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Original Research Article

Efficient biosynthesis of creatine by whole-cell catalysis from guanidinoacetic acid in *Corynebacterium glutamicum*

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ABSTRACT

Creatine is a naturally occurring derivative of an amino acid commonly utilized in functional foods and pharmaceuticals. Nevertheless, the current industrial synthesis of creatine relies on chemical processes, which may hinder its utilization in certain applications. Therefore, a biological approach was devised that employs whole-cell biocatalysis in the bacterium *Corynebacterium glutamicum*, which is considered safe for use in food production, to produce safe-for-consumption creatine. The objective of this study was to identify a guanidinoacetate N-methyltransferase (GAMT) with superior catalytic activity for creatine production. Through employing whole-cell biocatalysis, a *gamt* gene from *Mus caroli* (*Mcgamt*) was cloned and expressed in *C. glutamicum* ATCC 13032, resulting in a creatine titer of 3.37 g/L. Additionally, the study employed a promoter screening strategy that utilized nine native strong promoters in *C. glutamicum* to enhance the expression level of GAMT. The highest titer was achieved using the P_{1676} promoter, reaching 4.14 g/L. The conditions of whole-cell biocatalysis were further optimized, resulting in a creatine titer of 5.42 g/L. This is the first report of successful secretory creatine expression in *C. glutamicum*, which provides a safer and eco-friendly approach for the industrial production of creatine.

1. Introduction

Creatine is an amino acid derivative that naturally occurs in the human body and serves a pivotal role in sustaining ATP levels within highly metabolically active tissues, notably muscle and brain. This crucial function is accomplished through the catalytic action of creatine kinase. Creatine and creatine phosphate, in conjunction with creatine kinase, function as ATP buffers within cells, maintaining cellular energy homeostasis. Additionally, creatine phosphate acts as a conveyor of high-energy phosphate [1], assisting in intracellular energy transfer, thereby promoting the efficient provision of energy to muscle and neuronal cells [2]. These molecules are gradually converted to creatinine at a rate of 1.7% per day and are excreted in urine [3]. Therefore, the body must obtain creatine from external sources to maintain a stable creatine level. Although the human body can synthesize 60–80% of its own creatine requirements, additional supplementation from dietary sources is necessary [4].

At present, creatine synthesis in industry relies on chemical methods, which are plagued by long reaction times, complex operational procedures, and relatively low product purity. Of particular concern is that

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these conventional approaches often necessitate the utilization of highly toxic cyanamide or, alternatively, yield suboptimal results, posing significant safety hazards and escalating production costs. This poses a particularly significant challenge for large-scale industrial manufacturing, as any inadvertent release of toxic agents can potentially cause severe casualties, thus conflicting with the principles of sustainable and environmentally-friendly production. In addition, creatine synthesized through chemical methods often possesses an unpleasant taste, typically characterized by bitterness, rendering it unsuitable for direct utilization as a food additive. This challenge necessitates the employment of de-bittering agents, resulting in intricate post-treatment procedures and a meager harvest rate of merely 50%. Given the extensive utilization of creatine in the food and pharmaceutical sectors, it is imperative to develop a green, safe, and environmentally-friendly method for creatine production.

Whole-cell biocatalysis presents a promising avenue for the efficient and sustainable production of creatine. This approach offers a multitude of advantages, including high productivity, cost-effectiveness, simplified downstream processing, and enhanced product recovery [5]. There have been numerous studies that have explored the application of whole-cell biocatalysis for the production of various compounds, such as fructooligosaccharides [6], rubusoside [7] and a range of other food products and additives, demonstrating its versatility and potential in the field. Previous studies have investigated the development of whole-cell biocatalytic systems for guanidinoacetic acid, a crucial precursor of creatine, employing *Escherichia coli* [8] and *Bacillus subtilis* [9]. Liu et al. reported that a betaine-driven methylation system was established in *E. coli* by introducing betaine-homocysteine methyltransferase from *Thioclava nitratireducens* to enhance the supply of S-adenosylmethionine (SAM) [10]. This system was used for whole cell catalytic production of creatine in *E. coli*, resulting in a titer of 2.16 mM after 32 h of reaction [10]. This is also the sole report to date on the biosynthesis of creatine. In recent years, Gram-positive *Corynebacterium glutamicum* has been widely recognized as a Generally Regarded as Safe (GRAS) organism and established as a model microbe due to its well-characterized genetic background and the availability of a comprehensive gene manipulation toolbox [11–13]. Consequently, *C. glutamicum* has emerged as a widely employed host for the large-scale production of industrially relevant medicines and food-grade chemicals [14–17], owing to its robust expression capabilities. These findings strongly suggest the immense potential of whole-cell biocatalysis in the production of creatine in *C. glutamicum*. Moreover, *C. glutamicum*, along with all other genera in this group, possesses a distinct cell wall organization primarily characterized by the presence of a substantial cell wall polymer complex composed of peptidoglycan, arabinogalactan and mycolic acids (MAP) [18]. MAP, which are exceptionally long hydroxylated fatty acids (up to C90 in mycobacteria), are exclusively found in the cell walls of these organisms. This unique structure endows them with exceptional resistance against diverse chemicals and extreme conditions, such as high temperature, pH, and detergents. This exceptional resilience renders *C. glutamicum* a promising platform for the production of chemicals under challenging conditions [19–21]. Therefore, the utilization of *C. glutamicum* as a whole-cell biocatalysis for creatine production is a feasible approach.

Creatine is generally synthesized in the kidneys and liver via metabolic pathways involving arginine, glycine, and methionine [2]. The enzymatic facilitation of this synthesis is carried out by arginine: glycine aminotransferase (AGAT) and guanidine acetate N-methyltransferase (GAMT), which catalyze the conversion of these precursors into creatine [3]. AGAT catalyzes the transfer of the amidine group from arginine to the N of glycine, while GAMT mediates the methyl transfer from SAM to the N-terminal nitrogen of guanidine acetate (GAA), resulting in the production of creatine [22]. Due to the absence of a *gamt* gene derived from microorganism, two animal-derived *gamt* genes were selected for exogenous introduction and expression in the target microorganism *C. glutamicum*. The strategy of promoter screening was employed to enhance creatine production, and the optimal whole-cell biocatalytic conditions were also optimized in this study, as shown in Fig. 1.

2. Materials and methods

2.1. Plasmids, strains, and cultivation conditions

The strains and plasmids used in this study are listed in Table 1. The strains *E. coli* DH5α and *C. glutamicum* ATCC 13032 were purchased from Nanjing Tsingke Biotechnology Company and Beijing Microbiological Culture Collection Center, respectively, while the plasmid pXMJ19 was acquired from MiaoLing Plasmid Platform. *E. coli* DH5α was employed as the cloning host, and *C. glutamicum* ATCC 13032 served as the expression host in this study. *E. coli* was cultured at 37 °C in Luria-Bertani (LB) medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. *C. glutamicum* was grown in BHI medium (Oxoid, UK) at 30 °C. Chloramphenicol (50 mg/L) was added to these media for positive selection of recombinants. Solid media were made by the addition of agar at the concentration of 15 g/L. Furthermore, a total of nine native strong promoters of *C. glutamicum* (P_{1676}, P_{1911}, P_{226}, P_{2008}, P_{0535}, P_{0575}, P_{1109}, P_{0976}, and P_{2129}) were synthesized by Nanjing Tsingke Biotechnology Company. They were further compared for their ability to increase the expression level of GAMTs. Cell density was estimated by measuring the absorbance at 600 nm (OD600) using a BioPhotometer Plus spectrophotometer (Eppendorf, Hamburg, Germany).

2.2. Plasmid construction and microbial transformation

These *gamt* genes from Antrostomus carolinensis (chuck-will's-widow) (*Acgamt*, Gene ID 104524763) and *Mus caroli* (Ryukyu mouse) (*Mcgamt*, Gene ID 110303213) were codon-optimized according to the codon preference of *C. glutamicum* and synthesized by Nanjing Tsingke Biotechnology Company, respectively. Next, the synthetic codon-optimized *gamt* gene was amplified a linear fragment by PCR using primer pairs *Acgamt*-L/R or *Mcgamt*-L/R listed in Table 2. The plasmid pXMJ19 was linearized by restriction digestion with *Bam*H I and *Kpn*I. The PCR product was then inserted into the linearized shuttle expression vector pXMJ19 using a ClonExpress® II One Step Cloning Kit. The resulting recombinant plasmids pXMJ19-Ac and pXMJ19-Mc were introduced into competent *E. coli* DH5α cells, respectively. Plasmids harboring the correct sequence were validated by DNA sequencing and subsequently electroporated into competent *C. glutamicum* cells using a charging voltage of 1.8 kV and a time constant of 5 ms. After electroporation, approximately 200 μL of chilled sorbitol (1 M) was immediately added to the ice-cold 0.1 cm electroporation cuvette. Positive transformants were selected on a BHI plate containing 50 mg/L of chloramphenicol and confirmed by DNA sequencing (see Table 3).

Similarly, the plasmid construction and microbial transformation were performed for promoter optimization. Plasmid construction primers used for optimizing the promoters are listed in Table 2. Briefly, the gene amplification products of nine strong promoters were cloned into the plasmid pXMJ19 double-digested by restriction enzymes *Bam*H I and *Hin*d III, respectively. Plasmids carrying the correct sequences were then transformed into competent *C. glutamicum* cells by electroporation. Transformants were selected and steaked onto fresh BHI plates that were supplemented with 50 mg/L of chloramphenicol (Table 1).

2.3. Whole-cell biocatalysis of creatine

The recombinant *C. glutamicum* 13032/pXMJ19-Ac and *C. glutamicum* 13032/pXMJ19-Mc preserved in frozen tubes were first activated using a streak culture method on BHI plates. Subsequently, single colonies were transferred to 10 mL of BHI liquid medium

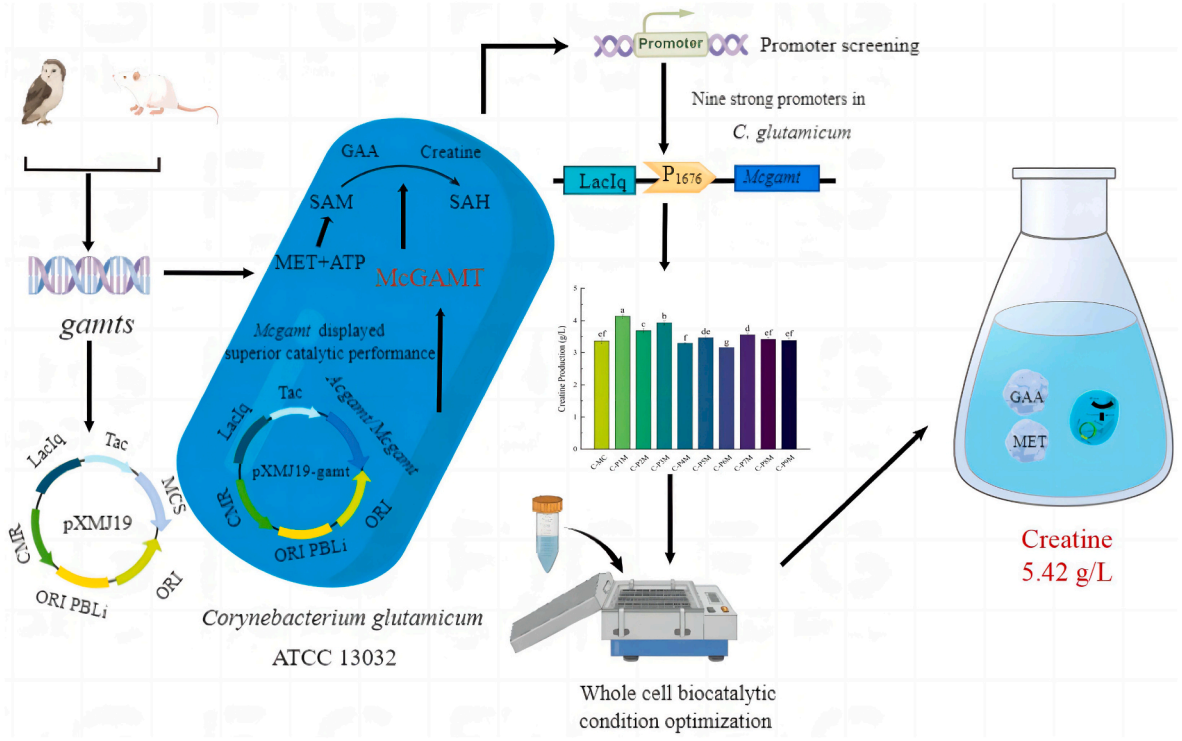

Fig. 1. Experimental flowchart of this study. GAMT: Guanidinoacetate N-methyltransferase; *gamt*: Guanidinoacetate N-methyltransferase gene; McGAMT: Guanidinoacetate N-methyltransferase from *Mus caroli* (Ryukyu mouse); AcGAMT: Guanidinoacetate N-methyltransferase from Antrostomus carolinensis (chuck-will's-widow); CMR: Chloramphenicol-resistance gene; LacIq: Lac repressor encoding gene; ORI: Origin of replication; PBLi: P1-like Bacteriophage; MCS: Multiple cloning site; GAA: Guanidinoacetic acid; SAM: S-adenosyl methionine; SAH: S-adenosyl-L-homocysteine; MET: Methionine; ATP: Adenosine triphosphate; Tac: Tryptophan operon repressor and activator protein-inducible and catabolite-repressible promoter.

containing 50 mg/L chloramphenicol at 30 °C and 200 r/min (IS-RDD3, Crystal Technology & Industries, USA) for 18 h. The colonies were then transferred to 50 mL of BHI liquid medium containing the same concentration of chloramphenicol at 1% (v/v) inoculum and incubated at 30 °C and 200 r/min for 3 h. The plasmid pXMJ19 used in this study contains the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *tac* promoter and the repressor gene *lacIq*, which confer the ability to modulate gene expression in response to IPTG induction. Thus, IPTG was added as an inducer to a final concentration of 0.5 mM at 3 h during cultivation for recombination screening as well as promoter optimization experiments. The repressor gene *lacIq* in the plasmid was not knocked out in the promoter optimization, allowing the replaced promoter to still be regulated by the Lac repressor and influencing the expression of the *gamt* gene. The resulting culture was incubated in a shaker at 30 °C for 12 h until the OD_{600} reached 1.0 (approximately 1.2 × 10^9 CFU/mL). Finally, the fermentation broth was centrifuged for 10 min at 4 °C and at 11000 r/min (Centrifuge 5430R, Eppendorf, GER), and then washed twice with 20 mL of 0.01 M PBS (phosphate buffer saline) buffer (136.89 mM NaCl, 2.67 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.0 adjusted with HCl). The pellet was resuspended using PBS buffer containing an initial concentration of 10 g/L guanidinoacetic acid and 10 g/L methionine. The above reaction system (5 mL) was performed for the whole-cell biocatalytic reaction at 30 °C under a constant temperature shaker at 220 rpm. Based on our preliminary experimental results, it was found that the highest titer of creatine was achieved at 24 h, and thus, 24-h was selected as the sampling time (data not shown). One milliliter of sample was collected after 24 h, centrifuged at 4 °C and 12000 r/min for 15 min, and the supernatant obtained was analyzed by HPLC.

2.4. Determination of creatine and SAM by high performance liquid chromatography (HPLC)

The concentration of creatine in each sample was determined using HPLC (LC-MS, Hitachi) on a ZORBAX Eclipse AAA reversed-phase column (3.5 μm), 4.6 × 150 mm system. The analysis was performed at 25 °C with a mobile phase of 10% methanol, 10% 0.2% H_3PO_4, 90% water at a flow rate of 1 mL/min and the titer of creatine was detected by monitoring at an absorbance of 210 nm [23]. The concentration of SAM in each sample was also determined using HPLC (LC-MS, Hitachi) on a WONDASIL C_{18} SUPERB (5 μm), 4.6 × 250 mm system. The analysis was performed at 25 °C with a mobile phase of 20% methanol, 80% buffer (10 mmol/L sodium heptanesulfonate and 50 mmol/L sodium acetate at pH 4.0) at a flow rate of 1 mL/min and the SAM concentration was detected by monitoring at an absorbance of 254 nm.

2.5. Optimization of whole-cell biocatalytic conditions

2.5.1. Effect of guanidinoacetic acid concentration on the whole-cell biocatalytic system

Under specified conditions of a substrate methionine concentration of 10 g/L and a pH value of 7.0, the whole-cell biocatalytic system was delicately manipulated to maintain substrate guanidinoacetic acid concentrations at 5, 10, 15, 20, and 25 g/L. The reaction was methodically executed at a controlled temperature of 30 °C for a duration of 24 h to ascertain the optimal concentration of substrate guanidinoacetic acid, HPLC was employed to precisely quantify the creatine titer within the transformation solution.

Table 1
Strains and plasmids used in this study.

Plasmids/Strains	Description	References
Plasmids		
pXMJ19	Shuttle vector of *C. glutamicum/E. coli*, IPTG-inducible promoter P_{tac}, Cm^R.	[26]
pXMJ19-P_{1676}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P_{1676} promoter.	This study
pXMJ19-P_{226}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P_{226} promoter.	This study
pXMJ19-P_{1911}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P_{1911} promoter.	This study
pXMJ19-P_{2008}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P2008 promoter.	This study
pXMJ19-P_{0536}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P_{0536} promoter.	This study
pXMJ19-P_{0575}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P_{0575} promoter.	This study
pXMJ19-P_{1109}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P_{1109} promoter.	This study
pXMJ19-P_{0976}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P_{0976} promoter.	This study
pXMJ19-P_{2129}-Mc	pXMJ19 derivate with *Mcgamt* cloned under the control of P_{2129} promoter.	This study
Strains		
E. coli DH5α		Nanjing Tsingke Biotechnology Company
C. glutamicum ATCC 13032		Beijing Microbiological Culture Collection Center
C–Ac	*C. glutamicum* 13032 harboring plasmid PXMJ19-Ac.	This study
C-Mc	*C. glutamicum* 13032 harboring plasmid PXMJ19-Mc.	This study
C–P1M	*C. glutamicum* 13032 harboring plasmid PXMJ191676-Mc.	This study
C–P2M	*C. glutamicum* 13032 harboring plasmid PXMJ19-226-Mc.	This study
C–P3M	*C. glutamicum* 13032 harboring plasmid PXMJ19-1911-Mc.	This study
C–P4M	*C. glutamicum* 13032 harboring plasmid PXMJ19-2008-Mc.	This study
C–P5M	*C. glutamicum* 13032 harboring plasmid PXMJ19-0536-Mc.	This study
C–P6M	*C. glutamicum* 13032 harboring plasmid PXMJ19-0575-Mc.	This study
C–P7M	*C. glutamicum* 13032 harboring plasmid PXMJ19-1109-Mc.	This study
C–P8M	*C. glutamicum* 13032 harboring plasmid PXMJ19-0976-Mc.	This study
C–P9M	*C. glutamicum* 13032 harboring plasmid PXMJ19-2129-Mc.	This study

2.5.2. Effect of methionine concentration on the whole-cell biocatalytic system

Under specified conditions, with a substrate guanidinoacetic acid concentration of 10 g/L and a pH value of 7.0, the concentrations of substrate methionine were meticulously adjusted to 5, 10, 15, 20, and 25 g/L within the whole-cell biocatalytic system. The reaction was precisely executed at 30 °C for a duration of 24 h. To ascertain of the optimal methionine concentration, HPLC was employed for the accurate detection of creatine titer within the transformation solution.

2.5.3. Effect of pH on the whole-cell biocatalytic system

In the presence of 10 g/L guanidinoacetic acid and 10 g/L methionine, the reaction system was maintained at 30 °C and executed within PBS buffers of varying pH levels (5.0, 6.0, 7.0, 8.0, and 9.0) for a duration of 24 h. Subsequently, HPLC was employed to quantitate the creatine titer in the resulting transformation solution, facilitating the identification of the optimal pH for the reaction.

2.5.4. Effect of reaction temperature on the whole-cell biocatalytic system

The whole-cell biocatalytic system was subjected to a series of temperatures (10, 20, 30, 40, and 50 °C) for a period of 24 h, while maintaining a consistent environment of 10 g/L guanidinoacetic acid, 10 g/L methionine, and a pH value of 7.0. Following this, HPLC was employed to precisely quantitate the creatine titer within the transformation solution, allowing for the identification of the optimal temperature for the reaction.

2.6. Determination of GAMT expression level and enzyme activity

The expression of GAMT was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, 12% Bis-Tris Gel) using a FastPAGETM Protein Preform Gel from Nanjing Tsingke Biotechnology Company. Safe Protein Fast Staining Solution (No-destaining) (Nanjing Tsingke Biotechnology Company) was used to stain of protein gels. After staining for 15 min, gels were washed with ddH_2O and observed through a gel imaging system (Quantum CX5). The total

Table 2
Primer sequences used in this study.

Primers	Sequences (5′–3′)	Expected band sizes (bp)
Acgamt-L	CGCGGATCCGCGTCTGTATGATACATATCCGCT	312
Acgamt-R	CGGGGTACCCCGGTCGGTGTAATCATTTTATG	
Mcgamt-L	CGCGGATCCGCGATGCATTCACTGACAATGTC	723
Mcgamt-R	CGGGGTACCCCGTGTTTTGTAACCAGCGGT	
P_{1676}–F	CCTGCAGGTCGACTCTAGAGTGGATCTGTATTTG	222
P_{1676}–R	AGTGAATGCATGTGGTGCTCATCTCCTACTTCT	
P_{226}–F	CCTGCAGGTCGACTCTAGAGATGCGACAGTACT	295
P_{226}–R	AGTGAATGCATCATGTCCATAGTCCTAACCAATCA	
P_{1911}–F	CCTGCAGGTCGACTCTAGAGGCTTGAACCGGCAT	192
P_{1911}–R	AGTGAATGCATCTGCTGCGTTCAATCCCTTTCAC	
P_{2008}–F	AGTGAATGCATGGCATTTACGTGTCGACCTC	92
P_{2008}–R	AGTGAATGCATCAGGACAGGGTAGAGAGGTCC	
P_{0536}–F	AGTGAATGCATGCGTTTCAGATCATGATTG	95
P_{0536}–R	AGTGAATGCATCATCCTCCGCTTTCCAGAAC	
P_{0575}–F	AGTGAATGCAT GCATGCATTTACGTGTCGAC	89
P_{0575}–R	AGTGAATGCATC ATGCAGGAAGGTGCGAGAACA	
P_{1109}–F	AGTGAATGCATATAGCGGACATTTGACG	206
P_{1109}–R	AGTGAATGCATCTGTTCCTTTAAGTTACGCTA	
P_{0976}–F	AGTGAATGCATGCATGCATTTACGTGTCGAC	81
P_{0976}–R	AGTGAATGCATC CCCAGGAGGCCCTTCAGATG	
P_{2129}–F	AGTGAATGCAT GCATGCATTTACGTGTCAGTAC	292
P_{2129}–R	AGTGAATGCATCCCTCACAATCGCCTATTGTT	

Table 3
Gene sequences used in this study.

Genes	Sequences (5′–3′)
Acgamt	ATTCTGTATGATACATATCCGCTGTCAGAAGAAACATGGCATACACATCAATTTACATTTATTAAAGATCATGCATTTAGACTGCTGCA ACCGGGCGGCGTTCTGACATATTGCAATCTGACATCATGGGGCGAACTGCTGAAAACAAAATCAGATATTGAAAAAATGTTTGAAGAA ACACAAATTGGCCATCTGGTTGAAGCGGGCTTTAAAAAAGAAAATATTAGAACAACAGTTATGGATCTGGTTCCGCCGCAAGATTGCA GATATTATTCATTTCATAAAATGATTACACCGACAATTATTAAACAT
Mcgamt	ATGCATTCACTGACAATGTCAAGCTCAGCAGCATCACCGCTGTTTGCACCGGGCGAAGATTGCGGCCCGGCATGGAGAGCAGCACCG GCAGCATATGATGCATCAGATACACATCTGCAAATTCTGGGCAAACCGGTTATGGAAAGATGGGAAACACCGTATATGCATGCACTG GCAGCGGCAGCGGCATCAAGAGGCGGCAGAGTTCTGGAAGTTGGCTTTGGCATGGCAATTGCAGCATCACGCGTTCAACAAGCACCG ATTGAAGAACATTGGATTATTGAATGCAATGATGGCGTTTTTCAAAGACTGCAAGATTGGGCACTGAGACAACCGCATAAAGTTGTTC CGCTGAAAGGCCTGTGGGAAGAAGTTGCACCGACACTGCCGGATGGCCATTTTGATGGCATTCTGTATGATACATATCCGCTGTCAGA AGAAGCATGGCATACACATCAATTTAATTTTATTAAAAATCATGCATTTAGACTGCTGAAAACGGGCGGCGTTCTGACATATTGCAATC TGACATCATGGGGCGAACTGATGAAATCAAAATATACAGATATTACAACAATGTTTGAAGAAACACAAGTTCCGGCACTGCAAGAAG CGGGCTTTCTGAAAGAAAATATTTGCACAGAAGTTATGGCACTGGTTCCGCCGGCAGATTGCAGATATTATGCATTTCCGCAAATGA TTACACCGCTGGTTACAAAACAT
P_{1676}	ATGCGACAGTACTTTTCATTAAGCCTAAGAAAATTCCTTTAATTGACACTTAATTGACCAATAAGAGTCGATTAGATTGCATTATTAG GTAATCTAGTGATTTAATGGAGAATAAGAGCAACTGGTGAAGAAAAGGCTTGATGAAAGAAGTTTTTTATCTAGCTAGATGTTCAAT CACGAGCTTTAAGAAAGTATGTCAATAACTTTGACATAACCTAAACACAATAAATTATGTAGTATTATGTGACACTAAGTTATTACAT TTATTATATGATTGGTTAGGACTATGGACATG
P_{226}	TGGATCTGTATTTGACGTGGTTCGAGACCCCGCGGTGTTGCACAAAGTGGAAGTCAGTGGAGGAATCCTCGAGCCTGAATGTGCTGC CTTGATGACCGAATTTTTCGAACTTCACAGGTAACGGATTTATATCAATTTCAGGGCGTGGCGAGCTTTTAGTGATTCACGCTCCTAC GGTGGGTATCACAAATACCTCAACTAGAAGTAGGAGATGAGCACCAC
P_{1911}	GCTTGAACCGGCATGAAAATCTCGTACCGGTTTTGGGCTCACAAGGCCATATAGGAACTTTGTAATTAGTTGCAGGTTCCAATTTTGG GTCAATGTAGCGTAATATTGTTCAAGGCCCATGTGCGGGCTGTGGAGGACGTGCATTCACGTTCTGGTCAAATGAAAAACGGTGAAA GGGATTGAACGCAGCAG
P_{2008}	GCATGCATTTACGTGTCGACCTCAACGCGATAGGTTTCAACCATAGTCGCCCTTGCTCACTCTAGACATGCCCATAAGCCTAGTACGT CATT
P_{0575}	GCATGCATTTACGTGTCGACTTATGTGTCGAGGTGAATCTCCGGTTCGCCCTTGCTCACTCTAGACATGTTCTCGCACCTTCCTGCAT
P_{0536}	GCGTTTTCAGATCATGATTGATTGGGCGTGCCTGCTTTTGTGTTTTTTAGGGACCCCAATGCGCGTGATTCAACTCATGTTTGATATG TGCTCCTAAGGTGTGTAACCTATATCGATGGTGTGCGTACATCTTGAGTGACGCAACCATTTTGAAGTGGAAAAACTTAAGGCCTCC CGCAGGGGAGTGTTCTGGAAAAGCGGAGGAT
P_{0247}	CTTGATTCAGGGTAGTTGACTAAAGAGTTGCTCGCGAAGTAGCACCTGTCACTTTTGTCTCAAATATTAAATCGAATATCAATATAT GGTCTGTTTATTGGAACGCGTCCCAGTGGCTGAGACGCATCCGCT AAAGCCCCAGGAACCCTGTGCAGAAAGAAAACACTCCTCTGGCTAGGTAGACACAGTTTATAAAGGTAGAGTTGAGCGGGTAACTG TCAGCACGTAGATCGAAAGGTGCACAAAG
P_{2129}	GCATGCATTTACGTGTCGACCTACACTTCTGGAGCGTTACGGTGCTCGCCCTTGCTCACTCTAGACATGTACTTAACAATAGGCGAT TGTGAGG
P_{1109}	ATAGCGGACATTTTTTGACGCAGATCACCTTACTCTGAAGGATAAGGATTCTTAGTGTCGGTGCACTTTTACTGATGTTTCACTGTGG AGGTCAACGACTCAAAGTCGAGAATTGGTGGCGCGTGTCACTGGAATTGACGCGTGAATGGGGTGGAAGTGGACGTCGAAAAGCAT TTTTGAGACGTTTATGTGAGCAATGTCCCATTTTCCCTGCTCACCTGTATGGGCACCCGCGGCGGAAGTGGAATTGCATATGGAGTTT TGATGATATTTAGCGTAACTTAAAGGAACA
P_{0976}	GCATGCATTTACGTGTCGACCCCCCTTTTGGGTGTCCAGATCGCCCTTGCTCACTCTAGACATCTGAAGGGCCTCCTGGG

protein content was determined using the Biuret method. The sample of 0.5 mL, to be tested, was reacted with 0.5 mL of distilled water and 4 mL of Biuret reagent. The absorbance of the sample was then measured at 540 nm. The total protein content in the sample was calculated based on the standard curve.

The expression level of GAMT was determined using a Mouse GAMT ELISA Kit (Shanghai Tongwei Biotechnology Co., Ltd.). GAMT activity was measured according to a previously described method [3]. Briefly, 0.5 mL of the sample was centrifuged at 100,000 *g* at 4 °C for 1 h, and the supernatants were then used to assess GAMT activity. The assay contained 35 mM Tris buffer (pH 7.4), 7 mM 2-mercaptoethanol, 50 μM SAM, and 0.2 mM GAA. After a 10-min pre-incubation of all analyzed components except GAA, the analysis was initiated by adding GAA. The blanks did not contain GAA. The homogenate was incubated at 37 °C and the reaction was stopped using 75 μL of 15% (wt/vol) TCA. Immediately afterwards, 72 μL of 1 M Tris (pH 7.4) was used for neutralization. The precipitated protein was centrifuged at 10,000 *g* for 5 min at room temperature, and then creatine was measured using the HPLC method described.

2.7. Statistical analysis

Each group of bioconversion experiments was performed with three replicates. Origin 2022 and SPSS 20.0 were used for data processing and analysis. Analysis of variance (ANOVA) was used to determine significant differences among the results (means ± standard deviation). The significant difference and mean values were obtained by Duncan's test at a confidence level of $p < 0.05$.

3. Results

3.1. Construction of the creatine synthesis pathway in C. glutamicum

To convert guanidinoacetate and methionine to creatine, the enzyme GAMT was introduced into *C. glutamicum*. The sequences of *Acgamt* (312 bp) and *Mcgamt* (723 bp) were retrieved from NCBI and amplified by PCR using plasmids pUC57-Ac and pUC57-Mc as templates, respectively. The recombinant *E. coli* pXMJ19-Ac and *E. coli* pXMJ19-Mc were obtained by transferring the homologous recombinant ligated products into *E. coli* DH5α. The recombinant plasmids pXMJ19-Ac and pXMJ19-Mc obtained from *E. coli* culture were electro-transformed into *C. glutamicum* ATCC 13032. PCR identification of the transformant colonies revealed that the size of the target bands was consistent with expectations, suggesting the successful construction of recombinant *C. glutamicum* 13032/pXMJ19-Ac and *C. glutamicum* 13032/pXMJ19-Mc (Fig. 2). The two strains were used for whole-cell biocatalysis and the titers of creatine were detected by HPLC. Under the condition that the concentration of substrate guanidinoacetic acid and methionine was 10 g/L, the catalytic reaction was carried out after 24 h of whole-cell biocatalytic reaction, *C. glutamicum* 13032/pXMJ19-Ac and *C. glutamicum* 13032/pXMJ19-Mc produced 2.82 ± 0.67 g/L and 3.37 ± 0.83 g/L creatine, respectively. Therefore, the *C. glutamicum* 13032 harboring plasmid pXMJ19-Mc (C-Mc) with a higher titer of creatine was selected for further experiments.

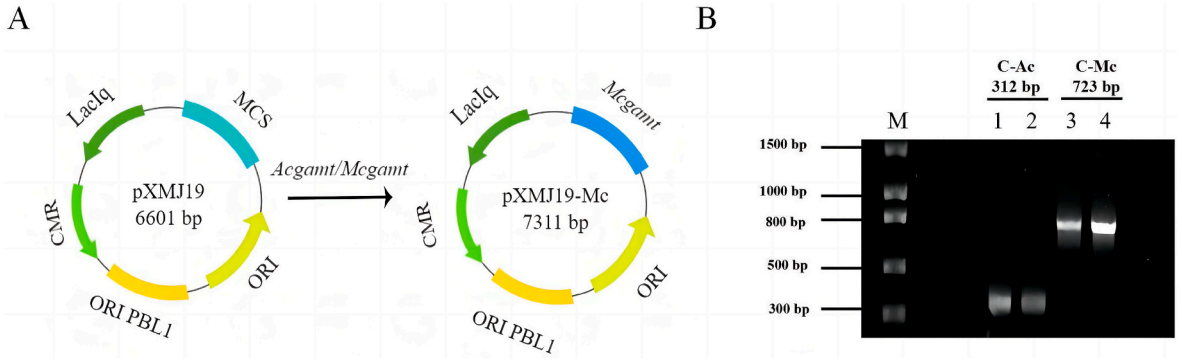

Fig. 2. (A) Plasmid mapping in creatine synthesis pathway construction, ligating *gamts* from two sources to the coding region of plasmid pXMJ19. (B) Transformant validation profiles of *C. glutamicum* ATCC 13032/pXMJ19-Ac (Lanes 1 and 2) and *C. glutamicum* ATCC 13032/pXMJ19-Mc (Lanes 3 and 4). The bands were consistent with the expected size.

3.2. Linking strong promoters to increase creatine titer

To improve the creatine titer in whole-cell biocatalysis, nine strong promoters, P_{1676} (295bp), P_{1911} (192bp) and P_{226} (222bp), P_{2008} (92bp), P_{0536} (206bp), P_{0575} (89bp), P_{1109} (292bp), P_{0976} (81bp) and P_{2129} (95bp), were selected according to a previous study in *C. glutamicum*, nine strong promoters were screened from 90 wild-type promoters of *C. glutamicum*, which played an important role in amino acid expression [24]. The template plasmid synthesized by the company was used for PCR amplification to obtain the target fragments of nine promoters. The purified PCR products were ligated with the linearized vector pXMJ19-Mc according to the instructions of homologous recombination kit. The homologous recombinant ligated products were transferred into *E. coli* DH5α to obtain recombinants. The PCR identification results of transformant colonies indicated that the size of the target bands was consistent with expectations, and further confirmation revealed successful ligation of the promoter (Fig. 3B). The results showed that after whole-cell biocatalysis, the production levels of creatine in *C. glutamicum* harboring plasmid pXMJ191676-Mc (C–P1M), *C. glutamicum* harboring plasmid pXMJ19-226-Mc (C–P2M), *C. glutamicum* harboring plasmid pXMJ19-1911-Mc (C–P3M) *C. glutamicum* harboring plasmid pXMJ192008-Mc (C–P4M), *C. glutamicum* harboring plasmid pXMJ190536-Mc (C–P5M), *C. glutamicum* harboring plasmid pXMJ190575-Mc (C–P6M), *C. glutamicum* harboring plasmid pXMJ191109-Mc (C–P7M), *C. glutamicum* harboring plasmid pXMJ190976-Mc (C–P8M) and *C. glutamicum* harboring plasmid pXMJ192129-Mc (C–P9M) were 4.14 ± 0.08 g/L, 3.69 ± 0.07 g/L, 3.93 ± 0.06 g/L, 3.29 ± 0.07 g/L, 3.47 ± 0.03 g/L, 3.16 ± 0.06 g/L, 3.55 ± 0.06 g/L, 3.42 ± 0.05 g/L, and 3.37 ± 0.08 g/L, respectively. The most effective promoter was P_{1676}, which increased creatine titer by 22.8% compared to the control (Fig. 3C).

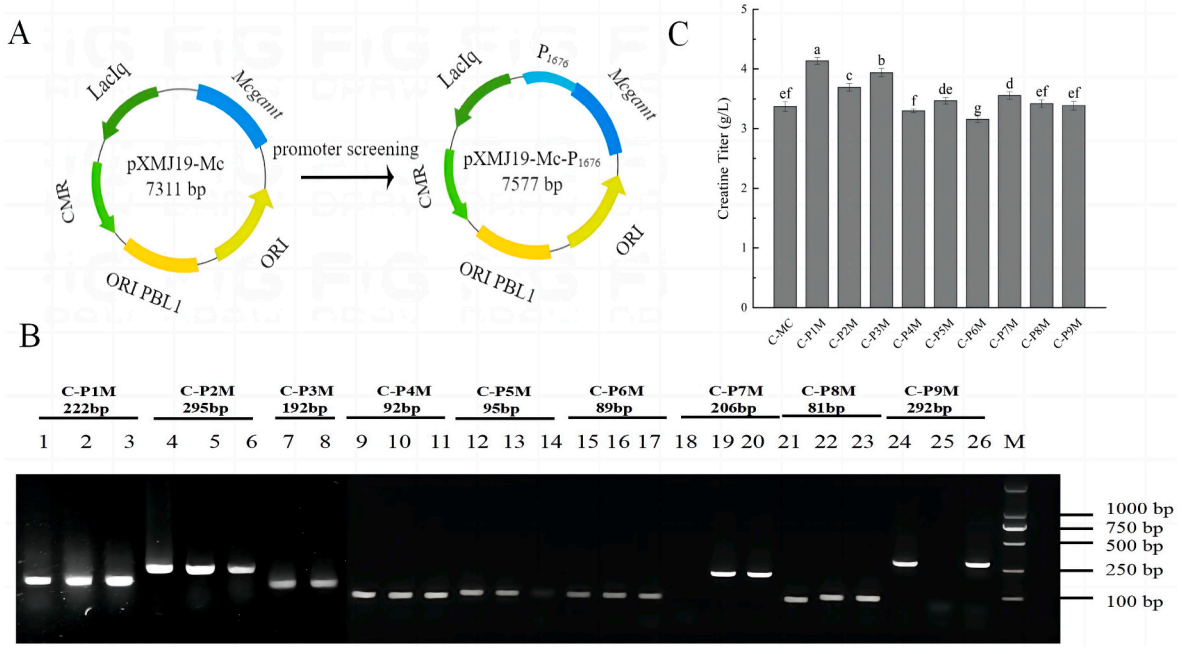

Fig. 3. (A) Schematic diagram of the promoter-optimized plasmids with the nine promoters ligated in front of the target genes *Acgamt* and *Mcgamt*. (B) Transformant validation profiles for C–P1M (Lanes 1, 2, and 3), C–P2M (lanes 4, 5, and 6), C–P3M (Lanes 7 and 8), C–P4M (Lanes 9, 10, and 11), C–P9M (Lanes 12 and 13), C–P6M (Lanes 14, 15, and 16), C–P5M (Lanes 17 and 18), C–P8M (Lanes 19, 20, and 21), and C–P7M (Lanes 22 and 23), The bands were consistent with expectations. (C) The creatine titers of different recombinant strains constructed in this study were compared. The results are the mean of the repeated experiments and the error bars indicate the standard error. The significant difference and mean values were obtained by Duncan's test at a confidence level of $p < 0.05$. Different letters represent significant differences between the two groups, and the same letter indicates no significant difference. All experiments were performed in triplicates.

3.3. Optimization of whole-cell biocatalytic conditions

To further enhance the creatine titer, we carefully investigated the reaction temperature, pH, and the substrate concentrations (guanidinoacetic acid and methionine) in the reaction system to determine the optimal conditions for whole-cell biocatalysis of creatine using *C. glutamicum*.

As the pH increased, the creatine titer also increased. The highest titer of creatine (4.21 ± 0.05 g/L) was observed at pH 8.0 of the reaction system. However, there was no significant difference in creatine titer between pH 7.0 and 8.0 (Fig. 4A). The creatine titer also increased with the rise of temperature, reaching a maximum of 4.14 ± 0.08 g/L at 30 °C. However, further increase in temperature showed a decreasing trend in titer (Fig. 4B). By properly increasing the substrate concentration, the enzymatic reaction rate can be accelerated and product synthesis can be promoted. The creatine titer exhibited a positive correlation with the concentration of guanidinoacetic acid, reaching a maximum of 5.07 ± 0.06 g/L at the gunidinoacetic acid concentration of 20 g/L. However, beyond this concentration, the titer decreased with a further increase in substrate concentration (Fig. 4C). A similar trend was observed for methionine, with creatine titer increasing as methionine concentration increased, reaching a maximum of 4.82 ± 0.05 g/L at the methionine concentration of 15 g/L. However, further increase in methionine concentration led to a decrease in creatine titer (Fig. 4D). This may be attributed to the toxic effect of methionine on the cells. Therefore, we determined the optimal reaction conditions for whole-cell biocatalysis to be: adjusting the pH of the reaction system to 8.0, maintaining the substrate guanidinoacetic acid concentration of 20 g/L, and maintaining the substrate methionine concentration of 15 g/L. The reaction was carried out in a constant temperature shaker at 30 °C and 220 rpm for 24 h, resulting in a maximum creatine titer of 5.42 ± 0.05 g/L.

3.4. Determination of SAM and GAMT expression

Prior to the commencement of whole-cell biocatalysis, samples were assessed the GAMT expression level and enzyme activity. As shown in Fig. 5A, the SDS-PAGE analysis results revealed that the presence of a band corresponding to the GAMT protein at approximately 25.9 kDa, which is consistent with the theoretical value. The strain connected to the promoter P_{1676} exhibited the highest GAMT expression level of 32.63 mg/gDCW (Fig. 5B). Additionally, the enzyme activities of C-MC, C–P1M, C–P2M, and C–P3M were 158.37 U/L, 196.36 U/L, 169.33 U/L and 181.93 U/L, respectively. Several studies have demonstrated that SAM serves as the primary methyl donor in the creatine synthesis pathway [1,2,10]. The requirement for sufficient SAM is particularly crucial when employing heterologous SAM-mediated methyltransferases for the synthesis of methylated chemicals [10]. Therefore, a limited intracellular concentration of SAM may hinder methylation efficiency and ultimately affect the production of methylated compounds. Fig. 5D and E reveal that there were no significant differences in SAM concentration among the four experimental strains (C-Mc, C–P1M, C–P2M, and C–P3M) ($p < 0.05$). Notably, the highest concentration of SAM was observed when 15 g/L methionine was added. The respective SAM concentrations of the four strains were C-Mc: 110.07 mg/L, C–P1M: 105.33 mg/L, C–P2M: 109.25 mg/L, and C–P3M: 108.52 mg/L.

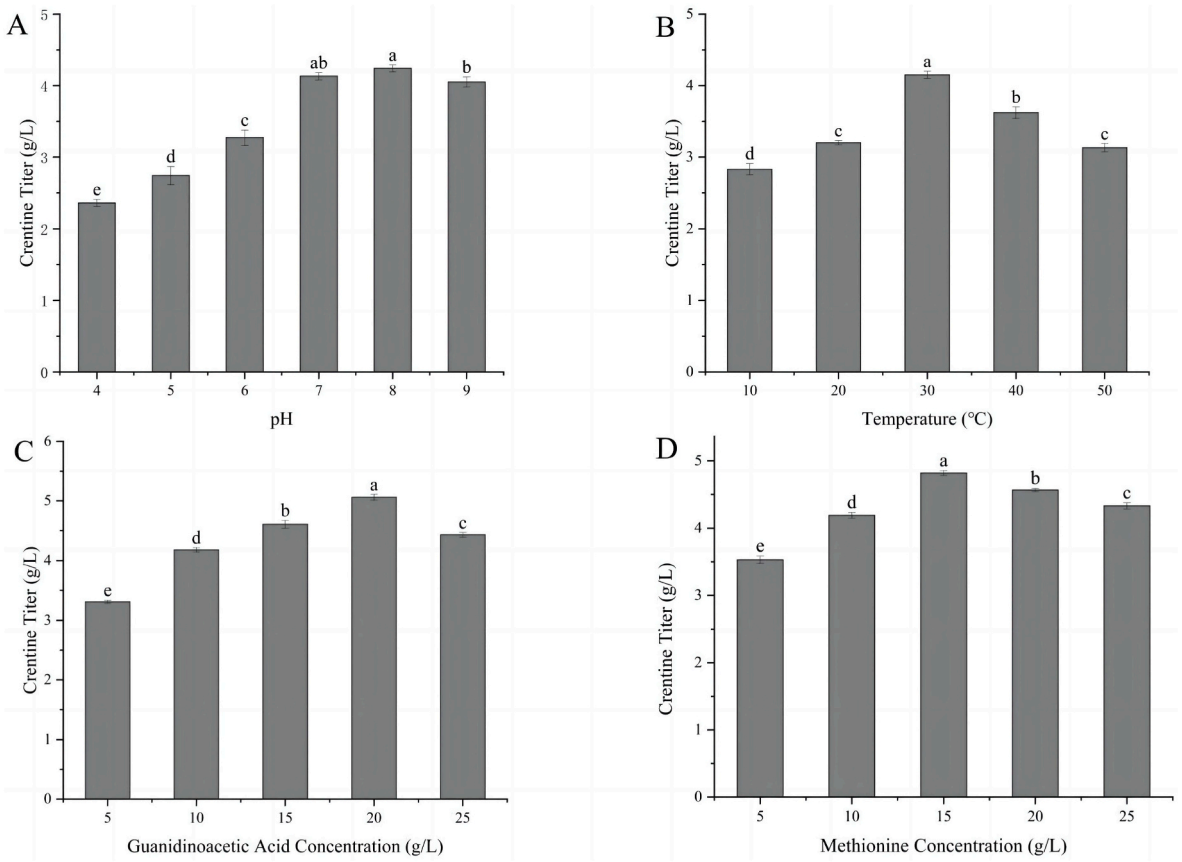

Fig. 4. Effects of reaction pH (A), temperature (B), and substrate concentrations of guanidinoacetic acid (C) and methionine (D) on creatine titers. The results are the mean of the repeated experiments and the error bars indicate the standard error. The significant difference and mean values were obtained by Duncan's test at a confidence level of $p < 0.05$. Different letters represent significant differences between the two groups, and the same letter indicates no significant difference. All experiments were performed in triplicates.

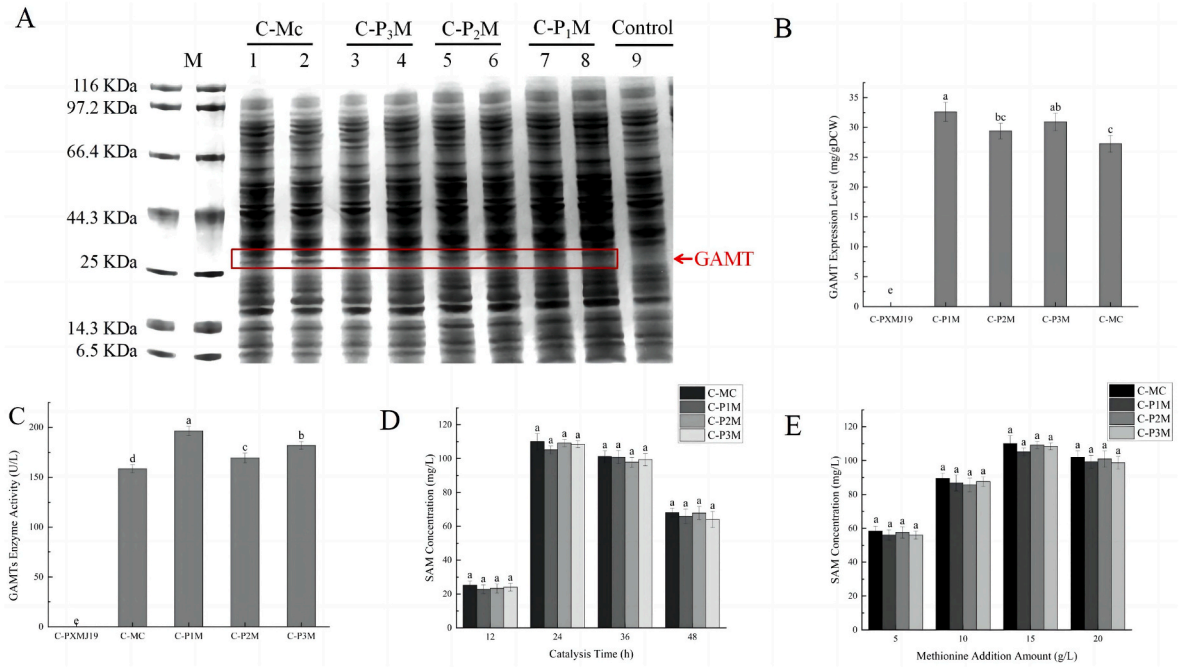

Fig. 5. (A) SDS-PAGE analysis of GAMT expression in different strains. (B) Determination of GAMT expression levels in the reaction system prior to the commencement of whole-cell biocatalysis. (C) Detection of GAMT activities prior to the commencement of whole-cell biocatalysis. (D) Detection of SAM concentrations during the whole cell biocatalysis period. (E) Effect of different methionine additions on SAM concentration in the whole cell catalytic system. The results are the mean of the repeated experiments and the error bars indicate the standard error. The significant difference and mean values were obtained by Duncan's test at a confidence level of $p < 0.05$. Different letters represent significant differences between the two groups, and the same letter indicates no significant difference. All experiments were performed in triplicates.

4. Discussion

Creatine is widely utilized in functional foods and pharmaceuticals. The utilization of microbial production offers a safer and more eco-friendly production method as it mitigates environmental issues such as pollution compared to conventional organic chemical production. For the biocatalytic production of creatine, we employed *C. glutamicum* as a whole-cell biocatalyst. By optimizing the creatine synthesis pathway and the system of whole-cell biocatalysis, the titer of creatine was enhanced.

The expression of genes encoding key enzymes is crucial in the development of whole-cell biocatalysis. In most fermentation processes, the expression levels of genes encoding key enzymes need to be optimized to mitigate the metabolic burden on the producing host. This is because overexpression of genes encoding key enzymes may cause poor cell growth and can impact the expression of the target material [25]. However, in the whole-cell biocatalytic platform, the expression of the target gene can reach its maximum level as it is not necessary to consider the metabolic burden on the host. Creatine is synthesized de novo through the metabolism of arginine, glycine, and methionine. The enzymes AGAT and GAMT are involved in its synthesis, with GAMT directly catalyzing the reaction between SAM and GAA to produce creatine [26]. Given the lack of reports on GAMT coding genes in microorganisms, two common GAMTs from Antrostomus carolinensis (chuck-will's-widow) and *Mus caroli* (Ryukyu mouse) were cloned and expressed in *C. glutamicum*. After achieving successful expression, the promoter was selected to enhance creatine production. Among these selected promoters, P_{1676} exhibited the best boosting effect, representing the most potently expressed natural promoter identified in previous investigations within *C. glutamicum* ATCC 13032 [26].

In this study, it was observed that the titer of creatine was significantly elevated at the pH of 8, surpassing the levels observed at other pH values. *C. glutamicum* exhibited remarkable pH homeostasis, maintaining its intracellular pH at 7.5 ± 0.5 despite exposure to external pH values ranging from 6.0 to 9.0 [27]. However, a significant decline in the growth rate of *C. glutamicum* cells was observed when the external pH fell below 6.0 or rose above 9.0 [27]. This literature demonstrates that *C. glutamicum* can maintain high productivity when the pH of the external environment is between 6.0 and 9.0. Therefore, in future potential industrial production, the pH of the reaction system can be controlled within this range of 7.0–8.0 to achieve the optimal production. The highest creatine titer was obtained at a reaction temperature of 30 °C, probably because this temperature was most suitable for the maximum enzyme activities of related enzymes such as GAMT and MetK [22,28].

In addition, ATP plays a pivotal role in supplying adenosyl for the biosynthesis of SAM. Several studies have alluded to the fact that an adequate intracellular ATP supply could lead to augmented SAM production, and ATP regeneration from adenosine serves to sustain the adenylate pool size and bolster the accessibility of ATP, thereby improving the efficiency of methyl supply [2,10]. Thus, it is crucial to enhance the SAM production through dynamic control of ATP concentration in future studies. It is well-known that an appropriate increase in substrate concentration can accelerate the rate of enzymatic reactions and promote product synthesis. In this study, creatine production increased with the elevation of guanidinoacetic acid concentration, peaking at 5.07 g/L at 20 g/L. However, creatine production subsequently declined when the guanidinoacetic acid concentration surpassed a certain threshold. Furthermore, creatine production reached a maximum of 4.82 g/L at a substrate methionine concentration of 15 g/L, beyond which further increases in methionine concentration resulted in decreased creatine production. The underlying reasons for this phenomenon remain unclear and warrant further investigation. It is plausible that excessively high concentrations of methionine may exert toxic effects on cells, thereby impacting creatine production. Moreover, a significant creatine production was achieved by introducing GAMT into *C. glutamicum*, a host that demonstrated enhanced suitability for creatine expression in our study. This achievement was further optimized

through rigorous promoter screening and whole-cell biocatalytic condition optimization. Following 32 h of whole-cell biocatalysis, a creatine titer of 5.17 g/L was obtained, which represents a substantial increase compared to the previously reported level of 0.297 g/L [8]. This breakthrough underscores the potential of our approach in advancing creatine production methods.

5. Conclusions

In conclusion, this study established the feasibility of synthesizing creatine through whole-cell biocatalysis in *C. glutamicum*. Through the exogenous introduction of GAMTs and screening of potent strong promoters, we successfully generated recombinant *C. glutamicum* capable of creatine production. Subsequently, we optimized the whole-cell biocatalytic reaction system, achieving a maximum creatine titer of 5.42 g/L after 24 h of whole-cell biocatalysis. However, the addition of substrates containing methionine and guanidinoacetic acid increases the economic burden of the whole-cell biocatalytic process. Therefore, considerable efforts are warranted to enhance the efficiency of creatine biosynthesis without relying on methionine, possibly through metabolic engineering and modulating the biodegradation pathways of creatine. Overall, this study presents a safer and eco-friendly approach for the industrial-scale production of creatine.

CRediT authorship contribution statement

Chunjian Li: performed the experimental work, analyzed, the data, prepared and revised this manuscript. **Pengdong Sun:** analyzed, the data, prepared and revised this manuscript. **Guoqing Wei:** analyzed, the data. **Yuqi Zhu:** prepared and revised this manuscript. **Jingyuan Li:** prepared and revised this manuscript, All authors reviewed and approved the final version of the manuscript. **Yanfeng Liu:** conceived and designed the experiments. **Jian Chen:** conceived and designed the experiments. **Yang Deng:** conceived and designed the experiments, prepared and revised this manuscript.

Declaration of competing interest

The authors indicate that they have no conflicts of interest.

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