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Article

Construction of an *Escherichia coli* Strain Capable of Utilizing Steamed Rice as the Sole Carbon and Energy Source by Extracellular Expression of Amylase and Its Use for the Production of Putrescine

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Abstract: The *amyE* gene encoding α-amylase from *Bacillus subtilis* 168 was fused to several genes, the products of which are membrane proteins to express AmyE extracellularly. Genes of CapA, a subunit of the capsular poly-γ-glutamate synthetase of *Bacillus subtilis* subsp. *natto*; YiaT^{R232}, the first 232 residues of YiaT, an outer membrane protein of *Escherichia coli* K-12; and PgsA, cytidine 5′-diphosphate-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase of *B. subtilis* subsp. *natto*, were used. The *Escherichia coli* strain harboring the plasmid carrying the *pgsA-amyE* fusion expressed a very high level of amylase not only on the extracellular surface of the cells but also in the medium and the intracellular space and solubilized steamed rice in two days. The N-terminal amino acid sequence of the amylase purified from the culture medium (Ser-Ala-Glu-Thr-Ala) indicated that it was cleaved at the signal peptide cleavage site of AmyE. The strain SH2204, transformed with pKN11 carrying *speA* (arginine decarboxylase gene), *speB* (agmatinase gene) and *argA*$^{ATG\ Y19C}$ (*N*-acetylglutamate synthase gene) and pMAN63 carrying *pgsA-amyE* produced 1.2 mM putrescine from 5 mM arginine by adding 0.5 mM IPTG in eight days. Discarding steamed rice is not only a food loss, but also a waste of renewable, biogenerated resources due to the burning of it with petroleum, increasing carbon dioxide in the atmosphere. The *E. coli* strain developed in this study can help solve this problem because it can produce an important chemical using steamed rice as the sole carbon and energy source.

Keywords: α-amylase; starch; renewable; biogenerated resource; putrescine

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1. Introduction

Escherichia coli has been used to produce a variety of useful substances. Recently, attempts have been made to make *E. coli* produce chemicals that are conventionally produced from petroleum, such as cadaverine [1,2], succinate [3,4], butanol [5–7], adipic acid [8,9], propanol [10,11], ethanol [12–14] and methane [15]. *E. coli* requires some carbon sources, such as sugars, to grow. However, *E. coli* cannot utilize starch because it cannot uptake such a large molecule, nor does it secrete amylase into the extracellular space to break it down into smaller sizes. There have been reports that *E. coli, Corynebacterium glutamicum* and *Lactobacillus casei* strains expressing amylase on the extracellular surface of the cells using anchor proteins utilized and grew on soluble starch or corn starch cooked at a low temperature as the sole carbon source [16–20]. Starch is a long-chain compound of glucose linked primarily by α-1,4 glucoside linkage but sometimes branched by α-1,6 glucoside linkage and is insoluble in water. Starch is cleaved and shortened by acid to produce soluble starch. Although the main component of steamed rice is starch, it is not refined and is in the form of rice grains. Unsold boxed lunches on the shelves of convenience stores and supermarkets that are nearing their expiration date, as well as leftover food from restaurants, are discarded and burned using petroleum, even though they are renewable, biogenerated resources, which increases carbon dioxide in the atmosphere. In Japan,

steamed rice, the staple food, is served on separate plates or bowls from other dishes in restaurants and packed in a special compartment in lunch boxes. This makes it easy to collect steamed rice separately. Unfortunately, there are no reports of *E. coli* that can utilize and grow on steamed rice as its sole carbon and energy source. Therefore, we decided to breed *E. coli* strains that could utilize steamed rice as the sole carbon and energy source. Our first goal of this research is to express high levels of amylase extracellularly using protein fusion of membrane protein and amylase.

We have successfully expressed *E. coli* SpeA (arginine decarboxylase) using *Bacillus natto* CapA (a subunit of the capsular poly-γ-glutamate synthetase) and *E. coli* GGT (γ-glutamyltranspeptidase) using *E. coli* YiaT^{R232} (the first 232 residues of an outer membrane protein of YiaT) to produce agmatine [21] and γ-Glu-Val-Gly [22], respectively. In these cases, SpeA and GGT were expressed on the extracellular surface of *E. coli* cells, and CapA and YiaT^{R232} served as anchor proteins. As we described in the previous paper [22], there are two *pgsA* genes in *B. subtilis* subsp. *natto*. One encodes a subunit of the capsular poly-γ-glutamate synthetase (which we call *capA* in this report) and the other encodes cytidine 5′-diphosphate-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (which we call *pgsA* in this report). This causes confusion because the same term is used to name the distinct enzyme genes. In fact, we mistakenly started to use the gene coding cytidine 5′-diphosphate-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase instead of a subunit of the capsular poly-γ-glutamate synthetase. In this report, we use the term "*pgsA*" to indicate the gene encoding cytidine 5′-diphosphatediacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase according to the KEGG genome database and GenBank. To our surprise, we obtained unexpectedly high amylase activity using PgsA-AmyE protein fusion and report this in this paper.

Petroleum is not only used as a fuel but about 20% of total petroleum consumption is used as a raw material for chemical products (https://oil-info.ieej.or.jp/whats_sekiyu/1-10.html, (accessed on 18 April 2023) in Japanese). Chemical fibers have been mainly produced from petroleum. Nylon 4,6, one of the fiber materials, is a type of polyamide synthesized by polycondensation of adipic acid and putrescine. Due to its excellent properties, such as its heat resistance and solvent resistance, plastics made from it have various applications. As the number of electric vehicles increases, the demand for nylon 4,6 for interiors, motors, controllers and electronic circuit boards is expected to continue to grow. We have developed an *E. coli* strain KT160 that can produce putrescine [23]. Figure 1 summarizes which parts of the polyamine metabolic pathway of *E. coli* were modified to obtain strain KT160. SpeA (arginine decarboxylase), SpeB (agmatinase) and ArgA$^{ATG\ Y19C}$ (*N*-acetylglutamate synthase; the first enzyme of arginine biosynthetic pathway), the initiation codon of which was substituted for the inefficient GTG by ATG and has the arginine-insensitive mutation Y19C overexpressed from the T5 promoter to increase the synthesis of arginine and its conversion to putrescine. ArgR (the repressor of the arginine biosynthetic pathway) was knocked out to release the arginine biosynthetic pathway from repression. The genes of PuuA (γ-glutamylputrescine synthetase), PatA (putrescine aminotransferase), SpeD (*S*-methionine decarboxylase) and SpeG (spermidine *N*-acetyltransferase), which metabolize putrescine, were knocked out to stop the consumption of putrescine. To prevent the re-import of excreated putrescine into the cells, the genes encoding the transporters PuuP and PotE were also deleted. In addition, the YifE^{Q100TAG} mutation, the mechanism of which for increasing putrescine yield is still unclear, was also introduced. As a result, strain KT160 synthesized a lot of putrescine but it cannot use steamed rice as its sole carbon and energy source. Our second goal of this research is to demonstrate the feasibility of producing putrescine using *E. coli* grown on steamed rice as the sole carbon and energy source.

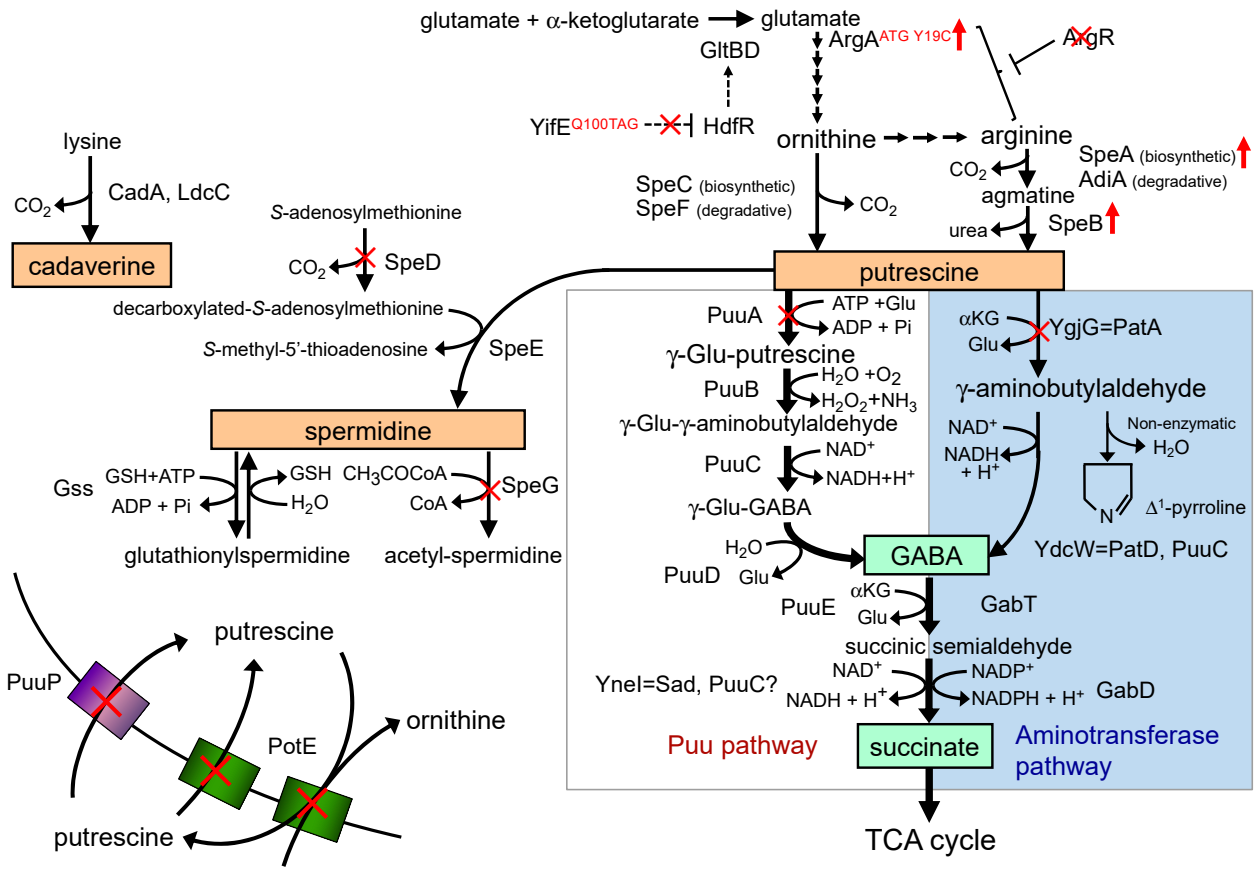


Figure 1. Metabolic pathways of polyamines in *E. coli*. Mutations introduced into strain KT160 used for putrescine production in [23] are indicated by red symbols and letters. Red arrows pointing upward indicate the overexpressed enzymes, red cross marks indicate the knocked-out genes and red letters on ArgA and YifE indicate the point mutations introduced.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids and Oligonucleotides

The bacterial strains and plasmids and oligonucleotides used in this study are listed in Tables 1 and 2, respectively. The plasmids were constructed based on pACYC184 and pQE-80L. Strain KT160 [23], which produces a large amount of putrescine outside the cells, was transformed with pMAN63 to obtain SH2287.

Table 1. Strains and plasmids used in this study.

Strains and Plasmids	Genotype	Source and Reference
(*Escherichia coli* K-12)		
AN7	pAN7/DH5α	This study
AN14	pMAN63 pSH1733/SH2204	This study
DH5α	F$^-$ *supE44* Δ*lacU169*(Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	
KT160	pKN11/SH2204	[23]
MG1655	F$^-$ *rph-1*	Carol A. Gross
SH2204	F$^-$ *rph-1* Δ*argR*::FRT Δ*patA*::FRT Δ*potE*::FRT Δ*speD*::FRT Δ*speG*::FRT Δ*argA*::FRT Δ*puuPA*::FRT *yifE*^{Q100TAG} *zie-296*::Tn*10*	[23]
SH2287	pMAN63 pKN11/SH2204	This study
(plasmids)		
pACYC184	p15A replicon *cat*$^+$ *tet*$^+$	Stanley N. Cohen, [24]

Table 1. *Cont.*

Strains and Plasmids	Genotype	Source and Reference
pAN1	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*pgsA*$^{+}$-*amyE*(Δsignal)	This study
pAN2	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*pgsA*$^{+}$-*amyE*ΔsignalΔpropeptide	This study
pAN5	p15A replicon *cat*$^{+}$ T5p-*yiaT*^{R232}-*amyE*ΔsignalΔpropeptideΔ12bp	This study
pAN7	p15A replicon *cat*$^{+}$ *tet*^{S}-T5p-*pgsA*$^{+}$-*amyE*-His$_9$-*rrnB*T1 *lacI*^{q}	This study
pFT06	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*capA*$^{+}$-*amyE*$^{+}$	This study
pKN11	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*speA*$^{+}$-*speB*$^{+}$-*argA*$^{ATG\ Y19C}$	[23]
pMAN36	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*pgsA*$^{+}$	This study
pMAN39	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*pgsA*$^{+}$-*amyE*$^{+}$	This study
pMAN63	p15A replicon *cat*$^{+}$ *tet*^{S}-T5p-*pgsA*$^{+}$-*amyE*$^{+}$-*rrnB*T1 *lacI*^{q}	This study
pMAN95	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*yiaT*^{R232}-*amyE*ΔsignalΔpropeptideΔ12bp	This study
pMAN99	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*capA*$^{+}$-*amyE*ΔsignalΔpropeptideΔ12bp	This study
pMAN100	p15A replicon *cat*$^{+}$ T5p-*capA*$^{+}$-*amyE*ΔsignalΔpropeptideΔ12bp	This study
pQE-80L	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-(His)$_6$	Quiagen
pSH1733	ColE1 replicon *bla*$^{+}$ *lacI*^{q} T5p-*argA*$^{ATG\ Y19C}$	[23]
(*Bacillus subtilis*)		
168	*trp*	Pasteur Institute
subsp. *natto* OK2	*bio*	Fujio Kawamura, [25]

Table 2. Primers used to construct plasmids in this study.

No.	Name	DNA Sequence
1	pQE_EcoRI_ATG_pgsA	5′-cccgaattcattaaagaggagaaattaactATGTTTAACTTA-CCGAATAAAATCAC-3′
2	PstI_natto_pgsA_Cterm	5′-cccctgcagGTTAGATGTTTTTAACGCTTCC-3′
3	Bsub168 alpha-amylase_C	5′-attaagcttTCAATGGGGAAGAGAACCGC-3′
4	Bsub 168 alpha-amylase Hind3_N	5′-GCCAAGCTTATTTGCAAAACGATTCAAAAC-3′
5	pgsA C-comp	5′-GTTAGATGTTTTTAACGCTTCCCAG-3′
6	amyE deltasignal_F	5′-CCGGCGGCTGCGAGTGCTGAAACGG-3′
7	amyE deltasignal_F2	5′-CTTACAGCACCGTCGATCAAAAGCG-3′
8	pQE80L-yiaTR232_infusion-F	5′-caatttcacacagaattattaaagaggagaaattaactATGTTA-ATTAATCGCAATATTG-3′
9	amyE(136bp)-F	5′-TCGATCAAAAGCGGAACCATTCTTCATGCA-TGG-3′
10	yiaTR232-amyE(136bp)_infuR	5′-GGTTCCGCTTTTGATCGAACGATCAATCATC-GGGCTGTCG-3′
11	pQE80L-amyE_infuR.new	5′-gctaattaagcttggcTCAATGGGGAAGAGAACCGC-TTAAGC-3′
12	EcoRI_RBS_ATG-ywtBF	5′-gaattcattaaagaggagaaattaactATGAAAAAAGAA-CTGAGCTTTCATGAAAAGCTGC-3′
13	RBS_EcoRI-T5pR	5′-agttaatttctcctctttaatgaattctgtgtgaaattgttatccgctcac-aattg-3′
14	amyE(136bp)-ywtB_infuR	5′-GGTTCCGCTTTTGATCGATTTAGATTTTAGTT-TGTCACTATGATC-3′
15	amyE(136bp)-F	5′-TCGATCAAAAGCGGAACCATTCTTCATGCA-TGG-3′

Table 2. *Cont.*

No.	Name	DNA Sequence
16	infuF_EcoRI-RBS-ywtB(25bp) new	5′-gataacaatttcacacagaattcattaaagaggagaaattaact-ATGAAAAAAGAACTGAGCTTTCATG-3′
17	infuR_amyE(15bp)-ywtB(25bp)	5′-GAATCGTTTTGCAAATTTAGATTTTAGTTT-GTCACTATGATC-3′
18	amyE-F	5′-TTTGCAAAACGATTCAAAACCTCTTTACTG-CCG-3′
19	pACYC184_T5p-F	5′-gcgaccacacccgtcctgtggatcgacgtctaagaaaccattattat-catgacattaacc-3′
20	pACYC184_T5p-R	5′-ttctcctctttaatgaattctgtgtgaaattgttatccgctcacaattg-3′
21	EcoRI_RBS_ATG_iPCR-F	5′-gaattcattaaagaggagaaattaactATG-3′
22	rrnBT1terminator_lacI-R	5′-cccgactggaaagcgggcagtgaggcggatttgtcctactcagga-gagcg-3′
23	lacI-F	5′-tcactgcccgctttccagtcgggaaacctgtcg-3′
24	pACYC184_lacIqp-R	5′-ccgccgccgcaaggaatggtggacaccatcgaatggtgcaaaac-ctttcgcgg-3′
25	BamH1-tamakiN	5′-cccggatccgaggcctttcgtcttcacctcgag-3′
26	Hind3-amyE-C	5′-cccaagcttggcTCAATGGGGAAGAGAACCGCTT-AAGC-3′
27	H5_TGA_BglII_KpnI	5′-CATCACCACCATCATTGAagatctgagcatggtaccc-ttgaggc-3′
28	H5_amyE_comp	5′-GTGATGATGATGATGGGGAAGAGAACCGC-TTAAGCCCCAGTC-3′

The numbers of the primers correspond to the numbers of the primers shown in Figure 2 and Table 3. Capital letters indicate the amino acid coding regions and stop codons. Single underlines and double underlines indicate the restriction endonuclease recognition sites and the α-amylase coding regions, respectively.

2.2. Construction of Plasmids Express α-Amylase in the Extracellular Space of E. coli

Figure 2A (left) shows schematics of the important parts of the plasmids used to express α-amylase. YiaT, the role of which is not known, is an outer membrane protein of *E. coli*. Han and Lee [16] reported that lipase and amylase were successfully expressed on the extracellular surface of *E. coli* using the Met1 to Arg232 region of YiaT as an anchor. The Arg232 residue is located in the fifth extracellular loop of YiaT. As described above, CapA is identical to PgsA by Ashiuchi et al. [26], which has been used as an anchor by other researchers [17,18]. The PgsA used in this study is a completely different protein from those that have been used as an anchor protein before by several researchers [17,18]. The *pgsA* and *capA*, *amyE* and *yiaT* genes were amplified using genomic DNA from *B. subtilis* subsp. *natto*, *B. subtilis* 168 and *E. coli* K-12 MG1655, respectively, as templates and the primers listed in Table 2 using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). pAN1, pAN2 and pAN7 were obtained using inverse PCR using KOD-plus mutagenesis kit (Toyobo, Osaka, Japan). For pMAN99 and pMAN 100, the amplified DNAs were treated with the In-Fusion HD cloning kit (Takara-Bio, Kyoto, Japan). For pMAN63, pMAN95 and pFT06, the amplified DNAs were digested with restriction endonucleases and then treated with the In-Fusion HD cloning kit. For pAN5, pMAN36 and pMAN39, the amplified DNA was digested with restriction endonucleases and then ligated to the plasmid digested with the same restriction endonucleases.

2.3. Comparison of Amylase Activity by the Iodine-Starch Test

Each plasmid-containing strain was grown in 10 mL LB medium Miller (Becton Dickinson, Sparks, MD, USA) supplemented with 100 μg/mL ampicillin and/or 20 μg/mL chloramphenicol at 37 °C for 16 h with reciprocal shaking at 120 rpm. The culture solution was collected and centrifuged at 5000× *g* for 5 min at 4 °C. The supernatant was collected in a tube and used as the culture supernatant sample. The cells were washed once with 10 mL of 100 mM Tris-HCl (pH 7.2) and resuspended in 10 mL of the same buffer. The OD_{600} of

the cell suspension was measured. Then, half of the cell suspension was sonicated with an ultrasonic generator (201M, Kubota, Tokyo, Japan) at 200 W for 10 min and centrifuged at 8000× *g* for 5 min at 4 °C. The supernatant was collected for use as a cell-free extract. Culture supernatants, cell suspensions and cell-free solutions were diluted with 100 mM Tris-HCl (pH 7.2) to correspond to that prepared from a culture with OD_{600} = 2.0.

Each sample (400 µL) was mixed with 400 µL of 2% (*w/v*) soluble starch dissolved in 100 mM Tris-HCl (pH 7.2) and incubated at 37 °C for 54 h. An amount of 10 µL of 1 N HCl was added to 100 µL of the reaction mixture and the reaction was terminated by vortexing followed by centrifugation at 8000× *g* at 4 °C for 10 min. An amount of 90 µL of the supernatant was mixed with 10 µL of 2% iodine-1% potassium iodide solution. An amount of 50 µL of the mixture was transferred to the new tube and photographed.

2.4. Western Blot Analysis of α-Amylase Expressed by Strain AN7

Plasmid pAN7, which expresses PgsA-AmyE with 9 additional His residues at the C-terminus, was obtained using inverse PCR using KOD-plus mutagenesis kit with pMAN63 as the template and primers 27 and 28. Strain AN7 (pAN7/DH5α) was grown in 50 mL of LB with 20 µg/mL chloramphenicol at 37 °C with reciprocal shaking at 120 rpm for 90 min. IPTG was added to a final concentration of 0.5 mM and the strain was allowed to grow for 18.5 h. An amount of 1 mL of the culture was centrifuged at 8000× *g* for 5 min at 4 °C. The collected cells were suspended in 500 µL of 100 mM Tris-HCl (pH 7.4), vortexed and then centrifuged at 8000× *g* for 5 min at 4 °C. After removing the supernatant, the precipitate was suspended in 100 µL of loading buffer, boiled for 5 min, centrifuged at 8000× *g* for 5 min and the supernatant was used as the cell sample. The proteins in the culture medium were concentrated as follows. The remaining culture was centrifuged at 5000× *g* for 5 min at 4ºC. Ammonium sulfate was added to the collected supernatant to 80% saturation. The solution was centrifuged at 5000× *g* for 10 min at 4 °C. The precipitate was dissolved in 1 mL of 100 mM Tris-HCl (pH 7.4) and dialyzed against the same buffer. The dialyzed sample was concentrated to 100 µL using an Amicon Ultra centrifugal filter (Ultracel 30K; Millipore, Billerica, MA, USA). An amount of 10 µL of the concentrated sample was mixed with the same volume of loading buffer and boiled for 5 min to use as the extracellular sample. An amount of 10 µL of the cell and extracellular samples were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using rabbit anti-His-tag antibody (Medical & Biological Laboratories, Tokyo, Japan) as the first antibody and goat peroxidase-linked anti-rabbit IgG(H+L) (Jackson Immuno Research Laboratories, West Grove, PA, USA) as the second antibody. POD Immunostain Set (Fujifilm Wako, Osaka, Japan) was used to stain the bands reacted with the second antibody.

2.5. Purification of α-Amylase from the Culture Medium of AN7 and Determination of Its N-Terminal Amino Acid Sequence

The remaining concentrated culture supernatant prepared above was subjected to 1.5 mL of Ni-Sepharose 6 Fast Flow (GE Healthcare Bio-Sciences, Uppsala, Sweden) column chromatography. Amylase was eluted with 20 mM Tris-HCl containing 0.5 M NaCl and 200 mM imidazole (pH 7.4), concentrated using an Amicon Ultra centrifugal filter and then was subjected to SDS-polyacrylamide gel electrophoresis followed by blotting onto a PVDF membrane (Immobilon P; Millipore, Billerica, MA, USA), staining with 0.025% Coomassie brilliant blue R-250 in 40% methanol and destaining with 60% methanol. After drying, the stained band was cut off and sent to Hokkaido System Science (Sapporo, Japan) for N-terminal amino acid sequencing by Edman degradation.

2.6. Production of Putrescine in 1 × M9 Steamed Rice Medium

An amount of 45.5 g of polished rice (Akita-komachi brand) and a stirrer bar (5 cm) were placed in a 300-mL conical beaker and washed twice with deionized water. After 54.6 mL of deionized water was poured into the conical beaker, the lid was covered with aluminum foil and the conical beaker was autoclaved at 121 °C for 20 min. After

cooling, the rice was mixed with 100 mL of 2 × M9 minimal medium and ampicillin and chloramphenicol were added at the final concentrations of 100 μg/mL and 20 μg/mL, respectively. Strain SH2287 was grown overnight in 50 mL LB medium at 37 °C. Cells were collected by centrifugation at 5000× *g* for 10 min at 25 °C, washed twice with 1 × M9 buffer and then suspended in 1 mL of 1 × M9. The 1 × M9 steamed rice medium was inoculated with SH2287 prepared as above to an initial OD_{600} of 0.05 and incubated at 37 °C with stirring at 750 rpm. After 2 days of incubation, arginine and isopropyl-β-D-1-thiogalactopyranoside (IPTG) were added.

At the indicated time, 300 μL of the culture was sampled and the reaction was terminated by mixing with 30 μL 100% trichloroacetic acid. After allowing the sample to stand for 10 min, the sample was centrifuged at 8000× *g* for 10 min at 4 °C. 4 μL of the supernatant was mixed with 196 μL of HPLC mobile phase. After centrifugation at 8000× *g* for 5 min at 4 °C, the supernatant was filtrated using the Millex-LH Syringe Driven Filter Unit (Millipore, Billerica, MA, USA). The filtrate was analyzed by HPLC.

2.7. Measurement of Putrescine

An amount of 5 μL of the filtrates prepared as described above was injected into an HPLC (LC-20, Shimadzu, Kyoto, Japan) equipped with a fluorescence detector (RF-10A_{XL}, Shimadzu) and TSKgel Polyaminepak column (4.6 × 50 mm) (Tosoh, Tokyo, Japan). The instrument was used as described previously [27,28]. The means of the putrescine concentrations of three independent cultures are shown in the results with standard deviations calculated by the STDEV function in Excel.

3. Results and Discussion

3.1. Comparison of Amylase Activity Expressed from Various Plasmids

There is some controversy as to which amino acid residues make up the signal peptide and the pro-peptide of *Bacillus subtilis* α-amylase, as shown in the upper left of Figure 2A. Culture supernatants, cell suspensions (intact cells) and cell-free solutions from each strain were mixed with soluble starch. After 54 h of incubation, the samples were subjected to the iodine-starch test and the amount of starch remaining was compared with the color. As starch is digested, the color of the reaction solution with iodine changes from dark bluish purple to brown, to golden, to yellow. As shown in Figure 2A, pMAN95 fused YiaT^{R232} to Ser46 of AmyE without inserting any additional amino acid residues resulting from the linker. On the other hand, the plasmid of Han and Lee [16] inserted Ser- and Arg-residues in between because of the restriction recognition site. DH5α transformed with pMAN95 expressed amylase on the extracellular surface as reported by Han and Lee [16]. DH5α transformed with pMAN99, which fused CapA to Ser46 of AmyE, expressed amylase in the medium but virtually no activity on the extracellular surface. DH5α transformed with pFT06, which fused CapA to Phe2 of AmyE, did not express amylase. On the other hand, to our surprise, DH5α transformed with pMAN39, which fused PgsA to Phe2 of AmyE, showed very high amylase activity in its culture supernatant, extracellular surface and cell-free solution. In contrast, DH5α transformed with pAN1 plasmid without a signal peptide and DH5α transformed with pAN2 plasmid without a signal peptide and propeptide showed no amylase activity. DH5α was transformed with pMAN36, which contains only *pgsA* without a stop codon at the regular position but has a stop codon after the addition of Leu-Gln-Pro-Ser-Leu-Ile-Ser. This transformant is a negative control and it had no amylase activity as expected. pMAN63 has the same construct from the T5 promoter-*pgsA-amyE* region as pMAN39, but the base plasmid is pACYC184 instead of pQE-80L. The color of the reaction mixture used DH5α transformed with pMAN63 is obviously brighter yellow than that of DH5α transformed with pMAN39. Therefore, we decided to use pACYC184-based plasmids.

As DH5α transformed with pMAN39 expressed a large amount of amylase outside, inside and on the extracellular surface of the cells, we wondered how the amylase expressed from this plasmid was processed and excreted into the medium. Plasmid pAN7

was designed to add a His-tag to the C-terminus of amylase (Figure 2B, top). After we confirmed that strain AN7 (pAN7/DH5α) showed very high amylase activity in the culture supernatant (Figure 2B, middle), the total cells and proteins collected from the culture supernatant of AN7 were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with anti-His-tag antibody. As shown in Figure 2B (bottom left), both samples showed bands between 70K and 55K. The molecular size of these bands suggested that the signal peptides were cleaved off in these cases. To confirm this, the amylase excreted in the culture supernatant was purified with the Ni-Sepharose column (Figure 2B, bottom right) and subjected to the N-terminal amino acid sequencing. The obtained N-terminal amino acid sequence, Ser-Ala-Glu-Thr-Ala, indicates that the signal peptide is cleaved between Ala31 and Ser32. This signal peptide cleavage site coincides with the site predicted by both SOSUI and SignalP programs.

DH5α transformed with each of pMAN63 and pACYC184 was grown in 1 × M9 steamed rice medium to compare rice lysis. As shown in Figure 2C, DH5α transformed with pMAN63 obviously lysed rice on day 2, while the DH5α transformed with pACYC184, which does not express PgsA-AmyE, remained as swollen steamed rice in 1 × M9 steamed rice medium.

These results indicate that our first goal of constructing an *E. coli* strain that expresses high levels of amylase extracellularly using a membrane protein and grows on steamed rice was achieved.

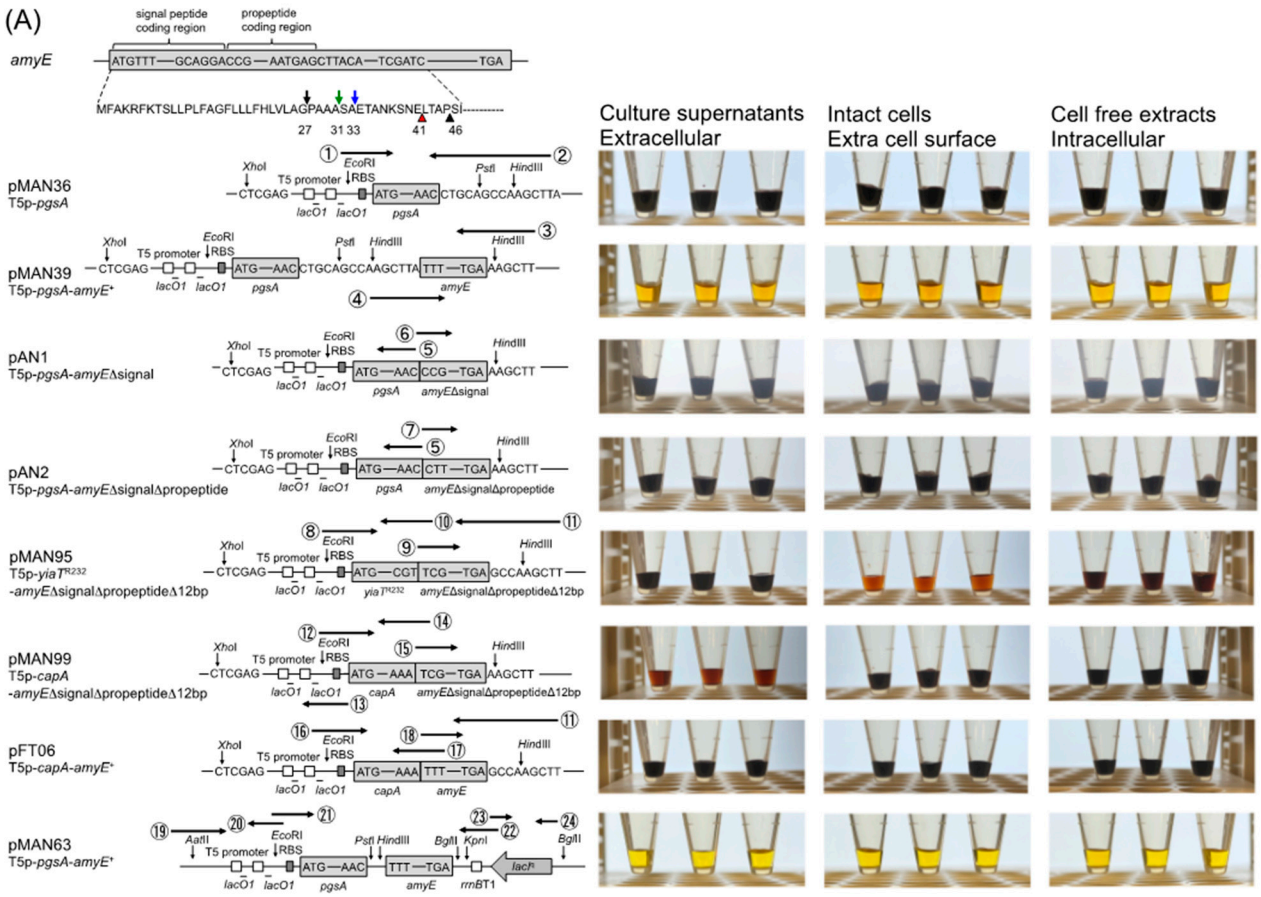


Figure 2. *Cont.*

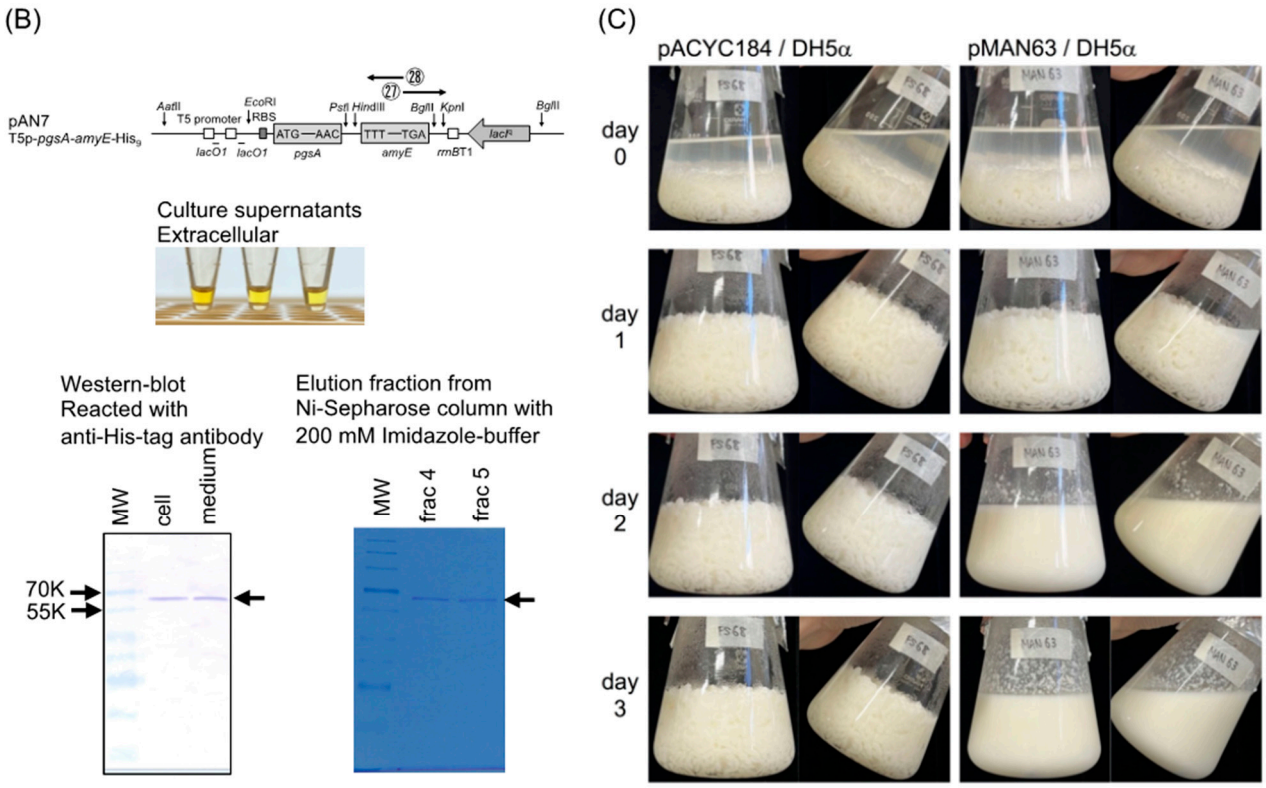


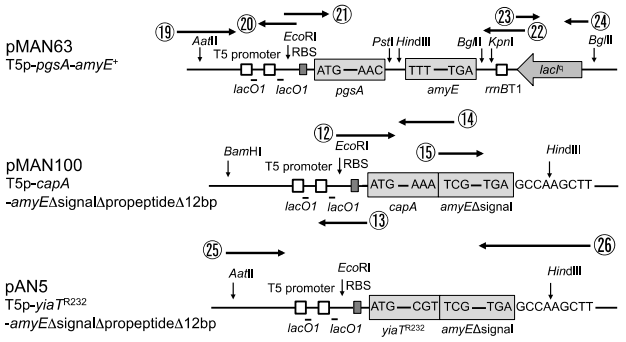
Figure 2. (**A**) (Left) Schematics of the important parts of the *amyE* gene of *Bacillus subtilis* and translated sequence of the N-terminal part of AmyE (top) and plasmids used in this study. Downward black, green and blue arrows indicate signal peptide cleavage sites predicted by the Bsubcyc website, SOSUI and SignalP programs and Yamazaki et al. [29], respectively. Upward red arrow indicates propeptide cleavage site indicated by Bsubcyc website and Yamazaki et al. [29] and the N-terminal of the purified mature extracellular AmyE was Leu-42, as shown by Mäntsälä and Zalkin [30]. The coding regions for the signal peptide and propeptide shown above the sequence are described based on Bsubcyc. Upward black arrow indicates the position of the N-terminal of AmyE connected to YiaT^{R232} by Han and Lee [16]. pMAN63 is based on pACYC184 (Cam^{R}) and the other plasmids are based on pQE-80L (Amp^{R}). (Right) Comparison of the iodine-starch reaction of the DH5α strains carrying the plasmids shown on the left using culture supernatants, intact cells and cell-free extracts. (**B**) Schematic of pAN7 (top) and the result of iodine-starch test of AN7 (middle). The result of Western blot analysis of AN7 cells and culture supernatant of AN7 and the result of SDS-PAGE of fractions 4 and 5 from Ni-Sepharose column (bottom). The MW marker used was the clearly stained protein ladder (Takara-Bio; Kyoto, Japan). (**C**) Comparison of the steamed rice media with pMAN63/DH5α and pACYC184/DH5α on day 0, 1, 2 and 3.

3.2. Comparison of Putrescine Production Expressed from Various Plasmids

Since pMAN63 gave a better result than pMAN39, the *tet*^{R} gene region of pACYC184 was replaced by the T5 promoter-*capA-amyE* region of pMAN99 and the T5 promoter-*yiaT*^{R232}-*amyE* region of pMAN95 to give pMAN100 and pAN5, respectively. Strain KT160 contains pKN11 in SH2204. Although SH2204 is a Δ*argA* strain, pKN11 contains *argA*$^{ATG\ Y19C}$, which has an efficient initiation codon ATG instead of GTG and a Y19C mutation that results in desensitization to arginine, so that KT160 is not arginine auxotrophic as long as ArgA is expressed from the plasmid. KT160, which produces a large amount of putrescine [23], was transformed with pMAN63, pMAN100 and pAN5, respectively. Putrescine production by these transformants was compared with 1 × M9 steamed rice medium with the addition of ampicillin and chloramphenicol, but without the addition of arginine and IPTG as described in Materials and Methods. As shown in Table 3, KT160 transformed with pMAN63 had the highest putrescine production.

Table 3. Effect of amylase-expressing plasmid on putrescine yield.

Plasmid Used to Transform KT160	Yield of Putrescine (μM)	SD
pMAN63	292	3.4
pMAN100	229	17
pAN5	246	8.3



KT160 (pKN11/SH2204) was transformed with each plasmid. Day 9 yields are compared. SD: standard deviation.

3.3. Effect of Arginine Supplementation on Putrescine Production

The effect of the addition of arginine on the 1 × M9 steamed rice medium on putrescine production was investigated.

By adding arginine to the medium, the yield of putrescine increased (Figure 3). The results suggested that the arginine productivity of KT160 transformed with pMAN63 was not sufficient. We did not add IPTG because pMAN63 allowed *E. coli* to produce a lot of amylase without the addition of IPTG. In addition, we were concerned that too much extracellular expression of amylase could affect the stability of the cell membrane.

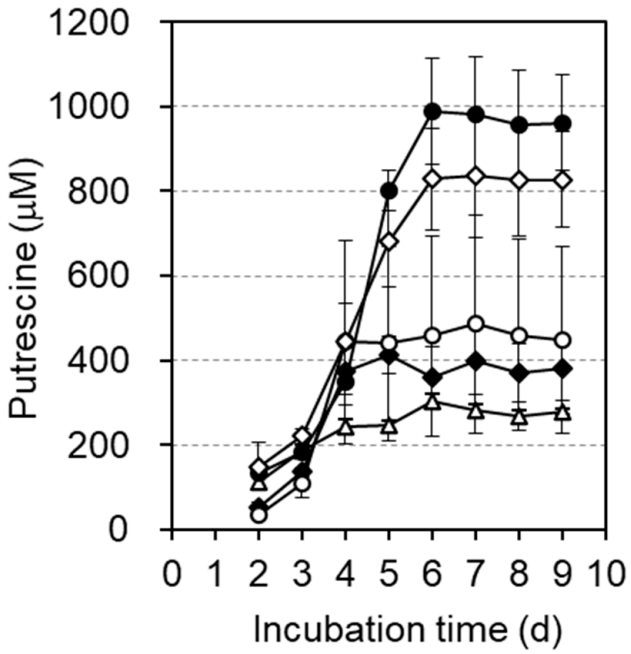


Figure 3. Effect of arginine addition on putrescine production by SH2287 (pMAN63/KT160). Arginine solution (pH 7.0) was added to the medium on day 2 at final concentrations of 0 (light triangles), 1 (black diamonds), 2 (light circles), 5 (black circles) and 10 mM (light diamonds). The mean values of three conical beakers are shown with the standard deviation.

3.4. Effect of IPTG Addition on Putrescine Production

Various concentrations of IPTG were added to the medium and the effects were observed in the presence of 5 mM arginine. Up to the addition of 0.5 mM of IPTG, there was a positive effect on putrescine production (Figure 4).

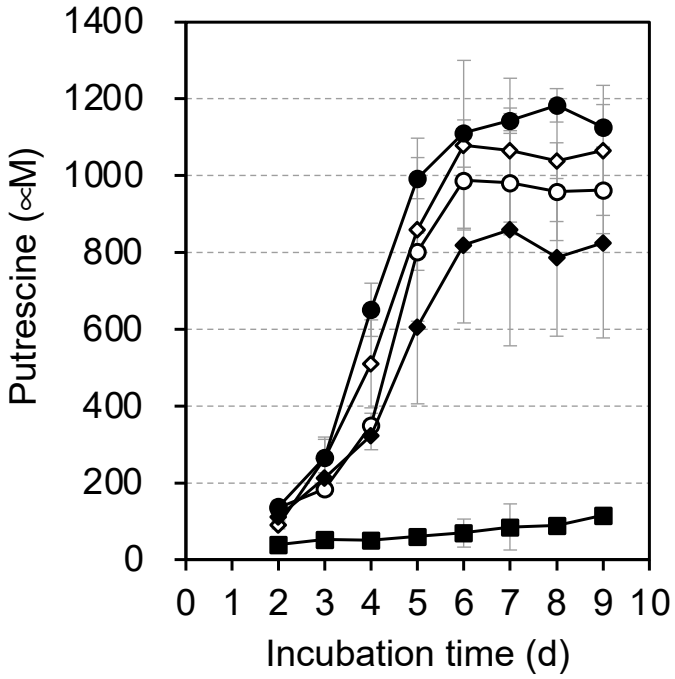


Figure 4. Putrescine production by AN14 (pSH1733/KT160; closed squares) and effect of IPTG addition on putrescine production by SH2287. IPTG was added to the medium for SH2287 at final concentrations of 0 (light circles), 0.1 (light diamonds), 0.5 (black circles) and 1 mM (black diamonds) with the addition of 5 mM arginine on day 2. The mean values of three conical beakers are shown with the standard deviation. The IPTG 0 mM graph (light circles) is a reuse of data from Figure 3. AN14 (black squares) was grown with the addition of 5 mM arginine and 0.5 mM IPTG as a control.

The yield of putrescine was improved to 1.2 mM by the addition of 0.5 mM IPTG. When we used KT160 for putrescine production, we obtained a maximum of about 1 mM putrescine using M9 galactose medium (data not reported). In addition, AN14, which expresses only ArgA$^{ATG\ Y19C}$ from pSH1733 and SpeA and SpeB only from the genome, produced very low levels of putrescine even in the presence of 5 mM arginine. In contrast, SH2287, expressing SpeA and SpeB with ArgA$^{ATG\ Y19C}$ from pKN11, produced significantly more putrescine.

4. Conclusions

We conclude that the supply of carbon and energy from steamed rice works in our developed system. Although there is a need to improve *E. coli* strains to produce more putrescine, this is the first report of an *E. coli* strain using steamed rice as the sole carbon and energy source to produce a chemical. Since the addition of IPTG leads to higher costs for industrialization, it is necessary to improve the method of inducing gene expression that does not require IPTG, such as the use of a temperature-sensitive repressor.

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Abbreviations

HPLC: high-performance liquid chromatography; IPTG: isopropyl-β-D-1-thiogalactopyranoside; OD, optical density; Puu: putrescine utilization; PVDF: polyvinylidene difluoride; SDS: sodium dodecyl sulfate.

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