Title:

Production of Itaconic Acid in *Escherichia coli* Expressing Recombinant α -Amylase Using Starch as Substrate

Running title:

ITACONIC ACID PRODUCTION FROM STARCH USING *E. COLI*

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26 **Abstract**

27 Several studies on fermentative production of a vinyl monomer itaconic acid from hydrolyzed
28 starch using *Aspergillus terreus* have been reported. Herein, we report itaconic acid
29 production by *Escherichia coli* expressing recombinant α -amylase, using soluble starch as its
30 sole carbon source. To express α -amylase in *E. coli*, we first constructed recombinant
31 plasmids expressing α -amylases by using cell surface display technology derived from two
32 amylolytic bacteria, *Bacillus amyloliquefaciens* NBRC 15535^T and *Streptococcus bovis* NRIC
33 1535. The recombinant α -amylase from *S. bovis* (SBA) showed activity at 28°C, which is the
34 optimal temperature for production of itaconic acid, while α -amylase from *B.*
35 *amyloliquefaciens* displayed no noticeable activity. *E. coli* cells expressing SBA produced
36 0.15 g/L itaconic acid after 69 h cultivation under pH-stat conditions, using 1% starch as the
37 sole carbon source. In fact, *E. coli* cells expressing SBA had similar growth rates when grown
38 in the presence of 1% glucose or starch, thereby highlighting the expression of an active
39 α -amylase that enabled utilization of starch to produce itaconic acid in *E. coli*.

40

41 **Introduction**

42 In recent years, many techniques for the production of organic acids by using
43 microbial biomass as the sustainable feedstock have been developed (1). Among such organic
44 acids, itaconic acid attracts considerable attention as a promising monomer for synthetic
45 polymers, owing to its unique structure consisting of one vinylidene and two carboxyl groups
46 (2, 3). Biobased polymers of itaconic acid with immense potential in carbon neutrality and
47 biodegradability have been recently reported (4, 5).

48 Itaconic acid is industrially produced by the fungus *Aspergillus terreus* from sugars
49 such as glucose and sucrose (6). The highest itaconic acid yield is achieved by fermentation of
50 glucose, which is an expensive substrate for commercial production. Therefore, cheaper
51 alternative substrates such as starch and molasses have been used for itaconic acid production
52 by *A. terreus* (7). Amongst them, corn starch represents a pure, inexpensive, and stable carbon
53 source on mass supply (8). Alternatively, production of itaconic acid from raw starch may
54 represent a cheaper and a sustainable alternative; however, itaconic acid production from raw
55 starch by using *A. terreus* is not a feasible option due to its weak the starch-hydrolyzing
56 activity. To resolve this problem, Kirimura et al. (9) bred new *koji* molds exhibiting
57 **glucoamylolytic** activity and the ability to produce itaconic acid from starch by interspecific
58 protoplast fusion between *A. terreus* and *Aspergillus usamii*. As a result, *A. terreus* exhibited
59 fivefold and 70% increase in itaconic acid production when grown on soluble starch and 120
60 g/L of glucose, respectively. Since this first report on direct production of itaconic acid from
61 starch, many groups have reported hydrolysis of raw starch by acid treatment prior to
62 production of itaconic acid (10, 11). For example, Yashiro et al. (12) demonstrated an itaconic
63 acid production yield of more than 60 g/L by using *A. terreus* TN-484 in a medium containing
64 140 g/L of corn starch that was pre-treated with nitric acid at pH 2 before heat sterilization.

65 **In general, it takes a relatively long time to cultivate fungi for itaconic acid**

66 production. Compared to fungi, *E. coli* seems to be a more suitable producer of itaconic acid
67 because of its high growth rate; furthermore, gene manipulation of *E. coli* is easier than that of
68 fungi (13). Additionally, Jantama et al. (14) demonstrated that pH-stat cultivation of *E. coli*
69 mutants with glucose resulted in a high production of succinic acid (86.5 g/L; 120 h
70 cultivation) and malic acid (69.2 g/L; 144 h cultivation). These results suggest that *E. coli* has
71 the potential to produce dicarboxylic acids, such as succinic acid, malic acid, and itaconic
72 acid.

73 The *cis*-aconitate decarboxylase gene (*cad*) encodes the key enzyme that catalyzes
74 the decarboxylation of *cis*-aconitate in the tricarboxylic acid (TCA) cycle (15). Expression of
75 *cad* under its inducible T7 promoter in an overnight culture of *Escherichia coli* in
76 Luria–Bertani (LB) medium resulted in production of 0.08 g/L itaconic acid (16). In a similar
77 vein, Okamoto et al. (17) reported the expression of T7-inducible *cad* gene along with
78 inactivation of isocitrate dehydrogenase gene (*icd*) in *E. coli*. Under this condition, *E. coli*
79 produced 4.34 g/L itaconic acid after 105 h cultivation in LB medium supplemented with
80 glucose under pH-stat condition. To reduce the production cost, it will be beneficial if itaconic
81 acid can be directly produced by *E. coli* using starch as a substrate, instead of glucose.
82 Recently, cell surface display represents a unique expression system to decorate protein on the
83 cell surface (18). For instance, displaying α -amylase (EC 3.2.1.1) on the cell surface (19-22)
84 enables saccharification of starch and utilization of the resulting sugar to produce the desired
85 end products. This technique can be applied for the direct production of itaconic acid from
86 starch by using α -amylase-expressing *E. coli* because *E. coli* can intrinsically assimilate
87 maltose and maltodextrins, which are produced by α -amylase from starch, via
88 maltose/maltodextrin system (23). We propose to develop a process for direct production of
89 itaconic from starch in which the recombinant *E. coli* with cell surface display of α -amylase
90 makes it possible to saccharify starch near the cell surface and utilize the resulting sugar to

91 produce itaconic acid. However, the itaconic acid-producing *E. coli* construct harbors two
92 plasmids pETHis-cad and pLysS containing ColE1 and p15A origins, respectively. Thus, the
93 α -amylase expression plasmid must show compatibility with these plasmids in the same
94 bacterial host.

95 Here we report, for the first time, the direct production of itaconic acid from starch as
96 the sole carbon source by using *E. coli* producing heterologous α -amylase. In this study, two
97 α -amylases from *Bacillus amyloliquefaciens* NBRC 15535^T (BBA) and *Streptococcus bovis*
98 NRIC 1535 (corresponding to strain *S. bovis* 148) (SBA) were selected because both
99 α -amylases have a strong ability to be adsorbed onto starch and hydrolyze it (19, 20, 24, 25),
100 and both enzymes have been demonstrated to express in *E. coli* so far (26, 27). We compared
101 their activities at 28°C, which is the optimal temperature for itaconic acid production in *E.*
102 *coli*.

103

104 **Materials and Methods**

105 *Strains and media*

106 Bacterial strains and plasmids used or constructed in this study are listed in Table 1.
107 All *E. coli* strains were grown in LB medium or a glucose-free M9 (12 g/L Na₂HPO₄, 6 g/L
108 KH₂PO₄, 1 g/L NaCl, 2 g/L NH₄Cl, 0.24 g/L MgSO₄, 3.4 mg/L thiamine, 0.01 g/L CaCl₂)
109 minimal medium. When required, 0.5% glutamate, 1% glucose, or 0.1% or 1% soluble starch
110 was added to the medium. Antibiotics were used at the following concentrations: 100 mg/L
111 spectinomycin, 50 mg/L chloramphenicol, and 50 mg/L carbenicillin, when needed.
112 Restriction and ligation enzymes were purchased from Roche Applied Science (Penzburg,
113 Upper Bavaria, Germany), Thermo Fisher Scientific (Waltham, MA, USA), New England
114 Biolabs (Beverly, MA, USA), and TOYOBO Co., Ltd. (Osaka, Japan). Recombinant *E. coli*
115 cultivated in LB medium at 37°C for 18 h was inoculated in fresh LB medium, to an optical

density at 600 nm (OD₆₀₀) of 0.1. The cultures were subsequently grown at 28°C or 37°C until it reached OD₆₀₀ of 0.4–0.6; isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM for *cad* and/or α-amylase gene (*amyA*) expression.

Construction of the recombinant plasmids

A compatible expression vector for cell surface display of amylase was constructed as described herein. A cloning vector pGBM1 (28) was digested with *Eco*RI and *Hind*III, and subcloned into pUC18 (TOYOBO), resulting in a plasmid pGU18. The fragment of pGBM1 containing the marker *Spc*^r and pSC101 origin was amplified by PCR by using KOD plus DNA polymerase (TOYOBO), pGU18 as a template, and a primer set of M13-U (5'-CGACGTTGTAAAACGACGGCCAGT-3') and M13-R (5'-TTTCACACAGGAAACAGCTATGAC-3'), under the following conditions: denaturation at 94°C for 4 min; followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 50 for 15 s, and polymerization at 68°C for 4 min. Next, the fragment of pVUB3 expression vector (29) containing *lacIq*['], *trc* promoter, *oprI*['], and *rrnBT1T2* was amplified by PCR using KOD plus DNA polymerase, pVUB3 as a template, and a primer set of VUB3-F (5'-TTAGAATTCGAGCTCGAGCTGATACCGCTCGCCGC-3') and VUB3-R (5'-GGTGAATTCCTCATGAGCGGATACATATTTGAATG-3'). The amplification conditions for the reactions included an initial denaturation step at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, and polymerization at 68°C for 3 min. The amplified fragment of pGBM1 was phosphorylated and ligated with the amplified fragment of pVUB3 to create pGV3.

Plasmid for *amyA* expression was designed by first constructing a set of primers for PCR-based amplification by studying the deposited sequences of *amyA* genes from *Bacillus amyloliquefaciens* NBRC 15535 (GenBank database accession no. J01542) and *Streptococcus*

bovis NRIC 1535 (GenBank database accession no. AB000829). The respective *amyA* genes were amplified by PCR by using KOD plus DNA polymerase, NBRC 15535 or NRIC 1535 genome DNA as the templates, and a primer set of amyA-F1 (5'-AAAAGATCTCCGTAAATGGCACGCTGATGC-3') and amyA-R1 (5'-AAGCTGCAGCCCGCACTCAGCTTGGAGGTG-3') for NBRC 15535 or amyA-F2 (5'-CGAGATCTCAGATGAACAAGTGTCAATGAA-3') and amyA-R2 (5'-CTGCTGCAGAAGCTACTTCTTAGGGAAAGG-3') for NRIC 1535. The amplification conditions were as follows: denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, and polymerization at 68°C for 1 min. The amplified *amyA* genes from NBRC 15535 and NRIC 1535 were cloned at *Bgl*II and *Pst*I sites of pGV3 plasmid to create pGV3-BAA and pGV3-SBA, respectively.

Construction of pETHis-cad plasmid for *cad* expression and recombinant *E. coli* with inactivation of isocitrate dehydrogenase gene was carried out as described elsewhere (17). DNA isolation and manipulation were performed according to the standard protocol (30).

Fermentation condition

The recombinant *E. coli* cells were first grown in 100 mL preculture LB medium in a 500-mL Erlenmeyer flask for 18 h at 37°C and 150 rpm. After the cultures reached OD₆₀₀ of 0.4–0.6, IPTG was added at a final concentration of 1 mM for *cad* and *amyA* expression. Next, the cells were harvested and washed twice with minimal medium without any carbon source. The cells were resuspended in the M9 medium supplemented with one of the following combinations of carbon sources such as 0.5% glutamate, 0.5% glutamate and 1% starch, or 0.5% glutamate and 1% glucose to OD₆₀₀ of 0.1 before transferring them into a jar fermenter. All batch cultivations were performed in a 2-L jar fermenter M-1000B (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) with a working volume of 1.5 L at an agitation speed of 300 rpm,

temperature at 28°C, aeration rate at 1.5 L/min, and pH maintained at 6.8 by automatic addition of 1M NH₄OH or 1M phosphoric acid. The cultures were induced with a final concentration of 0.1 mM IPTG at OD₆₀₀ of 0.4–0.6 for *cad* and *amyA* expression.

Analytical method

Itaconic acid produced in the culture supernatant was quantified using a HPLC system (Prominence Shimadzu, Kyoto, Japan) equipped with a SCR-102H column (Shimadzu). Itaconic acid and acetic acid were eluted using 0.1% perchloric acid solution with a flow rate of 0.9 mL/min. The absorption of the eluate was monitored at 210 nm. A commercial itaconic acid reagent (Wako Pure Chemical Industries) was used as a control for itaconic acid quantification.

Glucose and citric acid concentrations in the culture supernatant were measured using commercial kits: Glucose CII-Test Wako (Wako Pure Chemical Industries) and Enzytec Citric Acid (R-Biopharm AG, Darmstadt, Germany), respectively. Sugar concentration in the culture supernatant was determined by the phenol-sulfuric acid method (31). Glucose-free M9 medium containing 0.5% glutamate and appropriate concentrations of starch was used as the control for residual sugar concentrations. Protein concentration was measured using the protein assay reagent Coomassie Brilliant Blue (CBB) solution (Nacalai Tesque Inc., Kyoto, Japan), based on the Bradford method (32), with bovine serum albumin as a standard.

Expression of α -amylase proteins was confirmed by SDS-PAGE (12.5% polyacrylamide gel) by using 10 μ L of cells suspension. Before application to the gel, the cell concentration was adjusted to an OD₆₀₀ of 1 with Laemmli buffer (33) and then denatured by heating at 100°C. The α -amylase activity of the soluble fraction in the homogenate of *E. coli* was determined at 28°C or 40°C by using a commercial kit, α -Amylase Assay Kit (Kikkoman Corp., Chiba, Japan) with 2-chloro-4-nitrophenyl 6⁵-azido-6⁵-deoxy- β -maltopentaoside as the

substrate according to the manufacturer's instructions. One unit (U) of α -amylase activity was defined as the release of 1 μ mol of 2-chloro-4-nitrophenol within 1 min. The plate assay for α -amylase activities was performed as follows: *E. coli* JM109 cells harboring pGV3-BAA or pGV3-SBA were inoculated on LB agar plate supplemented with 0.1% soluble starch and 1mM IPTG. After incubation for 18 h at 28°C, the plate was stained with iodine solution (0.12% iodine + 0.4% potassium iodide). Appearance of clear zones around recombinant colonies indicate starch hydrolysis. Cad-specific activity in *E. coli* transformants was assayed at 37°C according to a previously described method (17).

Dry cell weight (DCW) was calculated from the optical density (OD₆₀₀) with a linear correlation factor (1 OD₆₀₀ = 0.36 g-DCW/L).

Specific growth rate (μ) was calculated as the slope of the regression line, from a plot between $\ln(X/X_0)$ and time (t) during the exponential growth period, where X (g-DCW/L) and X_0 (g-DCW/L) are the cell concentrations at t (h) and at the beginning of the exponential phase, respectively.

Results and Discussion

Construction of E. coli plasmids for amylase expression

The constructed plasmid pGV3 and its derivatives pGV3-BAA and pGV3-SBA harboring the respective *amyA* genes from *B. amyloliquefaciens* NBRC 15535^T or *S. bovis* NRIC 1535 are illustrated in Fig. 1. The pGV3 plasmid contains a region consisting of *lacIq*', *trc* promoter, a truncated *Pseudomonas aeruginosa* lipoprotein gene *oprI*', and *rrnBT1T2* terminator from pVUB3 (29) and a region comprising the pSC101 origin and the spectinomycin resistance gene from pGBM1 (28). In fact, pVUB3 and its parental plasmids containing *oprI*' gene was used to produce several types of heterologous proteins such as the *Leishmania major* surface protease gp63 and the African swine fever virus capsid protein p72

in the outer membrane of *E. coli* (29, 34). Similarly, pGV3 is capable of cell surface display of heterologous proteins in *E. coli* and is also compatible with plasmids containing ColE1 and p15A origins. SDS-PAGE analysis showed the presence of OprI'-AmyA fusion proteins expressed in *E. coli* harboring pGV3-BAA or pGV3-SBA (Fig. 2A). Treatment of intact *E. coli* cells with Proteinase K abolished amylase activity, and the culture supernatant of *E. coli* SO14 showed negligible amylolytic activity (0.07 ± 0.00 U/mL) at 28°C (data not shown). On the other hand, SDS-PAGE analysis revealed that inclusion body proteins corresponding to aggregated OprI'-AmyA fusion proteins were formed in the SO14 strain because of a high protein expression (data not shown). These results suggest that OprI' functioned as the anchor protein and OprI'-AmyA fusion proteins were displayed in the outer membrane. Okamoto et al. (17) have generated an itaconic acid-producing *E. coli* SO07 strain that carries the *cis*-aconitate decarboxylase gene (*cad*) and an inactivated isocitrate dehydrogenase gene (*icd*). The constructed plasmids pGV3-BAA and pGV3-SBA were individually maintained in the itaconic acid-producing *E. coli* strain (data not shown), which harbors the plasmids pETHis-*cad* and pLysS containing ColE1 and p15A origins, respectively.

The *E. coli* strain showed maximal production of itaconic acid at an optimal temperature of 28°C (17). To demonstrate itaconic acid production, we performed functional assays to determine the activities of the two α -amylases expressed in *E. coli* JM109. The α -amylase from *S. bovis* NRIC 1535 clearly showed amylolytic activity (4.42 ± 1.43 U/mg-protein) at 28°C, whereas that from *B. amyloliquefaciens* NBRC 15535^T showed negligible activity (0.01 ± 0.01 U/mg-protein; Fig. 2B). Similar results (Fig. 2C) were observed on plate assay, indicating higher activity of α -amylase from *S. bovis* NRIC 1535 at 28°C than from *B. amyloliquefaciens* NBRC 15535^T. *S. bovis* 148 produces two types of α -amylase, AmyI and AmyII. The latter enzyme, which was used in this study, shows high activity at temperatures between 30°C and 50°C (26). On the other hand, *B. amyloliquefaciens*

produces a thermostable α -amylase with the temperature optimum in the range of 50°C–70°C (35). Therefore, we concluded that SBA is more suitable for hydrolysis of starch to produce itaconic acid by using *E. coli*.

Direct production of itaconic acid from starch using by E. coli

We generated SBA-expressing *E. coli* SO17 strain by introducing pGV3-SBA into itaconic acid-producing *E. coli* SO07 strain. Expression levels of α -amylase in the resulting *E. coli* strain were determined by assaying the α -amylase activity at 40°C (13.2 ± 0.6 U/mg-protein; Fig. 3A). We next investigated the effect of co-expression of *amyA* with *cad* on the activity of Cad protein in *E. coli*. Thus, the *E. coli* strain expressing both genes showed less Cad activity (0.3 ± 0.1 U/mg-protein) compared to *cad* gene expression alone (0.7 ± 0.1 U/mg-protein; Fig. 3B). This result indicates that the production of α -amylase affects the expression of Cad in the *E. coli* strain by lowering its expression. In this particular case, both *amyA* and *cad* are expressed by IPTG-inducible *lac* and T7 promoters, respectively. Thus, these promoter activities may be reduced by simultaneous expression of *amyA* and *cad* in comparison to expression of *cad* alone. In addition, *E. coli* SO15 strain could not grow well with starch, resulting in no itaconic acid production from starch (data not shown).

To demonstrate direct production of itaconic acid from starch, we used a minimal medium supplemented with starch as the sole carbon source. The constructed *E. coli* SO17, which is auxotrophic for glutamate, showed no growth in the minimal medium without glutamate, but recovered upon addition of 0.5% glutamate (data not shown). Therefore, 0.5% glutamate was always supplemented with the minimal medium when culturing *E. coli* SO17 strain. This strain produced itaconic acid in minimal medium supplemented with 0.5% glutamate and either 1% starch or 1% glucose at the optimum temperature (28°C). The pH of culture was maintained at 6.8 during the entire fermentation process by using a pH stat.

Cultivation of *E. coli* SO17 strain in the absence of starch and glucose resulted in no itaconic acid production (data not shown), indicating that the presence of glutamate in the minimal medium does not influence the pathway.

The cultivation of *E. coli* with 1% starch resulted in μ of $0.07 \pm 0.02 \text{ h}^{-1}$, maximal cell growth of $1.32 \pm 0.39 \text{ g-DCW/L}$ after 69 h, and maximal itaconic acid production of $0.15 \pm 0.08 \text{ g/L}$ after 69 h (Fig. 4A), the levels of which remained unchanged thereafter. In the presence of 1% glucose, we observed *E. coli* levels of μ ($0.10 \pm 0.00 \text{ h}^{-1}$) and maximal cell growth ($1.81 \pm 0.71 \text{ g-DCW/L}$ after 106 h) and maximal itaconic acid production of $0.62 \pm 0.22 \text{ g/L}$ after 106 h (Fig. 4B). The activity of α -amylase in *E. coli* grown in minimal medium containing 1% starch ($79.7 \pm 1.9 \text{ U/mg-protein}$) and 1% glucose ($60.4 \pm 7.3 \text{ U/mg-protein}$; Fig. 3C) were similar. These show that the α -amylase functioned and then starch was utilized in the *E. coli* strain after hydrolysis by α -amylase.

To better understand the efficiency of itaconic acid production in glucose over starch, we compared the activities of Cad in strains that were grown in both substrates. We observed similar Cad activity in *E. coli* grown in the minimal medium containing 1% starch ($0.4 \pm 0.0 \text{ U/mg-protein}$) and 1% glucose ($0.4 \pm 0.1 \text{ U/mg-protein}$; Fig. 3D). This finding suggests that production of itaconic acid is directly correlated with the carbon source in the medium rather than with the Cad activity. It is well known that glucose mediates transcriptional activation of several genes of enzymes involved in glycolysis, such as the glyceraldehyde-3-phosphate dehydrogenase gene and the 3-phosphoglycerate kinase gene (36, 37). Along with the increase in concentration of itaconic acid, we observed higher yields of acetic acid and citric acid when the cells were cultured in a medium containing glucose as compared to that containing starch (Fig. 4). These findings suggest that glucose activated metabolic flux from glucose to itaconate in the *E. coli* strain and consequently enhanced itaconic acid production.

The itaconic acid level achieved in this study is low compared to that reported in the

previous study (17). This maybe due to the following reasons. First, Cad activity in *E. coli* was lower. Second, concentration of carbon source in the medium was lower. Third, the minimal medium was used instead of LB medium. Lastly, further metabolic engineering such as aconitase overexpression was not performed. Therefore, itaconic acid production in *E. coli* from starch can be enhanced by improving these points.

Fungi, including *A. terreus*, generally require simplified media without special ingredients, like the minimal medium used in this study (7). Therefore, the purification step of products from the cultures, which involves downstream processing, can be simplified. The technique described herein for itaconic acid production from starch by *E. coli* expressing α -amylase in the minimal medium may simplify the purification steps and reduce total incurred costs, including the production costs due to carbon source.

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Figure legends

FIG. 1. Construction of the recombinant plasmids pGV3, pGV3-BAA, and pGV3-SBA. The position and direction of transcription of recombinant genes are indicated by arrows. *Spc^r*, spectinomycin resistance gene.

FIG. 2. Expression of AmyA from *S. bovis* NRIC 1535 and *B. amyloliquefaciens* NBRC 15535 in *E. coli*. (A) SDS-PAGE analysis of AmyA expression in *E. coli* JM109. Arrows indicate bands corresponding to the fusion proteins of OprI'-AmyA of *S. bovis* NRIC 1535 (upper arrow, 85.7 kDa) and *B. amyloliquefaciens* NBRC 15535 (lower arrow, 63.2 kDa). Expression of AmyA in *E. coli* strains SO12, SO13, and SO14 was performed after cultivation in 2 mL of LB medium at 37°C for 18 h following IPTG induction (B) Specific AmyA activities in *E. coli* JM109, *E. coli* SO12, SO13, and SO14 were measured after cultivation in 2 mL of LB medium at 37°C for 18 h as described in the materials and methods section. This assay was performed at 28°C in triplicate, and the mean value is presented with error bars representing standard deviations from the mean. (C) Iodine staining of 0.1% starch-containing agar plates after inoculation with *E. coli* SO12, SO13, and SO14. Clear zones indicate hydrolysis of starch around recombinant colonies.

FIG. 3. Specific AmyA and Cad activities in engineered *E. coli* strains. (A) Specific AmyA and (B) Cad activities in *E. coli* SO15 and SO17 were measured after cultivation in 2 mL of LB medium at 28°C for 18 h. This assay was performed in triplicate, at 40°C for AmyA and at 37°C for Cad, and the mean is presented with error bars representing standard deviations from the mean. (C) Specific AmyA and (D) Cad activities in *E. coli* SO17 were measured after cultivation in 10 mL preculture LB medium in a 50-mL shaking flask for 18 h at 37°C with shaking. This assay was performed in duplicate, at 40°C for AmyA and at 37°C for Cad;

the mean value is presented with error bars representing standard deviations from the mean.

FIG. 4. Production of itaconic acid in engineered *E. coli* SO17 in different culture media. The recombinant bacteria was cultivated in minimal medium supplemented with (A) 0.5% glutamate and 1% starch, and (B) 0.5% glutamate and 1% glucose. Solid circles, cell growth; open circles, itaconic acid (IA) concentration; solid squares, acetic acid (AA) concentration; open squares, citric acid (CA) concentration; open triangle, residual sugar. This cultivation was performed in duplicate, and the average was represented with error bars indicating standard deviations.

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description ^a	Source
Strains		
<i>E. coli</i> JM109	Cloning and expression host	TOYOBO
<i>Bacillus amyloliquefaciens</i> NBRC 15535	Amylolytic bacterium	NBRC
<i>Streptococcus bovis</i> NRIC 1535	Amylolytic bacterium	NRIC
SO12	<i>E. coli</i> JM109, pGV3	This study
SO13	<i>E. coli</i> JM109, pGV3-BAA	This study
SO14	<i>E. coli</i> JM109, pGV3-SBA	This study
SO7	<i>E. coli</i> BW25113 (DE3), <i>Δicd</i> , pLysS, pETHis-cad	17
SO15	<i>E. coli</i> BW25113 (DE3), <i>Δicd</i> , pLysS, pETHis-cad, pGV3	This study
SO16	<i>E. coli</i> BW25113 (DE3), <i>Δicd</i> , pLysS, pETHis-cad, pGV3-BAA	This study
SO17	<i>E. coli</i> BW25113 (DE3), <i>Δicd</i> , pLysS, pETHis-cad, pGV3-SBA	This study
Plasmids		
pETHis-cad	T7 promoter, ColE1 ori, Amp ^r , <i>cad</i>	17
pLysS	p15A ori, Cm ^r	Novagen
pUC18	pMB1 ori, Amp ^r	TOYOBO
pGBM1	pSC101 ori, Spc ^r	NBRP, 28
pVUB3	pMB1 ori, trc promoter, <i>oprI'</i> , Amp ^r	NBRP, 29
pGV3	pSC101 ori, trc promoter, <i>oprI'</i> , Spc ^r	This study
pGV3-BAA	pGV3 containing <i>amyA</i> from NBRC 15535	This study
pGV3-SBA	pGV3 containing <i>amyA</i> from NRIC 1535	This study

^aAmp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Spc^r, spectinomycin resistance.

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Fig. 3 Okamoto *et al.*

Fig. 4 Okamoto *et al.*