH-$Tad1_{opt}Adi1_{opt}$ plasmid.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relative Characteristics	Resource/ Reference
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1,* Δ (*lac-proAB*)/*F'*[*tra*D36 $proAB^+$ $lacI^q$ *lacZ*ΔM15]	Takara Bio
E. coli NovaBlue	*endA1, hsdR17* ($rK12^-mK12^+$), *supE44, thi-1* *gyrA96*, *relA1, lacrecA1*/*F'*[$proAB^+$ $lacI^q$ *Z1*M15 Tn*10* TetR]*;* used for gene cloning	Novagen
C. glutamicum ATCC 13032	wild-type, biotin-auxotrophic	ATCC
C. glutamicum ATCC 13032 / pCH-$Irg1_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *Irg1* gene from *M. musculus*	This study

C. glutamicum ATCC 13032 / pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *Tad1* and *Adi* genes from *U. maydis*	This study
pMA-T-*Irg1*$_{opt}$	*AmpR*; *ColE1* origin, containing the *Irg1* gene from *M. musculus* codon-optimized for *C. glutamicum*	This study
pMK-RQ-*Tad1*$_{opt}$	*KanR*; *ColE1* origin, containing the *Tad1* gene from *U. maydis* codon-optimized for *C. glutamicum*	This study
pMA-T-*Adi1*$_{opt}$	*AmpR*; *ColE1* origin, containing the *Adi1* gene from *U. maydis* codon-optimized for *C. glutamicum*	This study
pCH	*KanR; E. coli-Corynebacterium* sp. shuttle vector	(42)
pCH-*Irg1*$_{opt}$	*KanR*; *ColE1* origin, pCH with PCR fragment containing the synthetic *Irg1* gene from *M. musculus*, codon-optimized for *C. glutamicum*.	This study
pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$	*KanR*; *ColE1* origin, pCH with PCR fragment containing the synthetic *Tadi1* and *Adi1* genes of *U. maydis,* codon-optimized for *C. glutamicum*.	This study

III.2.3. Cultural media and conditions

The bacterial strain was preserved as 25% (v/v) glycerol stocks at - 80 ºC. For all IA production experiments, BHI was mainly used for the first preculture where 2 mL of BHI liquid media with 4% (w/v) glucose was inoculated with a single colony of the desired *C. glutamicum* strain from a freshly streaked agar plate and incubated on a rotary shaker at 180 rpm for 8 h at 30 ºC. For both the second preculture and the main culture, modified CGXII (mCGXII) medium (1g/L urea, 1g/L KH_2PO_4, 1g/L K_2HPO_4, 42 g/L 3-morpholinopropanesulfonic acid (MOPS), 0.25 g/L $MgSO_4.7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4.7H_2O$, 0.1 mg/L $MnSO_4.H_2O$, 1 mg/L $ZnSO_4.7H_2O$, 0.2 mg/L $CuSO_4.5H_2O$, 20 mg/L $NiCl_2.6H_2O$, 0.2 mg/L biotin) with 4% (w/v) glucose and supplemented with a limited amount of nitrogen (1 g/L urea), as described previously was used for both the second preculture and the main culture. 10 mL of the second preculture in a 50 mL test tube was inoculated at a final optical density at 600 nm (OD_{600}) of approximately 0.2. After approximately 14 h of incubation at 30 ºC and 180 rpm, the cells were used to inoculate 20 mL of the mCGXII main culture in a 100 mL baffled flask to an OD_{600} of approximately 0.2. Additionally, to investigate the influence of different carbon sources on IA production, mCGXII media supplemented with different carbon sources (glucose, fructose, sucrose, maltose, mannose and sodium acetate) at a final concentration of 4% (w/v) were used.

III.2.4. Fed-batch fermentation using different carbon sources

Fed-batch experiments was conducted in 150 mL baffled flasks with initial volume of 20 ml of mCGXII media supplemented with different carbon sources (glucose, maltose and sucrose) at an initial concentration of 3% w/v. The pH was maintained at 7, and the initial OD_{600} of the second preculture, and that of the main culture was adjusted to 1. The experiment was conducted at 30 ºC and 180 rpm where the flasks were fed 4 times every 24 h using a feeding solution which composed of basal solution (5 g/L urea, 5 g/L KH_2PO_4, 5 g/L K_2HPO_4 and 210 g/L MOPS) , 200 g/L carbon source (glucose or maltose or sucrose), and 2mg/100ml biotin. The pH of the basal solution adjusted to 8 to control the acidity of the media resulted from the acids produced during fermentation.

III.2.5. Analysis of organic acids and carbon sources

Cultured broth samples were collected and centrifuged (15,000 rpm for 10 min at 4 °C). The concentration of IA, citric acid and succinic acid in the resultant supernatant was measured via high-performance liquid chromatography (HPLC) using a diode array detector SPD-20AV at UV210 nm (Shimadzu, Kyoto, Japan) equipped with a column (InertSustain 5 um, 150 mm X 4.6 mm I. D, GL Science). A mobile phase buffer of 50 mM NaH_2PO_4 (pH=2.1) at a flow rate of 1.0 mL/ min was used, and the temperature of the column oven was adjusted to 40 °C. The concentration of acetic acid and lactic acid in the culture supernatant was also analyzed using an HPLC fitted with a refractive index detector (RID-

10A; Shimadzu, Tokyo, Japan). An ICSepICE-COREGEL-87H column (300 mm x 7.8 mm of internal diameter, Transgenomic Inc., New Haven, CT, USA) was used and 5 mM H_2SO_4 buffer was used for elution at a flow rate of 0.6 mL/min at 80 ºC for 40 min. To evaluate the concentrations of sugars such as glucose, fructose, sucrose, mannose and maltose, a Shim-Pack SPR-Pb column (250 mm x 7.8 mm of internal diameter, Shimadzu, Kyoto, Japan) was used at a flow rate of 0.6 mL/min, with MilliQ serving as the mobile phase at 80 ºC for 40 min for each sample.

III.2.6. Statistical analysis

Each result is indicated by the average value with standard deviation as an error bar, which were calculated from three biological replicate experiments. The differences in data were compared using a paired Student's *t*-test via independent triplicated data. A *p*-value of < 0.05 was considered to be statistically significant.

III.3. Results and Discussion

III.3.1. Engineering of *C. glutamicum* for IA production

Identification of *cadA* in *A. terreus* (43), prompted much interest in producing IA in recombinant hosts such as *C. glutamicum.* Besides IA natural producers, *cadA* gene from *A. terreus* was mostly expressed for IA production in other non-natural IA producers (2, 27, 28, 29, 30). Furthermore, *C. glutamicum* is well known for its utility in the industrial production of several amino acids and other value-added chemicals. Therefore, recently many studies

have focused on its ability in the production of several organic acids (44).

In previous studies, IA was produced from metabolically engineered *C. glutamicum* ATCC13032, which expressed the *cadA* gene from *A. terreus* fused with *E. coli* maltose binding protein (*malE*), with a final titer of 7.8 g/L from glucose in shaking flask (30) and 29.2 g/L from sodium acetate by fed-batch fermentation in 42 L bioreactor (2).

On the other hand, many alternative pathways for IA production have been discovered in the fungus *U. maydis* and mammalian cells *M. musculus* (8, 13, 18, 19). Such distinct pathways were expressed previously for production of IA in bacteria including *E. coli* which expressed *Irg1* gene from *M. musculus* and resulted in a final IA titer of 0.56 g/L from glucose (13). *Pseudomonas putida* also was investigated for production of IA by expressing *trans*-pathway from *U. maydis* resulting in IA production with a final titer of 1.3 g/L and 1.4 g/L from *p*-coumarate and monomeric aromatic compounds produced from alkali-treated lignin, respectively (45).

Therefore, we also tried to develop such distinct pathways for improvement of IA production in engineered *C. glutamicum*. The *Irg1* gene, which was derived from *M. musculus* with a codon-optimized sequence, and the *trans*-pathway genes (*Adi1*/*Tad1*) derived from *U. maydis* strain, were expressed into wild type *C. glutamicum* ATCC13032 using the expression vector pCH (with HCE-high constitutive promoter) giving the two strains of *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$ and *C. glutamicum* ATCC13032 pCH-

Tad1$_{opt}$*adi1*$_{opt}$, which were investigated for IA production.

Our results are the first to confirm the possibility of expression of different genes such as *Irg1* from *M. musculus*, and *trans*-pathway (*Adi1*/*Tad1*) from *U. maydis* for the production of IA in *C. glutamicum*.

III.3.2. Evaluation of different carbon sources for production of IA using *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$.

To select the best carbon source for IA production and investigate the ability of *C. glutamicum* to produce IA from different substrates, various carbon sources were evaluated for the production of IA from the two constructed strains, *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$, and *C. glutamicum* ATCC13032 pCH-*Tad1*$_{opt}$*adi1*$_{opt}$. Most previous research used glucose mainly as a sole carbon source for production of IA. We tried to broaden the spectrum of carbon sources that can be used in IA production including, fructose which is contained in molasses and other carbohydrate raw material, sucrose which is a main constituent of various molasses, maltose which can be produced from starch liquefaction (46), mannose which can be found in residual stream of lignocellulosic process (47) and acetate which can be resulted from hydrolysis of lignocellulosic biomass (48).

We found that using different carbon sources can affect the final IA titer using both constructed strains.

In the case of *C. glutamicum* ATCC13032 pCH-*Irg1*$_{opt}$ constructed strain, six carbon

sources (glucose, fructose, sucrose, maltose, mannose and sodium acetate) were tested for production of IA under nitrogen-limiting conditions. The results revealed that the best carbon source for production of IA was sodium acetate with a final IA titer of 0.57 g/L, and the lowest carbon source was mannose with a final IA titer of 0.03 g/L, while sucrose, fructose, glucose and maltose yielded final IA titers of 0.23, 0.16, 0.15, 0.06 g/L, respectively (Fig. 2A).

It was obvious that all carbon sources were completely consumed in the media except in the cases of sucrose and mannose, which weren't totally consumed with 2.2 and 5.4 g/L remained in the media in case of sucrose and mannose, respectively (Fig.2B) similar to the case of the wild type where maltose was not completely consumed (Fig. 4A). Moreover, the results showed that the best cell growth reached was with the use of maltose as a carbon source while the lowest level of cell growth was attained using sodium acetate. Cell growth in the case of mannose increased in a slow manner (Fig. 2C). However, all the sodium acetate in the media was completely consumed, it resulted in the lowest cell growth, indicating that most of sodium acetate was used for IA production or by-products rather than for enhancement of cell growth.

Additionally, the analysis of organic acid revealed that there was more accumulation of organic acids in the cases of sodium acetate compared to other carbon sources (Table 2) especially lactic acid and succinic acid, while citric acid accumulated more in case of fructose

and maltose, respectively. However, we couldn't find a clear relation between accumulation of organic acids and usage of different carbon sources, which require further analysis in the future.

Moreover, in previous studies using *C. glutamicum* expressing the *cadA* gene, the usage of sodium acetate as a substrate was better than glucose in IA production and resulted in a final IA titer of 29.2 g/L via fed-batch fermentation (2, 30). Also, the *Irg1* gene was previously expressed in *E. coli* for production of IA from glucose, but this is the first study where the *Irg1* gene was expressed in *C. glutamicum* for the production of IA.

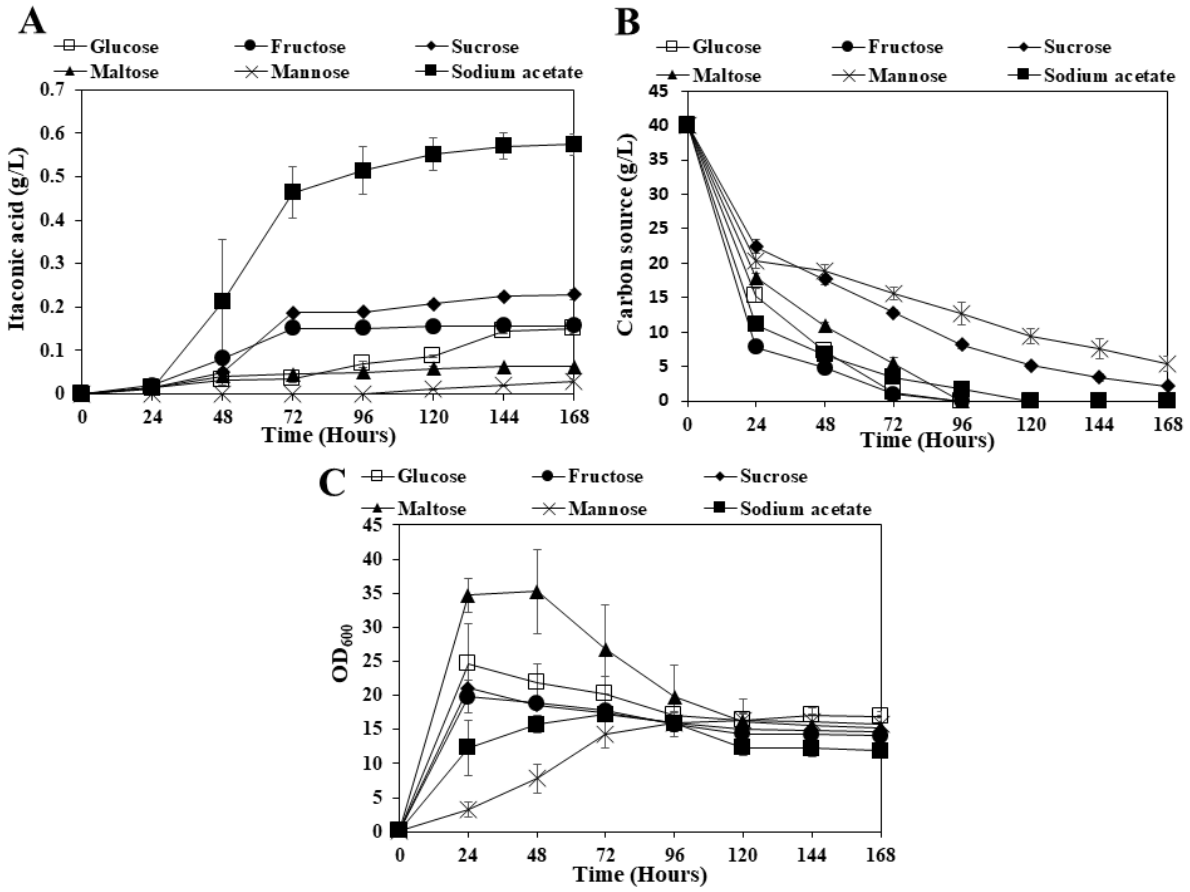

Fig. 2. Itaconic acid (IA) production by *C. glutamicum* ATCC 13032 pCH/*Irg1*$_{opt}$ strain from different carbon sources. IA production (A), carbon source consumption (B), and cell growth (OD_{600}) (C) are indicated. *C. glutamicum* ATCC 13032 pCH/*Irg1*$_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different carbon sources including glucose (open square), fructose (closed circle), sucrose (closed diamond), maltose (closed triangle), mannose (multiplication symbol), and sodium acetate (closed square) to obtain a final carbon source concentration of 40 g/L, under nitrogen-limited conditions. The data represent the average values and standard deviation calculated from three biological replicates.

Table 2. Concentration of organic acids produced by the recombinant *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$ using different carbon sources.

	Carbon source					
Product (g/L)	Glucose	Fructose	Sucrose	Maltose	Mannose	Sodium acetate
Itaconic acid	0.15±0.01	0.16±0.003	0.23±0.010	0.062±0.003	0.03±0.005	0.57±0.025
Lactic acid	0.03±0.06	0.45±0.050	0.29±0.031	0.233±0.010	0.13±0.005	0.66±0.018
Acetic acid	ND	ND	ND	ND	ND	ND
Citric acid	0.04±0.01	0.52±0.024	0.03±0.008	0.413±0.032	ND	0.27±0.019
Succinic acid	0.66±0.55	3.04±0.117	0.31±0.163	3.875±0.108	0.66±0.085	6.33±0.078
Total	0.88±0.63	4.17±0.194	0.86±0.212	4.588±0.153	0.82±0.095	7.83±0.14

[a] The recombinant strain *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$ was inoculated at an initial OD_{600} of 0.2 in mCGXII medium containing 40 g/L different carbon sources. Values were determined after 168 h of fermentation. The data represent the average values and standard deviations, which were calculated using three biological replicates.

III.3.3. Comparison of different carbon sources for production of IA using *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$

The same six carbon sources were used (glucose, fructose, sucrose, maltose,

mannose and sodium acetate) and tested for the production of IA from *C. glutamicum* ATCC13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ under nitrogen-limiting conditions. The expression of the *trans*-pathway (*Adi1*/*Tad1*) genes from *U. maydis* in *C. glutamicum* ATCC13032 strain led to a higher IA final titer of 3.73 g/L from maltose than from sucrose (3.55), glucose (3.48) and fructose (2.63) g/L (Fig. 3A). Unexpectedly, the use of sodium acetate led to a lower IA titer of 0.57 g/L followed by mannose with a final IA titer of 0.49 g/L **(**Fig. 3A). It also is obvious that all carbon sources were not completely consumed in the media with 1.7, 2.1, 4.2, 3.7, 5 and 0.44 g/L remained in the media in case of glucose, fructose, sucrose, maltose, mannose and sodium acetate, respectively (Fig. 3B). The best consumption recorded by glucose and sodium acetate. In the case of wild type, all carbon sources were completely consumed except for sucrose, maltose and mannose (Fig. 4). Furthermore, the results show that the best cell growth was reached using maltose followed by glucose as carbon sources. The lowest cell growth was attained using sodium acetate. Cell growth in the case of mannose increased slowly (Fig. 3C). It is clear that using sodium acetate as a carbon source led to the lowest IA final titer while most of sodium acetate was consumed in the media compared to other carbon sources. Consequently, we suggest that the difference in the behavior of the two constructed stains is probably related to the expression of two different pathways: *cis*-pathway (*Irg1* gene) and *trans*-pathway.

In previous study, the expression of *cadA* gene in *C. glutamicum* led to higher IA titer

by using sodium acetate as feedstock than by using glucose (2) which is similar to our study where expression of *Irg1* gene led to higher IA final titer by using sodium acetate as main carbon source than other sugars, however, by expression of *trans*-pathway, the lowest IA titer was achieved by using of sodium acetate as a feedstock. Consequently, the difference in IA production from sodium acetate is associated with expression of different genes. Also, expression of two genes (*Adi1*/*Tad1)* genes affect the production of IA from sodium acetate other than expression of one gene like *Irg1* or *cadA*. Consequently, we suggest that IA production can be affected by the used strain, the production pathway, and the carbon source used for production.

Also, however, the analysis of organic acids showed that the accumulation of organic acids was lower in sodium acetate and mannose compared to other sugars (glucose, fructose, sucrose and maltose) (Table 3), we couldn't propose that there is obvious relation between such accumulation of metabolites and using of different carbon sources and further analysis is needed in the future.

Furthermore, the only available study that investigated the production of IA by expressing *trans*-pathway (*Adi1*/*Tad1* genes) in *P. putida* and produced IA with a final titer of 1.3 g/L and 1.4 g/L from *p*-coumarate and monomeric aromatic compounds, respectively, proved that *trans*-pathway is better than *cis*-pathway (*cadA* gene) in production of IA. However, these titers much lower than our final IA titers from *C. glutamicum e*xpressing the

same *trans*-pathway which prove the ability of *C. glutamicum* to be a good strain for production of IA at high titers, that study also showed that the *trans*-pathway would improve IA production relative to the *cis*- pathway (*cadA* gene). Similarly, our study also shows that the *trans*-pathway (*Adi1*/ *Tad1*) genes is better than the *cis*-pathway (*Irg1*gene) for the production of IA in *C. glutamicum.*

Consequently, by comparing IA production between the two constructed strains, *C. glutamicum* ATCC13032 pCH-$Irg1_{opt.}$ and *C. glutamicum* ATCC13032 pCH-$Tad1_{opt}Adi1_{opt}$, it is obvious that the *C. glutamicum* strain expressing *trans*-pathway (*Adi1*/*Tad1*) genes is better at IA production with high titers of 3.73, 3.55 and 3.48 g/L from maltose, sucrose, and glucose respectively compared to 0.23, 0.15 and 0.06 g/L of IA from sucrose, glucose and maltose respectively, when using the *C. glutamicum* strain expressing the *Irg1* gene from *M. musculus*. Moreover, in our previous study that used engineered *C. glutamicum expressing cadA* gene using the same expression vector pCH (with HCE-high constitutive promoter) for production of IA from lignocellulosic biomass and glucose, it resulted in final IA titer of 0.34 g/L from glucose (49). This result confirms that the *trans*-pathway is better than *cis*-pathway (*cadA* or *Irg1* gene) for production of IA from glucose by engineered *C. glutamicum*.

Overall, the *trans*-pathway from *U. maydis* is shown to be better than *Irg1* gene from *M. musculus* which may be due to the production of thermodynamically favorable intermediate (*trans*-aconitate) as shown in previous work (45), where this *trans*-isomer

resulted via the *trans*-pathway forms 88% of aconitate at equilibrium and is a competitive inhibitor of the aconitase enzyme. Such characteristics help in accumulation of more aconitate, and therefore the flux could be increased to IA. In contrast, in case of *cis*-pathway (*cadA* gene or *Irg1* gene), *cis*-aconitate (the main intermediate in IA production) is formed by the conversion of citrate to *cis*-aconitate by aconitase enzyme, and cis-aconitate is converted to isocitrate also by aconitase enzyme which decreases the accumulation of aconitate, and therefore results in low IA production. Consequently, we propose that *trans*-pathway would enhance IA production in away better than *cis*-pathway by providing a thermodynamically favorable route to divert carbon flux from the TCA cycle.

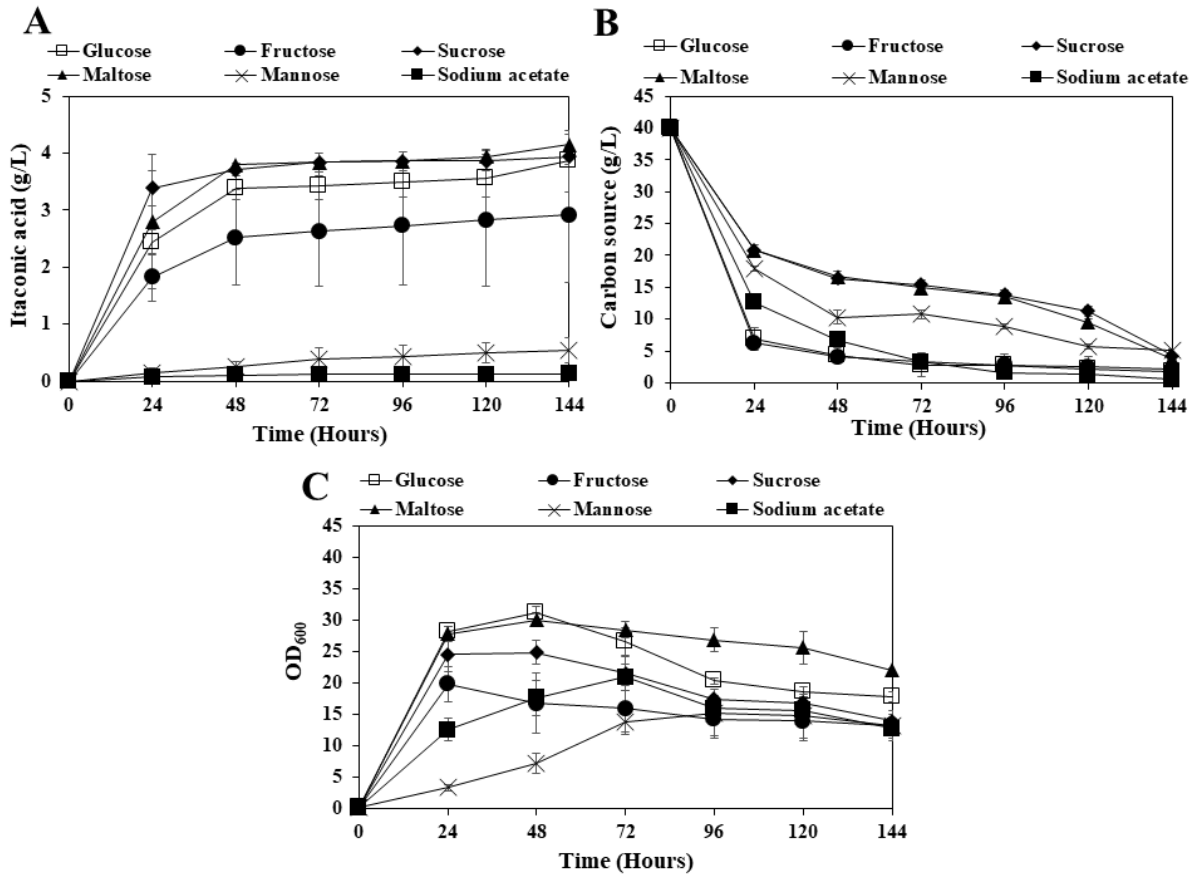

Fig. 3. Itaconic acid (IA) production by *C. glutamicum* ATCC 13032 pCH/*Tad1*$_{opt}$*Adi1*$_{opt}$ strain from different carbon sources. IA production (A), carbon source consumption (B), and cell growth (OD_{600}) (C) are indicated. *C. glutamicum* ATCC 13032 pCH/*Tad1*$_{opt}$*Adi1*$_{opt}$ strain was inoculated at initial OD_{600} of 0.2 in mCGXII media containing different carbon sources including glucose (open square), fructose (closed circle), sucrose (closed diamond), maltose (closed triangle), mannose (multiplication symbol), and sodium acetate (closed square) to obtain a final carbon source concentration of 40 g/L, and under nitrogen-limited conditions. The data represent the average values and standard deviation calculated from three biological replicates.

Table 3. Concentration of organic acids produced by the recombinant *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ [a] using different carbon sources.

	Carbon source					
Product (g/L)	Glucose	Fructose	Sucrose	Maltose	Mannose	Sodium acetate
Itaconic acid	3.48±0.49	2.63±1.07	3.55±0.13	3.73±0.17	0.49±0.20	0.12±0.08
Lactic acid	3.23±0.10	1.38±0.13	3.13±0.21	3.26±0.17	0.48±0.06	0.32±0.07
Acetic acid	1.04±0.14	2.36±0.58	ND	ND	ND	ND
Citric acid	0.13±0.02	0.48±0.26	0.10±0.02	0.17±0.05	0.04±0.02	0.20±0.01
Succinic acid	0.70±0.03	2.10±1.02	0.31±0.11	0.51±0.19	0.10±0.04	0.92±0.15
Total	8.58±0.78	8.95±3.06	7.09±0.47	7.67±0.58	1.11±0.32	1.56±0.31

[a] The recombinant strain *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt\ t}$ was inoculated at an initial OD_{600} of 0.2 in mCGXII medium containing 40 g/L different carbon sources. Values were determined after 168 h of fermentation. The data represent the average values and standard deviations, which were calculated using three biological replicates.

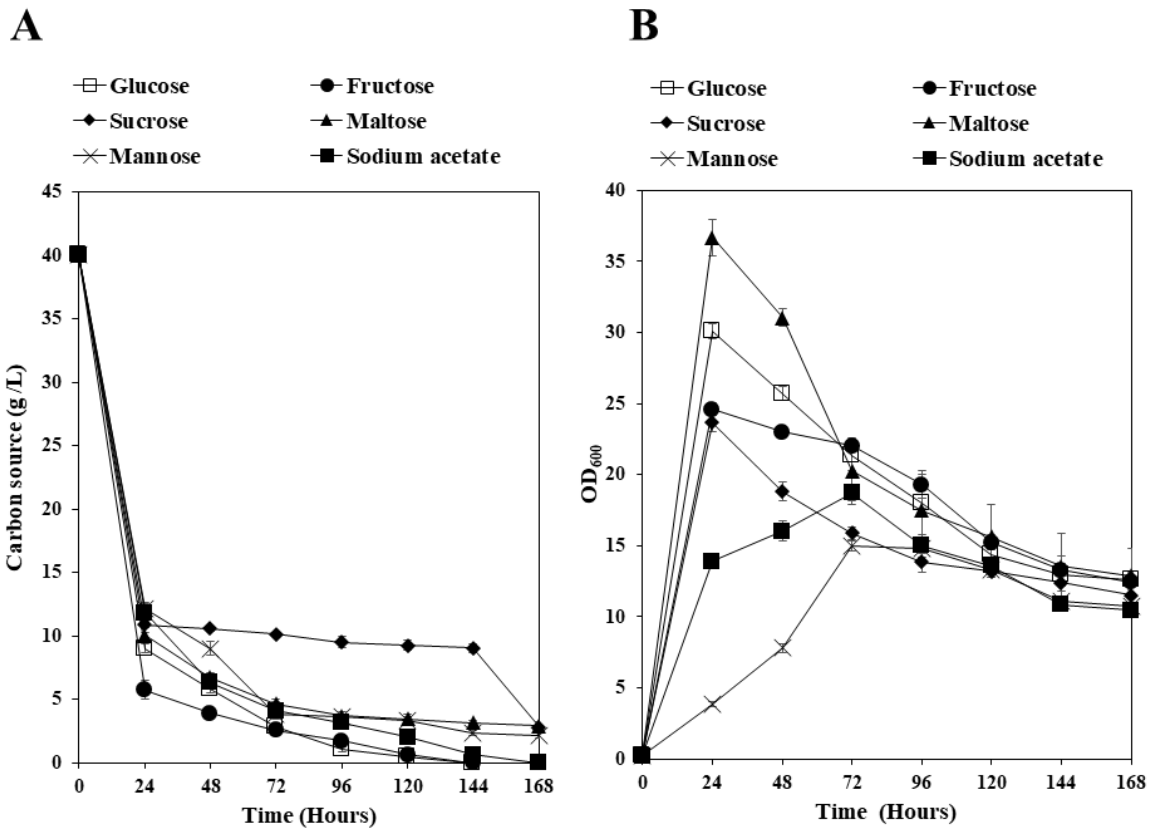

Fig. 4. Cell growth and carbon source consumption of wild type strain, *C. glutamicum* ATCC 13032 strain growing on different carbon sources. carbon source consumption (A) and cell growth (OD_{600}) (B) are indicated. *C. glutamicum* ATCC 13032 strain was inoculated at initial OD_{600} of o.2 in mCGXII media containing different carbon sources including glucose (open square), fructose (closed circle), sucrose (closed diamond), maltose (closed triangle), mannose (multiplication symbol), and sodium acetate (closed square) to obtain a final carbon source concentration of 40 g/L, and under nitrogen limiting conditions. The data represent the average values and standard deviation calculated from three biological replicates.

III.3.4. Fed-batch fermentation for production of IA in *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ using different carbon sources

To Further improve IA titer, fed-batch fermentation was performed using different carbon sources (glucose, maltose and sucrose) via the use of the *C. glutamicum* ATCC13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ strain.

Three carbon sources (glucose, sucrose and maltose) were utilized for fed-batch fermentation using mCGXII under nitrogen-limiting conditions and with an initial carbon source concentration of 3% w/v. The media was fed four times with a feeding solution consisting of basal solution, sugar (glucose or sucrose or maltose), and biotin. Cell growth for the *C. glutamicum* strain increased till 72 h, after which the cell growth tended to stabilize before decreasing after 120 h (Fig. 5). It also is obvious that all the carbon sources were completely consumed after 144 h (Fig. 5). Furthermore, the highest IA titer which of 12.25 g/L was achieved by feeding glucose as the carbon source followed by the titers achieved using maltose and sucrose at 11.34 and 11.02 g/L of IA, respectively (Fig. 5).

Those results represent the highest IA titer produced from glucose using *C. glutamicum*, while in other studies the highest IA titer reached from glucose was 7.8 g/L with molar yield of 0.40 mol/mol using a *C. glutamicum* strain expressing the *cadA* gene fused with maltose binding protein and 0.06 mol/mol by engineered *C. glutamicum* expressing *cadA* gene without fusion with maltose binding protein (30). In the present study, the final IA titer from glucose is 12.25 g/L with molar yield of 0.22 mol/mol when using a *C. glutamicum* strain expressing the *trans*-pathway (*Adi1*/*Tad1*) genes while the yield is 0.43, 0.42 mol/mol

from maltose and sucrose, respectively. This confirms the possibility for improvement of IA production by *C. glutamicum* stain expressing *trans*-pathway by applying more metabolic engineering experiments. Also, *trans*-pathway can be similarly expressed in other non-natural producers to improve IA production.

This study confirmed the possibility for the production of IA from *C. glutamicum* by developing two distinct pathways other than *cis*-pathway (*cadA* gene). Also, these pathways could be used for production of IA using various carbon sources and lead to different final concentrations of IA depending on the expressed pathway. However generally, expression of the *trans*-pathway in *C. glutamicum* improved IA production than *Irg1* gene with a final titer of 12.25 g/L and molar yield of 0.22 mol/mol from glucose via fed-batch fermentation. So, *trans*-pathway can be used in the future for improvement for IA production in other organisms among bacteria. Also, this pathway can be used for further improvement of IA production by *C. glutamicum*.

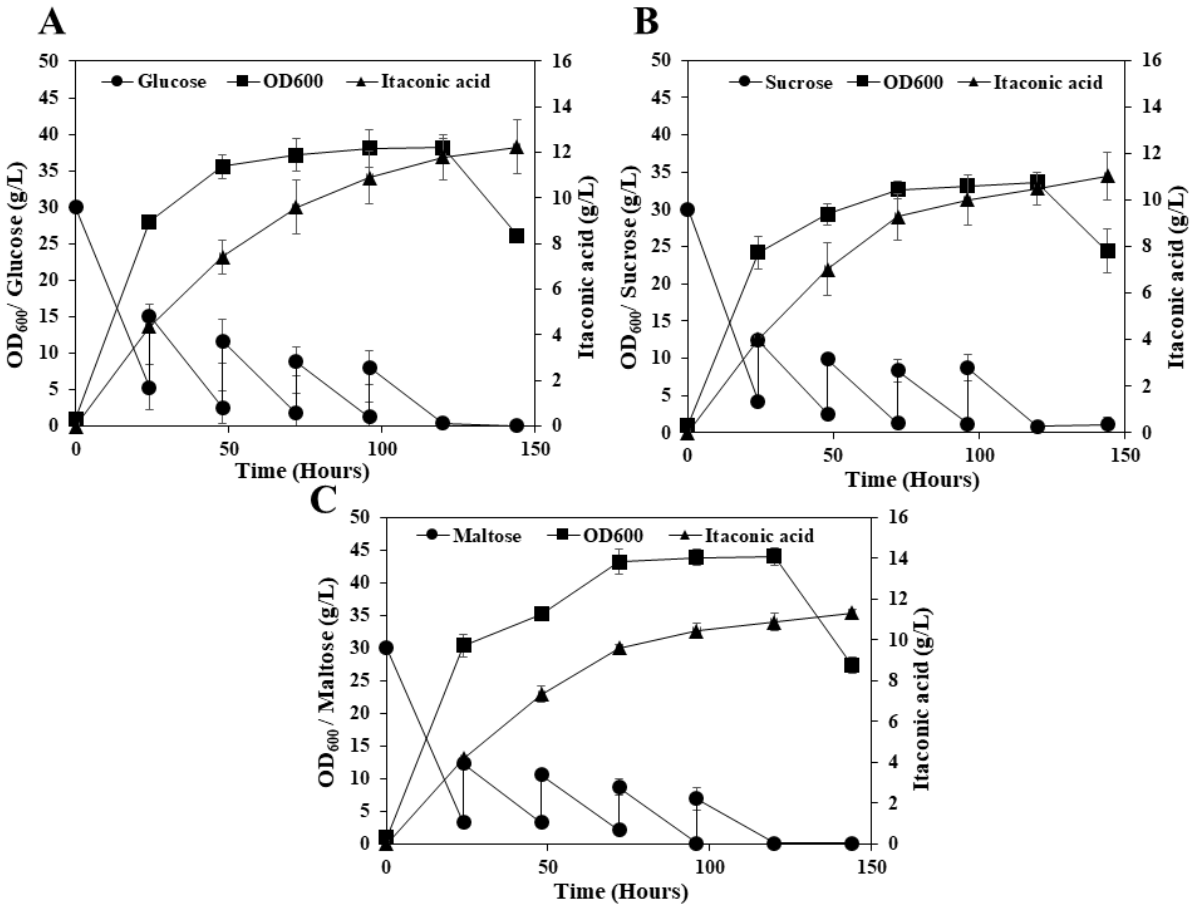

Fig. 5. Fed-batch fermentation for itaconic acid (IA) production by *C. glutamicum* ATCC 13032 pCH/*Tad1*$_{opt}$*Adi1*$_{opt}$ from glucose (A), sucrose (B), and maltose (C). Carbon source consumption (closed circle), cell growth (closed square), and IA production (closed triangle) are indicated. The strain *C. glutamicum* ATCC 13032 pCH/*Tad1*$_{opt}$*Adi1*$_{opt}$ was inoculated at initial OD_{600} of 1 in mCGXII medium containing glucose or sucrose or maltose as main carbon source to obtain an initial carbon source concentration of 3% (w/v), using nitrogen-limited conditions. The data represent the average values and standard deviation calculated from three biological replicates.

III.4. Conclusions

This study confirmed the possibility for the production of IA using *C. glutamicum* by

developing two distinct pathways other than *cis*-pathway (*cadA* gene). Also, these pathways could be used for production of IA using various carbon sources, and lead to different final concentrations of IA depending on the expressed pathway. However, expression of the *trans*-pathway in *C. glutamicum* improved IA production than *Irg1* gene with a final titer of 12.25 g/L and a molar yield of 0.22 mol/mol from glucose via fed-batch fermentation. Therefore, *trans*-pathway can be used in the future for improvement for IA production in other organisms among bacteria. Also, this pathway can be used for further improvement of IA production by *C. glutamicum*.

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CHAPTER IV

Utilization of sweet sorghum juice as a carbon source for enhancement of itaconic acid production in engineered *Corynebacterium glutamicum*

IV.1. Introduction

By increasing the awareness for sustainability, the usage of plant biomass as a renewable substrate for the production of chemicals and fuels has become one of the primary goals due to the global shortage of fossil fuels and to supply renewable energy and materials [1]. The juices or hydrolysates obtained from such lignocellulosic biomass can be used as alternative carbon sources by microorganisms for production of bio-based chemicals due to its high content of sugars such as glucose and sucrose [2].

Sweet sorghum (C_4 plant) is one of the most promising sugar crops that can be used as a feedstock for IA production as a replacement to the pure sugars [3]. The importance of sweet sorghum is related to the high content of sugars that can be found in the plant stalks, its highly adaptation to various soil types including clay and sandy soils, it can grow also in wide range of temperatures, can grow in a short period, the plant need from water and fertilizers is lower than other sugar crops, and shows high resistance to drought [4].

Sugar cane is also one of the most abundant sugar crops around the world and can be grown in a wide range of temperatures. After the juice extraction from sugar cane, the

remaining fibrous residual material called bagasse [5]. Sugar cane bagasse is considered as one of the most important agro-industrial residue matter that can be used as a raw material for several bioproducts including organic acids due to its availability, stable supply from sugar cane, and low pre-treatment cost [6] .

Itaconic acid (IA) is an unsaturated carboxylic acid which is a bio-derived potential platform chemical that was considered to be among the twelve most promising bio-based products. It has a unique chemical structure that allows its usage in various applications including styrene butadiene rubber latex SBR, unsaturated polyester resins, plastics, coatings and detergents [7, 8].

Lignocellulosic-based carbon sources were reported for IA production as a potential substrate due to their availability and low-cost production. Many studies have produced IA by using fermentable sugars released from lignocellulosic biomass (Table 1). Most of these trials, that used fermented sugars from lignocellulosic biomass for production of IA, used mainly the natural IA producer *A. terreus*. IA production from substrates other than pure sugars has many challenges for *A. terreus*, due to its sensitivity to the inhibitors that can be found in the fermentation medium and have unfavorable effect on cell growth.

Table 1. Attempts for production of IA from lignocellulosic biomass

Organism	Substrate	Titer [g/L]	Reference
A. terreus NRRL 1960	Organosolv beech wood	7.2	[9]
A. terreus AT-90	Undetoxified enzymatic hydrolysate of steam exploded corn stover	19.3	[10]
A. terreus DSM 23081	Wheat chaff	27.7	[11]
A. terreus CICC 40205	wheat bran hydrolysate	49.7	[12]
A. terreus M69	Corn stover hydrolysate activated with charcoal	33.6	[13]
A. terreus NRRL 1960	Bleached cellulose pulp	27.6	[14]
A. terreus AtYSZ-38	Bamboo residues	19.35	[15]
C. glutamicum HKC2029	Enzymatic hydrolysate of kraft pulp	6.15	[16]
C. glutamicum ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$	Sweet sorghum Juice	8.4	This study

Corynebacterium glutamicum, which is known as a workhorse in industrial biotechnology, is used in the production of various bioproducts such as amino acids, biofuels and organic acids. *C. glutamicum* can utilize several carbon sources and showed a high tolerance to inhibitors contained in hydrolysates of lignocellulosic biomass [17, 18]. Since fermented sugars obtained from lignocellulosic biomass were proven to be an alternative substrate for organic acid production, a realistic possibility of IA production is also expected

by using metabolically engineered *C. glutamicum* under optimized fermentation conditions. However, IA production from hydrolysates of lignocellulosic biomass by *C. glutamicum* has been investigated only once in our previous study, by using enzymatic hydrolysate of Kraft pulp giving final IA titer of 6.15 g/L [16].

There are three different known metabolic pathways for production of IA by natural producers. The first pathway depends mainly on *cis*-aconitate decarboxylase gene (*cadA*) and occurs in *A. terreus.* The second pathway which was found in *Mus musculus* mammalian cells, depends on immunoresponsive gene (*Irg1*) for IA production [19, 20]. Both first and second pathways called *cis*-pathway, where production of IA occurs through one step by conversion of *cis*-aconitic acid into IA by *cadA* or *Irg1* genes. The third pathway occurs in *U. maydis*, and IA is produced through two steps. Firstly, *cis*-aconitic acid is converted into its isomer, *trans*-aconitic acid by aconitase delta isomerase gene (*Adi1*). After that, *trans*-aconitic acid is converted to IA by trans aconitate decarboxylase gene (*Tad1*). This pathway is called *trans*-pathway and is thermodynamically favorable pathway for IA production [21, 22].

In this study, three engineered *C. glutamicum* strains expressing the three different metabolic pathways (*cadA* gene, *irg1*gene and *Adi1*/*Tad1* genes) for IA production were used for IA production from sweet sorghum juice and bagasse sugar lysate (BSL) to compare between the three pathways for IA production from plant-based biomass. Moreover, this is

the first study that used sorghum juice and BSL for IA production in engineered *C. glutamicum*. Sorghum juice has a high concentration of soluble sugars (glucose and sucrose) with no chemical inhibitors, while BSL has a high concentration of glucose with presence of some chemical inhibitors. *C. glutamicum* showed the ability to grow in the media containing BSL and sorghum juice without any growth inhibition and this confirms the high tolerance of *C. glutamicum* to fermentation inhibitors, however, sorghum juice proved to be better than BSL in IA production. Additionally, *C. glutamicum* strain expressing *Adi1*/*Tad1* genes from *U. maydis* which was used previously for IA from pure sugars, proved to be better also for IA production from sorghum juice and BSL which confirms that *trans*-pathway is better than *cis*-pathway in IA production by *C. glutamicum* not only from pure sugars but also from pant-based biomass.

IV.2. Materials and Methods

IV.2.1. Preparation of sorghum juice and bagasse sugar lysate (BSL)

Five different species of sweet sorghum were used in this study (A, B, C, D and E) obtained from Binex Inc. (Tokyo, Japan). The juice was extracted from the stalks using a juice extractor. Then, the juice was stored at - 20 °C. Before usage, the juice was thawed overnight and passed through two purification steps to get rid of all suspended materials and impurities. Firstly, the sorghum juice was centrifuged at 12,000 x g for 15 min at 4 °C. After that, the supernatant was collected and filtered to remove all fine particles by using Celite

503RV (Nacalai tesque, Japan) by using suction filtration. Then, the filtrate was collected and stored at - 20 °C. Sugar cane bagasse was treated by hydrothermal treatment to obtain BSL as mentioned previously [23].

IV.2.2. Fermentation experiments conditions

The bacterial strains used in this study are listed in Table 2. Three bacterial strains: *C. glutamicum* ATCC 13032 pCH_*cadA*_opt, *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$, and *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ were used for production of IA from five sorghum juices (A, B, C, D and E) which contained different concentrations of sucrose, glucose and fructose (Table 3). BSL was also used and contained 50 g/L glucose, 25 g/L xylose, furfural (4.5mM), 5-HMF (3.4 mM), acetic acid (66 mM), formic acid (7.2 mM), and lactic acid (7 mM). For all fermentation experiments, a single colony of *C. glutamicum* from freshly streaked BHI (Brain heart infusion) (Difco Laboratories, Detroit, MI, USA) agar plate was used to inoculate the first preculture of two mL BHI liquid medium and incubated on rotary shaker at 180 rpm for 8 h at 30 ºC. Then, the cells of the first preculture were used to inoculate 10 mL second preculture in 50 mL test tube of mCGXII (modified CGXII) (1g/L KH_2PO_4, 1g/L K_2HPO_4, 42 g/L 3-morpholinopropanesulfonic acid (MOPS), 0.25 g/L $MgSO_4.7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4.7H_2O$, 0.1 mg/L $MnSO_4.H_2O$, 1 mg/L $ZnSO_4.7H_2O$, 0.2 mg/L $CuSO_4.5H_2O$, 20 mg/L $NiCl_2.6H_2O$, 0.2 mg/L biotin) medium at a final OD_{600} of approximately 1 and incubated on rotary shaker at 180 rpm for 14 h at 30 ºC.

The mCGXII medium was supplemented with 4% (w/v) sorghum juice or BSL as main carbon sources and fermentation carried out under nitrogen-limited conditions (1 g/L urea), as described previously [24]. Then, the cells of the second preculture were used to inoculate 20 mL main culture in 100 mL baffled flask of the same mCGXII medium with 4% sorghum juice or BSL as sole carbon sources under nitrogen-limited conditions at a final OD_{600} of approximately 1. The pH was maintained at 7 for all fermentation experiments.

Table 2. Strains used in this study

Strain	Relative Characteristics	Resource/ reference
C. glutamicum ATCC 13032 pCH_*cadA*_opt	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *cadA* gene of *A. terreus*	[16]
C. glutamicum ATCC 13032 / pCH-$Irg1_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *Irg1* gene from *M. musculus*	[21]
C. glutamicum ATCC 13032 / pCH-$Tad1_{opt}Adi1_{opt}$	ATCC 13032 strain carrying the shuttle vector pCH with the synthetic codon-optimized *Tad1* and *Adi* genes from *U. maydis*	[21]

Table 3. Concentrations of sugars in different sorghum juices

Sorghum Juice	Glucose (g/L)	Fructose (g/L)	Sucrose (g/L)
A	76	66	5
B	70	60	5
C	45	25	5
D	70	57	2
E	20	10	15

IV.2.3. Fed-batch fermentation

Fed batch fermentation experiments were carried out using two sorghum juices (A and B) and BSL as main carbon sources. For all fed-batch experiments, initial volume of 20 ml of mCGXII in 150 mL baffled flask was used as main culture. The medium was supplemented with 3 % w/v sorghum juice or BSL and under nitrogen-limited conditions. The cells of the second preculture were used to inoculate the main culture medium at final OD_{600} of approximately 1. The pH was maintained at 7. The feeding was carried out four times every 24 h using a feeding solution which was composed of carbon source (Sorghum juice A or sorghum juice B or BSL), basal solution (5 g/L urea, 5 g/L KH_2PO_4, 5 g/L K_2HPO_4 and 210 g/L MOPS) and 20 mg/L biotin. The flasks incubated on rotary shaker at 30 ºC and 180 rpm.

IV.2.4. Analysis of IA and sugars

For all fermentation experiments, 2 mL culture samples were collected every 24 h and centrifuged at 15,000 rpm for 10 min at 4 °C. High-performance liquid chromatography

(HPLC) was used to analyze the concentration of IA and sugars in all samples. For IA, the samples were analyzed using a diode array detector SPD-20AV at UV210 nm (Shimadzu, Kyoto, Japan) equipped with a column (InertSustain 5 um, 150 mm x 4.6 mm internal diameter, GL Science, Tokyo, Japan). 50 mM NaH_2PO_4 (pH=2.1) served as a mobile phase buffer at a flow rate of 1.0 mL / min for each sample, and the temperature of the column oven was adjusted to 40 °C. For evaluation of sugars concentrations such as glucose, fructose and sucrose a cosmosil sugar-D column (250 mm x 4.6 mm of internal diameter, Nacalai tesque, Japan) was used at a flow rate of 0.5 mL/min, with Acetonitrile/water (75 % / 25 %) serving as the mobile phase at 30 ºC for 40 min for each sample.

IV.2.5. Statistical analysis

Each result is calculated from triplicate experiments and indicated by the average value with standard deviation as an error bar. Paired Student's *t*-test was used to compare the differences in data via independent triplicated data. A p-value of < 0.05 was considered to be statistically significant.

IV.3. Results and Discussion

IV.3.1. Comparison between different engineered strains of *C. glutamicum* for IA production.

In our previous studies [16, 21], three engineered strains of *C. glutamicum*, *C. glutamicum* ATCC 13032 pCH_*cadA*_opt, *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$, and *C.*

glutamicum ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ were constructed. The highest final IA titer achieved by using *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ using different pure sugars with final IA titer of 12.25, 11.34, and 11.02 g/L from glucose, maltose, and sucrose, and the results revealed that *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$. Depending on our previous results, we suggested that *C. glutamicum* expressing *Adi1*/*Tad1* genes (*trans*-pathway) is better in IA production than the other two strains expressing *cis*-pathway (*cadA* gene or *irg1* gene) . Consequently, we tried to broaden the range of substrates used for IA production by *C. glutamicum* to include lignocellulosic feedstock.

The use of plant biomass as a feedstock for the production of chemicals is in urgent need nowadays for more sustainability and supplying of renewable energy and materials. Till now, the industrial production of IA occurs by the natural producer *A. terreus* by using glucose as the main carbon source. Additionally, most of the reports that used lignocellulosic biomass for IA production were achieved by using also *A. terreus*. We tried to enhance IA production using renewable sources including sweet sorghum juice and BSL.

Purified juice of five different sorghum species (A, B, C, D and E) and BSL were used as main carbon sources for production of IA in three engineered *C. glutamicum* strains, *C. glutamicum* ATCC 13032 pCH_*cadA*_opt, *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$, and *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$. The results showed that *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ was better in IA production using all sorghum juices and BSL (Fig.

1A) with IA final titer of 3.29 g/L from BSL, followed by sorghum B (2.7 g/L), sorghum A (2.52 g/L), sorghum D (2.07g/L), sorghum E (1.37 g/L), and sorghum C (1.29 g/L). In the case of *C. glutamicum* ATCC 13032 pCH_*cadA*_opt, no IA produced using most sugar juices, only 0.04 g/L IA was produced from sorghum B and BSL (Fig.2A). On the other side, *C. glutamicum* ATCC 13032 pCH-$Irg1_{opt}$ produced IA from many sorgham juices and BSL, however, the final IA titer was very low with 0.05 g/L from sorghum B, 0.04 g/L from sorghum D and BSL, 0.03 g/L from sorghum A, and 0.02 g/L from sorghum C and E (Fig. 3A). Additionally, the best cell growth was achieved by using BSL as a main carbon source in case of *C. glutamicum* ATCC 13032 pCH_*cadA*_opt and *C. glutamicum* ATCC 13032 pCH-$Irg1_{opt}$ (Figs. S1B and S2B), while the highest cell growth in case of *C. glutamicum* ATCC 13032 pCH-$Tad1_{opt}Adi1_{opt}$ was achieved using sorghum D, B and BSl (Fig. 1B). This result indicates the ability of *C. glutamicum* to grow with high cell density in high concentration of inhibitors as found in BSL.

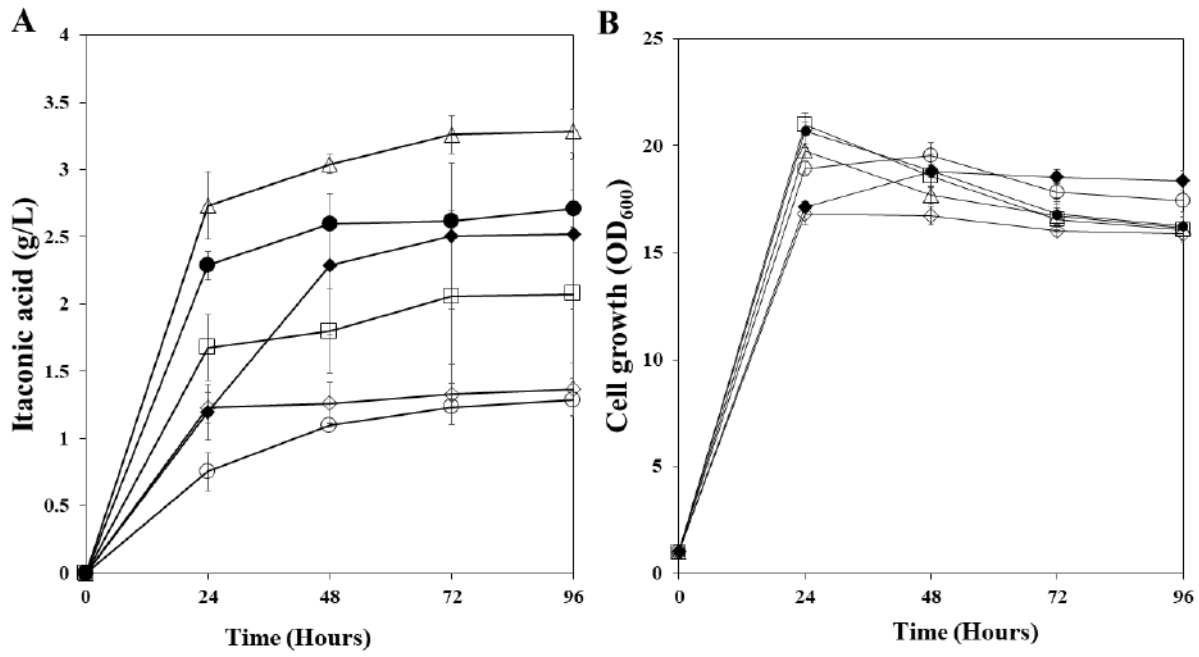

Fig. 1. Itaconic acid production using different sorghum juices and bagasse sugar lysate in *C. glutamicum* ATCC 13032 pCH/$Tad1_{opt}Adi1_{opt}$. The itaconic acid production (A) and cell growth (OD600) (B) are indicated. *C. glutamicum* ATCC 13032 pCH/$Tad1_{opt}Adi1_{opt}$ strain was inoculated at initial OD600 of 1 in mCGXII medium containing different sorghum juices or bagasse sugar lysate; sorghum A (◆), sorghum B (●), sorghum C (○), sorghum D (□), sorghum E (▯) and BSL (Δ) as the main carbon source and under nitrogen-limited conditions. The data represents the average values and standard deviations calculated from three biological replicates.

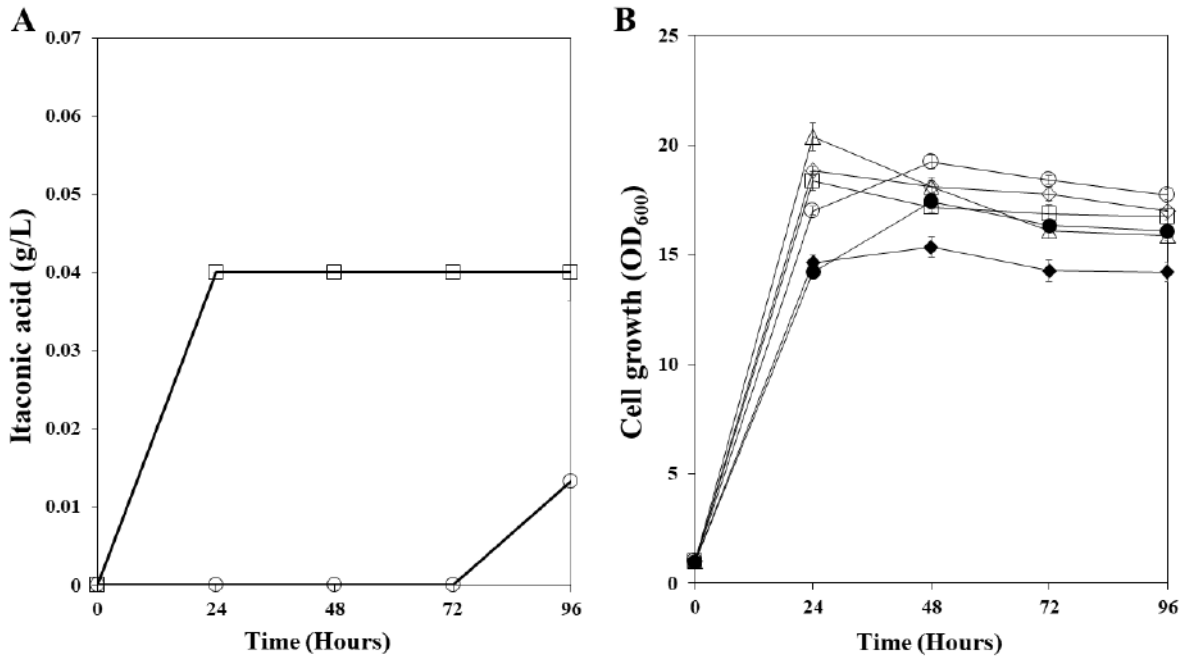

Fig. 2.

Itaconic acid production using different sorghum juices and bagasse sugar lysate in *C. glutamicum* ATCC 13032 pCH_*cadA*_opt. The itaconic acid production (A) and cell growth (OD600) (B) are indicated. *C. glutamicum* ATCC 13032 pCH_*cadA*_opt strain was inoculated at initial OD600 of 1 in mCGXII medium containing different sorghum juices or bagasse sugar lysate; sorghum A (◆), sorghum B (●), sorghum C (○), sorghum D (□), sorghum E (▯) and BSL (Δ) as the main carbon source and under nitrogen-limited conditions. The data represents the average values and standard deviations calculated from three biological replicates.

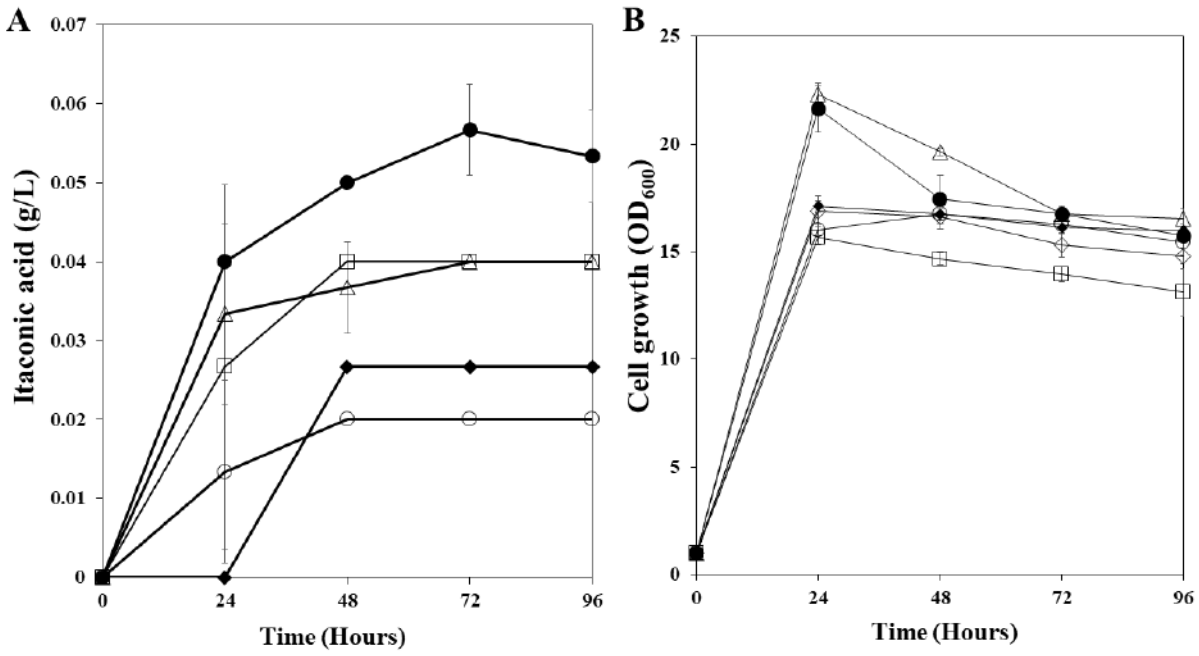

Fig. 3. Itaconic acid production using different sorghum juices and bagasse sugar lysate in *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$. The itaconic acid production (A) and cell growth (OD600) (B) are indicated. *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$ strain was inoculated at initial OD600 of 1 in mCGXII medium containing different sorghum juices or bagasse sugar lysate; sorghum A (◆), sorghum B (●), sorghum C (○), sorghum D (□), sorghum E (⍰) and BSL (Δ) as the main carbon source and under nitrogen-limited conditions. The data represents the average values and standard deviations calculated from three biological replicates.

It is clear also that all the carbon sources that present in all sorghum juices and BSL were completely consumed in the media in case of *C. glutamicum* ATCC 13032 pCH_*cadA*_opt, *C. glutamicum* ATCC 13032 pCH-$Irg1_{opt}$ and *C. glutamicum* ATCC 13032 pCH-$Tad1_{opt}Adi1_{opt}$ (Figs. 4, 5 and 6). Consequently, *C. glutamicum* ATCC 13032 pCH-$Tad1_{opt}Adi1_{opt}$ used the carbon sources in the media for growth and IA production, while the other two strains used all carbon sources for growth only.

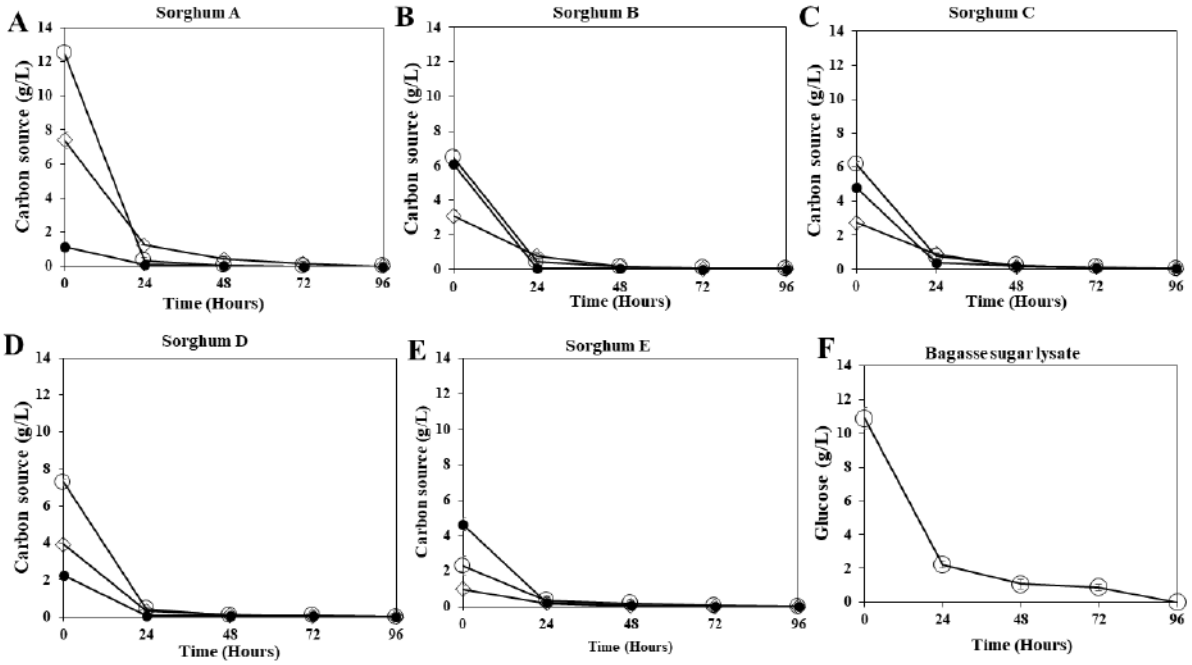

Fig. 4. Carbon source consumption by *C. glutamicum* ATCC 13032 pCH/$Tad1_{opt}Adi1_{opt}$ growing in different sorghum juices and bagasse sugar lysate. Glucose (○), fructose (□) and sucrose (●) are indicated. *C. glutamicum* ATCC 13032 pCH/$Tad1_{opt}Adi1_{opt}$ strain was inoculated at initial OD600 of 1 in mCGXII medium containing different sorghum juices or bagasse sugar lysate; sorghum A (A), sorghum B (B), sorghum C (C), sorghum D (D),

sorghum E (E) and BSL (F) as the main carbon source and under nitrogen-limited conditions.

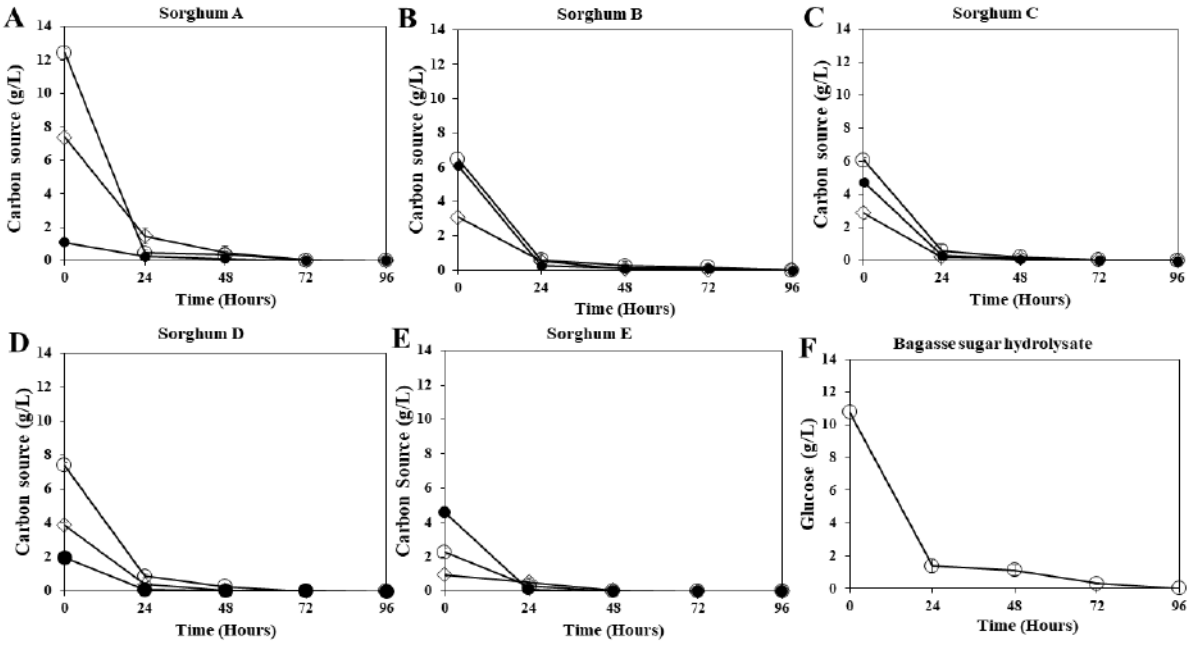

Fig. 5. Carbon source consumption by *C. glutamicum* ATCC 13032 pCH_*cadA*_opt growing in different sorghum juices and bagasse sugar lysate. Glucose (○), fructose (□) and sucrose (●) are indicated. *C. glutamicum* ATCC 13032 pCH_*cadA*_opt strain was inoculated at initial OD600 of 1 in mCGXII medium containing different sorghum juices or bagasse sugar lysate; sorghum A (A), sorghum B (B), sorghum C (C), sorghum D (D), sorghum E (E) and BSL (F) as the main carbon source and under nitrogen-limited conditions. The data represents the average values and standard deviations calculated from three biological replicates.

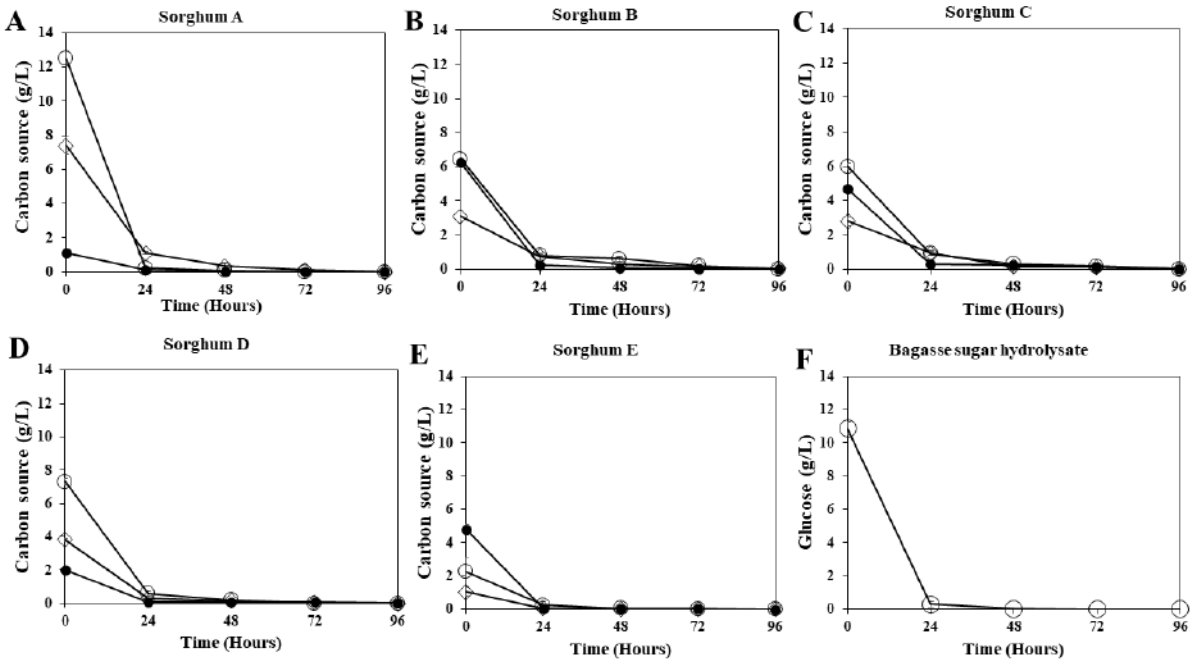

Fig. 6. Carbon source consumption by *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$ growing in different sorghum juices and bagasse sugar lysate. Glucose (○), fructose (□) and sucrose (●) are indicated. *C. glutamicum* ATCC 13032 pCH-*Irg1*$_{opt}$ strain was inoculated at initial OD600 of 1 in mCGXII medium containing different sorghum juices or bagasse sugar lysate; sorghum A (A), sorghum B (B), sorghum C (C), sorghum D (D), sorghum E (E) and BSL (F) as the main carbon source and under nitrogen-limited conditions. The data represents the average values and standard deviations calculated from three biological replicates.

IV.3.2. Fed-batch fermentation for IA production in *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ by using sorhum juice and BSL.

To further enhance the final IA production titer using sorghum juice and BSL and compare more between both of them for IA production, fed-batch fermentation was performed using the purified juice of sorghum A and B, and BSL as main carbon sources via

the use of *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$.

Sorghum A juice, sorghum B juice, and BSL were used for production of IA in *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$ using fed-batch fermentation. The medium was fed four times every 24 h with feeding solution. The IA final titer showed an increase in the final titer reaching to 8.4 g/L from sorghum A after 120 h (Fig. 7A) followed by sorghum B (6.07 g/L) (Fig. 7B), and the lowest final IA titer achieved by using BSL (4.02 g/L) (Fig. 7C).

By using batch fermentation, BSL was better than sorghum juice in IA production by *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$, while after fed-batch fermentation, sorghum juice proved to be better in IA production than BSL after 4 times feeding which indicates that accumulation of high concentrations of inhibitors in the fermentation medium containing BSL as a sole carbon source might affect the production of IA in *C. glutamicum*.

In our previous study, the enzymatic hydrolysate of kraft pulp was used for production of IA by engineered *C. glutamicum* strain expressing *cadA* gene fused with maltose-binding protein reaching to final IA titer of 6.15 g/L and *C. glutamicum* showed high ability for growth in high concentration of inhibitors in the medium. In this study, sorghum juice and BSL were used for the first time for production of IA, and our results showed the ability of *C. glutamicum* to growth in presence of high concentration of inhibitors in case of BSL, however, sorghum juice was better in IA production with final titer of 8.4 g/L which is better than our previous reached titer from kraft pulp. However, the final titer reached using

sorghum juice (8.4 g/L) is still lower than our previous final titer from glucose using the same *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$, it is higher than the titer obtained by using enzymatic hydrolysate of kraft pulp and can be improved in the future using more meatboloc engineering and fermentation approaches.

Moreover, current study confirmed our previous result assumption that expressing *trans*-pathway in *C. glutamicum* is better than *cis*-pathway for IA production from both pure sugars, sorghum juice and BSL. This due to the formation *trans*-aconitate, which is thermodynamically favourable isomer that accounts for 88 % of aconitate and help in increasing flux towards IA production.

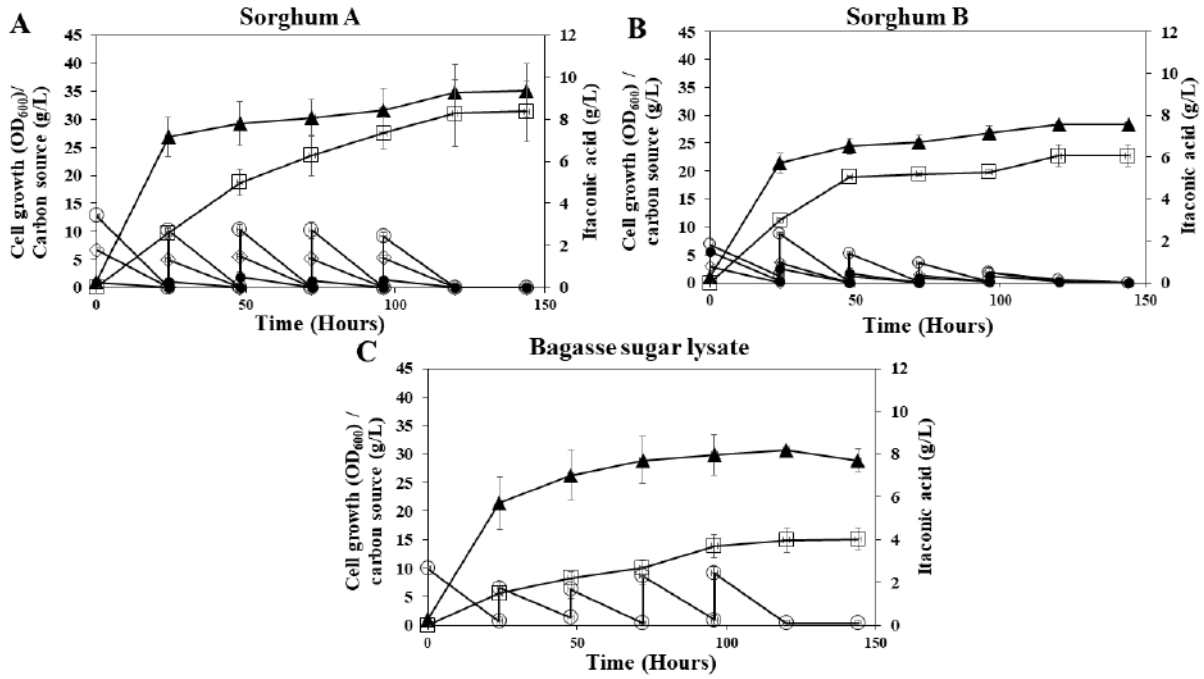

Fig. 7. Fed-batch fermentation for itaconic acid (IA) production from different types of sorghum juice and bagasse sugar lysate *C. glutamicum* ATCC 13032 pCH-*Tad1*$_{opt}$*Adi1*$_{opt}$. IA production (□), cell growth (OD_{600}) (▲), glucose consumption (○), fructose consumption (▽), and sucrose consumption (●) are indicated. The strain *C. glutamicum* ATCC 13032 pCH/*Tad1*$_{opt}$*Adi1*$_{opt}$ was inoculated at initial OD_{600} of 1 in mCGXII medium containing; sorghum A (A), or sorghum B (B), or BSL (C) as main carbon source under nitrogen-limited conditions. The data represents the average values and standard deviations calculated from three biological replicates.

IV.4. Conclusions

This study confirmed the possibility for the production of IA using *C. glutamicum* ATCC 13032 pCH/*Tad1*$_{opt}$*Adi1*$_{opt}$ strain expressing *trans*-pathway (*Adi1*/*Tad1*) genes from different plant-based biomass including sorghum juice and BSL. However, sorghum juice

was better than BSL in IA production with a final IA titer of 8.4 g/L. Therefore, expressing *trans*-pathway in *C. glutamicum* proved to be better in IA production from both pure sugars and different plant-based biomass. This study also suggests that *C. glutamicum* is a suitable microorganism for production of IA from various lignocellulosic biomass.

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CHAPTER V

General conclusions and Future Research

The day after day, there is massive increase in the need for the use of sustainable renewable products have taken us to more research to replace petroleum-based products by bio-ones. Itaconic acid (IA) is one of the most valuable products and is considered as a promising and a very clean alternative to petroleum based acrylic acid which can be used as a starting material for many polymers including poly-itaconic acid and can be used in many applications, IA synthesized naturally by many microorganisms including *Aspergillus terreus* which is the main Producer of IA industrially. *A. terreus* based IA production has many drawbacks, so we tried to produce IA using engineered *Corynebacterium glutamicum* as promising host for IA production from various substrates.

The effect of different carbon sources on the production of IA from *C. glutamicum* was investigated. Also, the effect of a mixture of substrates utilization via *C. glutamicum* on cell growth and IA production was evaluated. The heterologous expression of a codon optimized, *cis*-aconitate decarboxylase gene from *A. terreus* in a wild-type strain of *C. glutamicum* produced low IA production titer from numerous carbon sources such as glucose, fructose, sucrose, maltose and mannose. Sodium acetate, however, was the carbon source that led to the highest IA while maltose led to optimal cell growth titer. Higher concentrations of

sodium acetate, however, led to a decrease in cell growth and to a longer lag phase. Consequently, the combination of sodium acetate and maltose led to improved cell growth and IA production from the engineered strain *C. glutamicum* ATCC 13032 pCH-$cadA_{opt}$.

In our study, *C. glutamicum* was engineered by developing two distinct IA production pathways: *cis*-pathway that depends on *Irg1* gene from *M. musculus* and *trans*-pathway (*Adi1*/*Tad1*) genes from *U. maydis*, which were then used for IA production at high titers using a wide range of carbon sources. The results confirmed the potential of *C. glutamicum* for IA production using pathways other than the common *A. terreus cis*-pathway, that depends mainly on the *cadA* gene. We also found that the strain expressing *trans*-pathway (*Adi1*/*Tad1*) genes from *U. maydis* resulted in high IA titers by utilizing most carbon sources. On the other side, expressing codon-optimized *Irg1* gene from *M. musculus* in *C. glutamicum* resulted in a low IA titer, which reflects the great effect of different metabolic pathways in IA production by *C. glutamicum.*

The present study also investigated the production of IA from sugar hydrolysate of sorghum and sugarcane bagasse by engineered *C. glutamicum*, which showed its ability to produce IA from different lignocellulosic biomass with high final IA titer (8.4 g/L).

LIST OF PUBLICATIONS

CHAPTER II

Elkasaby, T., Hanh, D. D., Kawaguchi, H., Toyoshima, M., Ogino, C. and Kondo, A.: Co-utilization of maltose and sodium acetate via engineered *Corynebacterium glutamicum* for improved itaconic acid production. (Accepted for publication in Biotechnology and Bioprocess Engineering Journal)

CHAPTER III

Elkasaby, T., Hanh, D. D., Kawaguchi, H., Kondo, A., and Ogino, C.: Effect of different metabolic pathways on itaconic acid production in engineered *Corynebacterium glutamicum*, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2023.1005.1006 (2023).

CHAPTER IV

Elkasaby, T., Hanh, D. D., Kawaguchi, H., Ogino, C. and Kondo, A.: Utilization of sweet sorghum juice as a carbon source for enhancement of itaconic acid production in engineered *Corynebacterium glutamicum* (Under review in Enzyme and Microbial Technology Journal)

Co-authored publications

Hanh, D. D., Elkasaby, T., Kawaguchi, H., Tsuge, Y., Ogino, C. and Kondo, A.: Enhanced production of itaconic acid from enzymatic hydrolysate of lignocellulosic biomass by recombinant *Corynebacterium glutamicum*, J. Biosci. Bioeng. (2023).

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