Bioprocess and Biosystems Engineering (2023) 46:507–513
https://doi.org/10.1007/s00449-022-02835-4

RESEARCH PAPER

Enhanced production of maltobionic acid by a metabolically engineered *Escherichia coli* incapable of maltose utilization

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Received: 16 August 2022 / Accepted: 8 December 2022 / Published online: 20 December 2022

Abstract

To produce maltobionic acid (MBA) from maltose in *Escherichia coli*, we recombinantly expressed a glucose dehydrogenase gene (*gdh1*) from *Enterobacter cloacae* and a pyrroloquinoline quinone (PQQ) synthesis gene cluster (*pqqFABCDEMIH*) from *Pseudomonas taetrolens*. Although the recombinant *E. coli* strain (*E. coli* [pKK-ECGDH1 + pACYC-PQQ]) successfully produced MBA from maltose, the yield of MBA was rather low, indicating that *E. coli* has other maltose utilization pathways. Amylomaltase (MalQ) is the first enzyme in the maltose utilization pathway in *E. coli*. To investigate the potential role of MalQ on MBA production, *E. coli malQ* was inactivated. The culturing of the recombinant *E. coli* strain (*E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ]) in a flask resulted in higher MBA production titer, yield, and productivity (209.3 g/L, 100%, and 1.1 g/L/h, respectively) than those of *E. coli* [pKK-ECGDH1 + pACYC-PQQ] (162.1 g/L, 77.4%, and 0.5 g/L/h, respectively), indicating that the MalQ inactivation was highly effective in improving the MBA production ability of *E. coli*. After fermentation using 5-L bioreactor, MBA production titer, yield, and productivity of the recombinant *E. coli* strain were 209.3 g/L, 100%, and 1.5 g/L/h, respectively, which were 1.3-, 1.3-, 2.3-fold higher than those of *E. coli* [pKK-ECGDH1 + pACYC-PQQ] (167.3 g/L, 79.9%, and 0.65 g/L/h), respectively. Thus, our results provide an important foundation for efficient MBA production using recombinant *E. coli* strain.

Keywords Maltobionic acid production · *Escherichia coli* · Inactivation of maltose utilization pathway · Amylomaltase

Abbreviations

GDH1	Glucose dehydrogenase
HMCS	High-maltose corn syrup
HPLC	High-performance liquid chromatography
LBA	Lactobionic acid
MalQ	Amylomaltase
MBA	Maltobionic acid
PQQ	Pyrroloquinoline quinone
WCB	Whole-cell biocatalyst

Introduction

Maltobionic acid (MBA) is an aldonic acid obtained from the oxidation of maltose. MBA has been a component of the human diet since it was first discovered in honey [1]. MBA is a stereoisomer of lactobionic acid (LBA), a well-known aldonic acid that has been used for various industrial applications [2, 3]. Similar to LBA, MBA is also utilized in a variety of industries such as foods, cosmetics, and pharmaceuticals [1, 4, 5] because of excellent antioxidant, metal-chelating, and moisturizing properties [6–8].

MBA is almost exclusively produced by chemical oxidation. However, this chemical method requires harmful and costly metal catalysts and generates undesired by-products, such as maltulose and 2-keto MBA [9]. In contrast, biological MBA production methods have recently received considerable attention, because they do not require toxic catalysts and does not generate by-products. In a previous study, we succeeded in producing MBA at a high concentration (200 g/L) and productivity (9.52 g/L/h) using genetically modified *Pseudomonas taetrolens* [10]. Moreover, using this *P. taetrolens* strain as a whole-cell biocatalyst (WCB), we successfully produced MBA with a high production titer (200 g/L) and improved productivity (18.18 g/L/h) [11]. However, *P. taetrolens* cannot grow to a high cell density, which could be a weakness in the preparation of WCB for MBA production. Therefore, a new MBA-producing strain

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that can grow to a high cell density is required for efficient MBA production via whole-cell biocatalysis.

Among various bacteria, *E. coli* is a candidate MBA-producing species. It is the most extensively used bacterium for producing chemicals and proteins. This species can grow at high cell densities using inexpensive substrates such as glycerol. Moreover, the genetic tools for *E. coli* are plentiful, and their metabolic pathways are well understood [12, 13]. Therefore, *E. coli* can be used as a WCB for MBA production. Because *E. coli* cannot inherently produce MBA from maltose, we attempted to produce MBA by constructing a recombinant *E. coli* strain in this study. In our previous studies, we found that GDH from *P. taetrolens* could not only convert lactose into LBA but also maltose into MBA [10, 14]. The GDH uses PQQ as a cofactor. The PQQ synthesis gene cluster of *P. taetrolens* consists of nine genes (*pqqFABCDEMIH*). Using the GDH gene and PQQ synthesis gene cluster from *P. taetrolens*, we constructed a recombinant *E. coli* strain (*E. coli* [pKK-PTGDH + pACYC-PQQ]) with LBA-producing ability [15]. We found that GDH1 from *Enterobacter cloacae* also converts lactose to LBA [16]. Thus, we constructed another recombinant *E. coli* strain (*E. coli* [pKK-ECGDH1 + pACYC-PQQ]) showing LBA-producing ability using GDH1 from *E. cloacae* and the PQQ synthesis gene cluster from *P. taetrolens* [15]. In this study, we investigated whether this recombinant strain could also produce MBA. In addition, we tried to enhance the MBA-producing ability of *E. coli* by engineering the metabolic pathways of *E. coli*.

Material and methods

Materials

Maltose (EP grade, 92% purity) was bought from Daejung Chemical and Metals Co. Ltd. (Korea). MBA and other reagents were obtained commercially from Sigma-Aldrich (USA) and BD Difco (USA). All DNA manipulation tools including restriction and DNA-modifying enzymes, were bought from New England Biolabs (USA), Solgent (Korea), or Sigma-Aldrich.

Bacterial strains and plasmids

Escherichia coli JM109 was used as the host strain for MBA production. The strain was grown in Luria–Bertani (LB) medium at 37 °C for 24 h and was kept frozen in 30% glycerol at –80 °C. Plasmid pKK-ECGDH1 was used for expressing the GDH gene from *E. cloacae* in *E. coli*. The GDH was cloned into pKK223-3, the expression vector for *E. coli* which contains the *tac* promoter. Plasmid pACYC-PQQ was used for expressing a pyrroloquinoline quinone (PQQ) synthesis gene cluster (*pqqFABCDEMIH*) from *P. taetrolens* in *E. coli*. The PQQ synthesis gene cluster was cloned into pACYC184, the expression vector for *E. coli* which contains the *tet* promoter [15].

Knock-out of amylomaltase gene in *E. coli*

The deletion of *malQ* (GenBank accession number CAD6000741.1) in *E. coli* JM109 was carried out using the Quick and Easy Conditional Knockout Kit (Germany). To delete *malQ*, a Δ*malQ* loxP-neo-loxP cassette was made by PCR using the following pair of designed primers: MalQ_Del_F 5′-ATGGAAAGCAAACGTCTGGATAATGCCGCGCTGGCGGCGGGGATTAGCCCCAATTACATCTACCGTTCGTATAGCATACA-3′, MalQ_Del_R 5′CTACTTCTTCTTCGCTGCAGCTCTGCGCCGTCTGTCCAAATCCTTCAGCAACTTGTTCACTACCGTTCGTATAATGTATG-3′ to use FRT-PGK-gb2-neo-FRT as the template DNA.

The *malQ* gene of *E. coli* JM109 was replaced with the Δ*malQ* loxP-neo-loxP (chloramphenicol, Cm) cassette [17]. After being cultivated in LB medium, *E. coli* cells reached a cell density of 0.6 at an OD_{600nm}, and the pRED/ET recombinase gene was induced by adding 10% L-arabinose. After incubation for 1 h at 37 °C, the cells were harvested and rinsed thrice with ice-cold 10% glycerol. Approximately 400 ng of the PCR products were transformed via electroporation at 2.5 kV, 25 μF, and 200 Ω using a 2-mm cuvette. The deletion mutant was chosen in an LB medium containing 30 μg/mL Cm. The deletion of *malQ* was confirmed by PCR. The Cm cassette was removed from *E. coli* Δ*malQ* using a Cre expression plasmid pJW.

Culture conditions

To construct two recombinant *E. coli* strains, *E. coli* [pKK-ECGDH1 + pACYC-PQQ] and *E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ], pKK-ECGDH1 and pACYC-PQQ were transformed into *E. coli* JM109 and *E. coli* JM109 Δ*malQ* using a standard heat-shock transformation protocol.

Single colonies of *E. coli* strains were cultivated in 10 mL of nutrient broth (NB, 1 g/L beef extract, 2 g/L yeast extract, 5 g/L peptone, and 5 g/L NaCl). The cells were cultivated at 25 °C and 200 rpm for 24 h and then used as seed cultures for shaker flask and bioreactor experiments.

Shaker-flask culture experiments were conducted in 300-mL baffled flasks containing 50 mL of NB medium with 200 g/L maltose, 30 g/L $CaCO_3$, and proper antibiotics. Precultured bacteria were inoculated into 50 mL of NB medium and grown in a shaking incubator at 25 °C and 200 rpm.

Bioreactor conditions

Batch fermentation was carried out in a 5 L fermenter (BioCNS, Korea) containing 2 L of NB medium with 200 g/L maltose and 30 g/L $CaCO_3$ at 25 °C. The agitation speed was adjusted from 200 to 1000 rpm to maintain the dissolved oxygen (DO) level at 30%. The added $CaCO_3$ helped to keep the pH of the growth media between 6.0 and 7.2.

Analytical methods

Cell growth was assayed by a spectrophotometer (UV-2600, Shimadzu, Japan) at a wavelength of 600 nm (OD_{600nm}). The concentrations of maltose and MBA in the culture medium were determined using high-performance liquid chromatography (HPLC) equipment (Agilent 1260, USA). The HPLC system was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., New Haven, CT, USA) and a refractory index detector (1260 Infinity II RID, Agilent). HPLC analysis conditions were as follows: a mobile phase of 0.5 mM H_2SO_4 was used at a flow rate of 0.3 mL/min, and the column temperature was set to 75 °C.

Results

MBA production by recombinant *E. coli* strain

In this study, we first investigated whether *E. coli* [pKK-ECGDH1 + pACYC-PQQ] could produce MBA. As shown in Figure S1, *E. coli* [pKK-ECGDH1 + pACYC-PQQ] successfully produced MBA. However, in contrast to our previous results [10], the production yield (77.4%) of MBA from maltose obtained from this *E. coli* strain was lower than that obtained from *P. taetrolens* strain used in our previous study (100%; data not shown). This result implies that some maltose was not utilized for MBA synthesis in *E. coli*. Therefore, we attempted to identify the maltose utilization gene in *E. coli*. From a literature survey, we found that *malQ* is the first gene of the maltose utilization pathway in *E. coli* (Fig. 1) [18–20]. Therefore, in subsequent experiments, we attempted to inactivate *malQ* and investigated the effect of amylomaltase (MalQ) inactivation on MBA production.

Effect of MalQ inactivation on maltose utilization in *E. coli*

To investigate the effect of MalQ inactivation on maltose utilization in *E. coli*, the *malQ* gene in *E. coli* was knocked out, and this knockout strain (*E. coli* Δ*malQ*) was incubated in NB medium with maltose. In contrast to wild-type *E. coli*, *E. coli* Δ*malQ* cannot consume maltose (Fig. 2), confirming

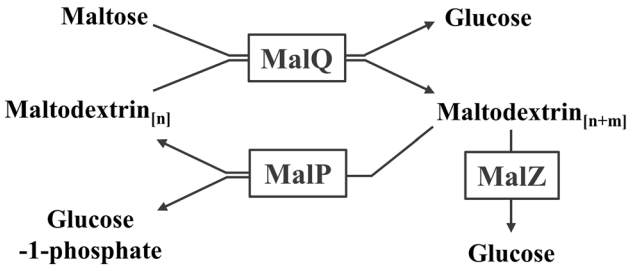

Fig. 1 Schematic representation of maltose utilization pathway in *E. coli*

that MalQ plays a critical role in maltose utilization in *E. coli*. Using *E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ], we attempted to produce MBA from maltose in subsequent experiments.

MBA production by recombinant *E. coli ΔmalQ* strain

Escherichia coli Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ] and *E. coli* [pKK-ECGDH1 + pACYC-PQQ] were cultivated in flasks containing NB medium containing maltose. The MBA production titer, yield, and productivity of *E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ] were 209.3 g/L, 100%, and 1.1 g/L/h, which were 1.3-, 1.3-, 2.2-fold higher than those of *E. coli* [pKK-ECGDH1 + pACYC-PQQ] (162.1 g/L, 77.4%, and 0.5 g/L/h), respectively (Fig. 3). This result indicates that all the available maltose was converted into MBA by *E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ], in contrast to *E. coli* [pKK-ECGDH1 + pACYC-PQQ]. Therefore, we found that *E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ] was more suitable for producing MBA from maltose.

To scale up MBA production, batch fermentation of *E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ] was conducted in a 5-L fermenter. After fermentation, the MBA production titer, yield, and productivity were 209.3 g/L, 100%, and 1.5 g/L/h, respectively (Fig. 4). This experiment established a successful MBA production scaled up in a 5-L fermenter.

Discussion

In the present study, we successfully produced MBA using a recombinant *E. coli* strain for the first time. Moreover, we established that the inactivation of MalQ, the first enzyme of the maltose utilization pathway in *E. coli*, could improve the MBA-producing capacity of the recombinant *E. coli* strain (Fig. 5). However, the MBA-producing ability of this *E. coli* strain was relatively low compared to that of *P. taetrolens* obtained in our previous study [10]. Therefore, further improvement in the MBA-producing ability of the

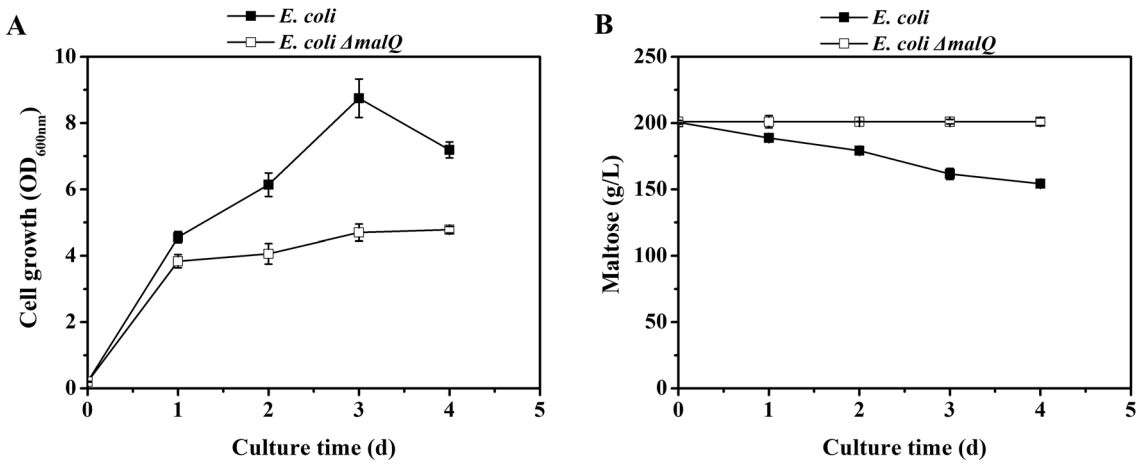

Fig. 2 Comparison of maltose consumption between the wild-type *E. coli* and *E. coli* Δ*malQ* in NB medium. Each strain was incubated in a 300 mL baffled flask containing 50 mL NB medium with 200 g/L maltose and 30 g/L $CaCO_3$ with 200 rpm rotary agitation at 25 °C. Time course of cell growth (**A**) and maltose consumption (**B**). Error bars represent the standard deviation derived from three independent experiments

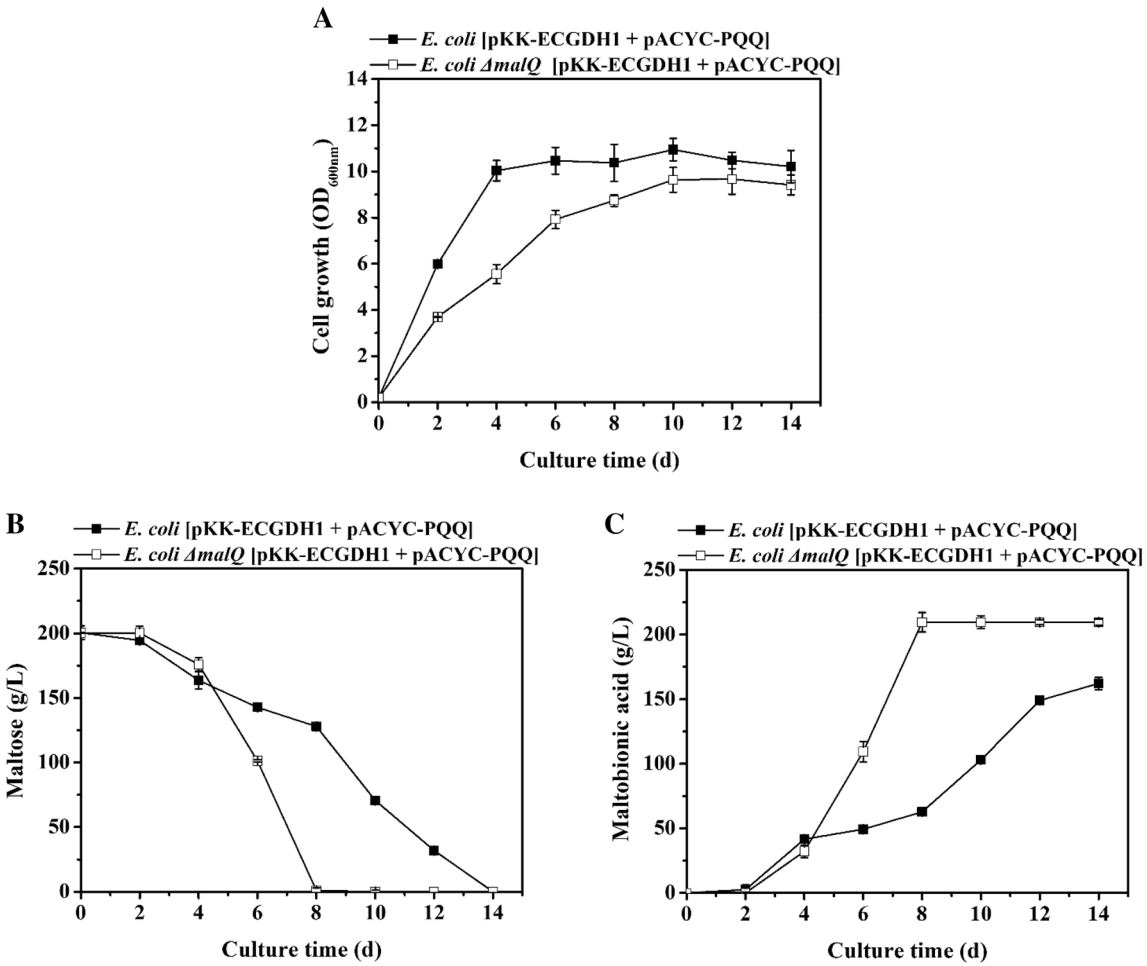

Fig. 3 Comparison of MBA production between *E. coli* [pKK-ECGDH1 + pACYC-PQQ] and *E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ] in NB medium. Each strain was incubated in a 300 mL baffled flask containing 50 mL NB medium with 200 g/L maltose and 30 g/L $CaCO_3$ with 200 rpm rotary agitation at 25 °C. Time course of cell growth (**A**), maltose consumption (**B**), and MBA production (**C**). *MBA* maltobionic acid

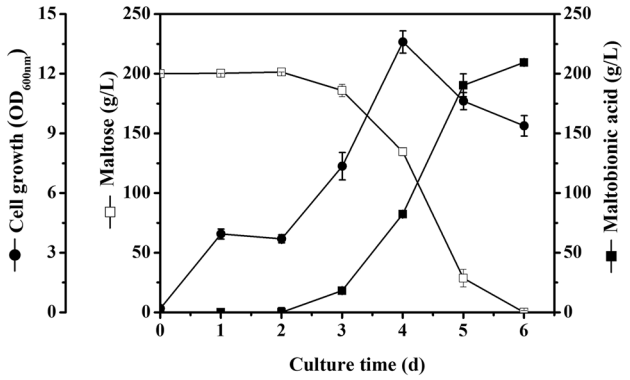

Fig. 4 Batch fermentation of *E. coli* Δ*malQ* [pKK-ECGDH1 + pACYC-PQQ] for MBA production. The *E. coli* cell was cultivated in a 5 L jar bioreactor containing 2 L NB medium with 200 g/L maltose and 30 g/L $CaCO_3$ at 25 °C. MBA, maltobionic acid

E. coli strain is needed. In a previous study, we enhanced the LBA-producing ability of an *E. coli* strain by changing the type of GDH [15]. To secure a new *E. coli* strain with improved MBA-producing ability, our future work will involve expressing various new GDHs recombinantly in *E. coli* and assessing their MBA-producing abilities. Further work will focus on improving the MBA-producing ability by the protein engineering of GDH1 from *E. cloacae*.

In addition, we are trying to improve the MBA-producing ability of the recombinant *E. coli* strain by enhancing the PQQ synthesis. In this study, the PQQ synthesis gene cluster was cloned into pACYC184, which was a low copy number plasmid having a weak constitutive *tet* promoter, and thus, the expression level of PQQ synthesis gene cluster might be rather low. Hence, the synthesis level of PQQ in the *E. coli* strain might also be low, which might be insufficient to activate all the GDH expressed in the *E. coli* strain for efficient MBA production. In our previous works, we tried to produce LBA using *E. coli* [pKK-ECGDH1], in which the PQQ synthesis gene cluster (pACYC-PQQ) was not co-expressed [15]. When 10 μM PQQ was added, *E. coli* [pKK-ECGDH1] successfully produced 209.3 g/L LBA with a productivity of 2.75 g/L/h. In contrast, *E. coli* [pKK-ECGDH1 + pACYC-PQQ] produced 209.3 g/L LBA with a productivity of 0.85 g/L/h, of which MBA productivity was only 30.9% of that obtained from *E. coli* [pKK-ECGDH1] with external PQQ addition. This result implied that PQQ was the limiting factor for LBA production. Although the accurate PQQ concentration synthesized in *E. coli* [pKK-ECGDH1 + pACYC-PQQ] was unknown, the PQQ concentration might be lower than 10 μM, implying the PQQ synthesis level of *E. coli* [pKK-ECGDH1 + pACYC-PQQ] was insufficient. From these results, enhancing the PQQ concentration in the *E. coli* strain by increasing the expression level of the PQQ synthesis gene cluster during MBA production might improve the MBA-producing ability of the *E. coli* strain. Thus, we are constructing a recombinant *E. coli* strain to increase the synthesis level of PQQ.

Recently, consumers have preferred plant-derived cosmetic components over animal-derived components because

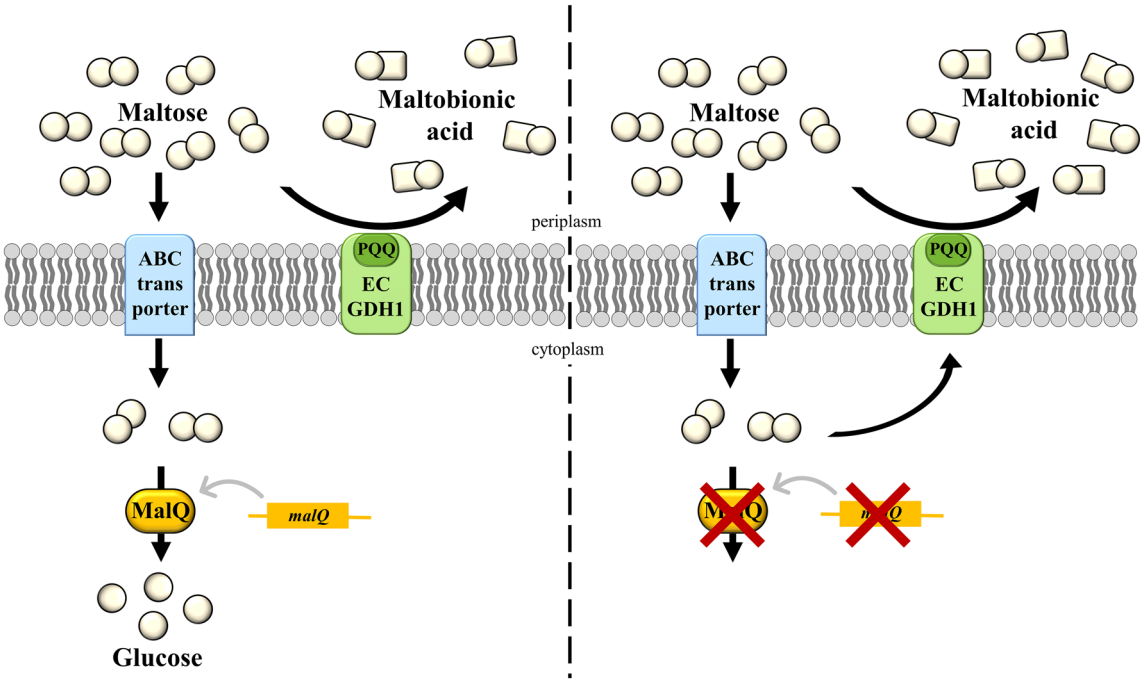

Fig. 5 Schematic representation of improvement of MBA production in the recombinant *E. coli* strain when MalQ was inactivated

of concerns regarding virus and prion contamination of animal-derived products. Consequently, materials derived from plant are generally regarded as safer for humans than animal-derived materials. LBA has been widely used in cosmetics as a moisturizing and peeling agent. However, LBA is generated from animal milk, because LBA is made from lactose. MBA is a plant-derived substance produced from maltose, a starch-derived disaccharide. Because the physicochemical properties of MBA are highly similar to those of LBA, MBA can also be used as a moisturizing and peeling agent in cosmetics. MBA is expected to be an alternative for LBA in the future because it can appeal to cosmetic consumers.

To further expand the use of MBA, lowering the price is highly desirable. The price of raw materials, particularly maltose, accounts for a substantial portion of the MBA production price. In our previous studies, we tried to produce MBA using cheap substrates. Using high-maltose corn syrup (HMCS), with a price approximately 1.1% of that of pure maltose, we successfully produced MBA [10]. Moreover, MBA can be produced using waste-cooked rice, which is significantly cheaper than HMCS and pure maltose [21]. Our study can help lower the production cost of MBA and extend the applications in industrial areas.

Conclusions

In the present study, a recombinant *E. coli* strain capable of producing MBA from maltose without the addition of PQQ was created by co-expressing the heterologous GDH gene and the PQQ synthesis gene cluster. The MBA-producing ability of this *E. coli* strain was enhanced by the inactivation of the maltose utilization pathway in *E. coli*. After batch fermentation of the *E. coli* strain using a 5-L fermenter, the MBA production titer, yield, and productivity of the strain were 209.3 g/L, 100%, and 1.5 g/L/h, respectively. Our future work will involve the cultivation of this recombinant strain at a high cell density and the production of MBA via whole-cell biocatalysis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00449-022-02835-4.

Author contributions CC: Investigation, validation, data curation, writing–original draft. GTE: Conceptualization, project administration, supervision, writing–original draft, writing–review and editing, funding acquisition, resources.

Funding This work was supported partly by the R&D programs of MOTIE/KEIT (20018375), KRICT (SS2242-10, BSF22-515), and Ulsan-KRICT (US22-03).

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no financial or commercial conflict of interest.

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