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Sustainable production of 2,3,5,6-Tetramethylpyrazine at high titer in engineered *Corynebacterium glutamicum*

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Abstract: The industrial amino acid production workhorse, *Corynebacterium glutamicum* naturally produces low levels of 2,3,5,6-tetramethylpyrazine (TMP), a valuable flavor, fragrance, and commodity chemical. Here, we demonstrate TMP production ($\sim$0.8 g L^{-1}) in *C. glutamicum* type strain ATCC13032 via overexpression of acetolactate synthase and/or α-acetolactate decarboxylase from *Lactococcus lactis* in CGXII minimal medium supplemented with 40 g L^{-1} glucose. This engineered strain also demonstrated growth and TMP production when the minimal medium was supplemented with up to 40% (v v^{-1}) hydrolysates derived from ionic liquid-pretreated sorghum biomass. A key objective was to take the fully engineered strain developed in this study and interrogate medium parameters that influence the production of TMP, a critical post-strain engineering optimization. Design of experiments in a high-throughput plate format identified glucose, urea, and their ratio as significant components affecting TMP production. These two components were further optimized using response surface methodology. In the optimized CGXII medium, the engineered strain could produce up to 3.56 g L^{-1} TMP (4-fold enhancement in titers and 2-fold enhancement in yield, mol mol^{-1}) from 80 g L^{-1} glucose and 11.9 g L^{-1} urea in shake flask batch cultivation.

One-Sentence Summary: *Corynebacterium glutamicum* was metabolically engineered to produce 2,3,5,6-tetramethylpyrazine followed by a design of experiments approach to optimize medium components for high-titer production.

Keywords: *C. glutamicum*, TMP, Overexpression, Hydrolysate, Medium optimization

Graphical abstract
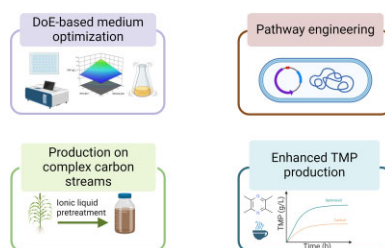
Introduction

2,3,5,6-Tetramethylpyrazine (TMP), a nitrogen-containing heterocyclic aromatic compound, is used widely as a flavor additive in the food industry (Kumar Verma et al., 2022; Mortzfeld et al., 2020). TMP production has been demonstrated using chemical and biological as well as chemical–biological hybrid approaches. Chemically, it can be synthesized using the Maillard reaction (Amrani-Hemaimi et al., 1995). Biologically, TMP and other related pyrazine production has been reported in bacteria such as *Bacillus* sp. (Gan et al., 2023; Jeong et al., 2019; Kłosowski et al., 2021; Larroche et al., 1999; Li et al., 2023; Liu et al., 2024; Meng et al., 2020; Tang et al.,

2023; Yin et al., 2018; Zhu & Xu, 2010; Zhu et al., 2010), *Lactococcus lactis* (Kim, 1991; Lee et al., 1994), *Corynebacterium glutamicum* (Demain et al., 1967; Dickschat et al., 2010; Eng et al., 2020; Fadel et al., 2018), *Escherichia coli* (Li et al., 2021; Peng et al., 2020; Xu et al., 2018), and fungi such as *Saccharomyces cerevisiae* and *Tolypocladium inflatum* (Cui et al., 2020; Fadel et al., 2022). High-titer TMP production at scale is well established and uses metabolic engineering (involving overexpression of pathway enzymes to improve precursor acetoin titers, deletion of competing pathways, and cofactor balancing) together with process optimization in natural and nonnative producers of TMP such as *Bacillus subtilis*

Table 1. Strains and Plasmids Used in This Study

Name	Genotype	Reference
Strains		
JBEI-145390	*Corynebacterium glutamicum* wild-type, biotin auxotroph	ATCC 13032
JBEI-7936	*C. glutamicum* BRC-JBEI 1.1.2, biotin auxotroph	Sasaki et al. (2019)
JBEI-252426	JBEI-145390 Δ*ldh*	This study
JBEI-241007	JBEI-145390 Δ*ldh* Δ*poxB*	This study
JBEI-234448	JBEI-145390 harboring JBEI-234435	This study
JBEI-234449	JBEI-145390 harboring JBEI-234436	This study
JBEI-234447	JBEI-145390 harboring JBEI-234434	This study
JBEI-234450	JBEI-145390 harboring JBEI-252429	This study
JBEI-234446	JBEI-145390 harboring JBEI-234433	This study
JBEI-234451	JBEI-145390 harboring JBEI-234439	This study
JBEI-252428	JBEI-7936 harboring JBEI-234439	This study
JBEI-252427	JBEI-252426 harboring JBEI-234439	This study
JBEI-241008	JBEI-241007 harboring JBEI-234439	This study
Plasmids		
JBEI-234435	pEC-XK99E-P$_{LacUV5}$-*alsS* (from *Bacillus subtilis*)	This study
JBEI-234433	pEC-XK99E-P$_{LacUV5}$-*alsS* (from *Lactococcus lactis*)	This study
JBEI-234436	pEC-XK99E-P$_{LacUV5}$-*budA* (from *Bacillus subtilis*)	This study
JBEI-234434	pEC-XK99E-P$_{LacUV5}$-*budA* (from *Lactococcus lactis*)	This study
JBEI-234429	pEC-XK99E-P$_{LacUV5}$-*alsS–budA* (from *Bacillus subtilis*)	This study
JBEI-234439	pEC-XK99E-P$_{LacUV5}$-*alsS–budA* (from *Lactococcus lactis*)	This study
JBEI-096766	pk18mobsacB—Δ*ldh*	Sasaki et al. (2019)
JBEI-096764	pk18mobsacB—Δ*poxB*	Sasaki et al. (2019)

(Shi et al., 2023), *Bacillus licheniformis* (Meng et al., 2020), and *E. coli* (Li et al., 2021).

We previously observed serendipitous production of TMP in the generally recognized as safe (GRAS) certified microbe, *C. glutamicum* (Eng et al., 2020). Functional genomics of the engineered strain revealed overexpression of TMP pathway genes among others, leading to enhanced production of TMP compared to the negligible levels in the wild-type (WT) (Banerjee et al., 2021). Since *C. glutamicum* could provide a robust industrially relevant microbial platform to produce this commodity chemical, we sought to specifically engineer *C. glutamicum* for a fully biological route to TMP production. In conjunction, we also assessed the role of minimal medium components in improving titer, rate, and yield (TRY) of TMP using established statistical methods to obtain a more systematic understanding of these parameters. Many studies have shown that changes in the minimal medium composition impact both growth and/or production in *C. glutamicum* (Becker et al., 2018; Freier et al., 2016; Sasaki et al., 2019; Unthan et al., 2014; Wolf et al., 2021; Yang et al., 2021). Therefore, identification of critical medium components and fine-tuning them further to maintain robust and stable production of TMP are valuable for further scale-up. Specifically, the type of nitrogen source added in two-stage cultivation processes has been known to dramatically affect the final titers of TMP (Peng et al., 2020; Xu et al., 2018). We hypothesize that a two-step metabolic engineering of *C. glutamicum* together with medium optimization could result in a multifold enhancement in TRY compared to multiedit engineering, which sometimes leads to poor performance of the host strain on scale-up.

We heterologously expressed TMP pathway genes—acetolactate synthase (*alsS*) and/or α-acetolactate decarboxylase (*budA*) from two different microbial hosts (*B. subtilis and L. lactis*) in *C. glutamicum*. The engineered *C. glutamicum* strain overexpressing *alsS* and *budA* from *L. lactis* stably produced ~0.8 g L^{-1} of TMP in the CGXII minimal medium supplemented with 40 g L^{-1} of glucose in a 24-deep well plate format. This engineered strain was also able to grow and produce TMP when supplemented with ionic liquid-pretreated sorghum hydrolysate. The medium components are also optimized leading to up to a four-fold increase in TMP titers (up to 3.5 g L^{-1}) using statistical tools compared to the control (0.8 g L^{-1}) in a microbioreactor. This production route to TMP in engineered *C. glutamicum* together with the lessons from the optimization studies can further be scaled up for sustainable production.

Materials and Methods
Chemicals and Reagents
Chemicals and reagents (molecular biology grade or higher) were purchased from Sigma-Aldrich (St Louis, MO, USA) or as indicated.

Strains and Plasmids
The strains and plasmids used in this study are listed in Table 1 and their sequences are available at http://public-registry.jbei.org (Ham et al., 2012). Isothermal DNA assembly (Gibson et al., 2009) using the HiFi DNA Assembly Master Mix was used according to manufacturer's protocols (NEBuilder, New England Biolabs, Ipswich, MA, USA) to assemble pathway gene overexpression plasmid constructs. Oligonucleotides for plasmid assembly and PCR were synthesized by Integrated DNA Technologies, Inc. (San Diego, CA) and listed in Supplementary Table S1.

For error-free polymerase chain reaction (PCR), Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) was used. Plasmids were initially transformed into chemically competent *E. coli* XL-1 Blue (New England Biolabs) and all sequences were confirmed by colony PCR and whole plasmid sequencing (Primordium Labs, Arcadia, CA, USA) followed by electroporation into *C. glutamicum* ATCC13032 as described previously (Sasaki

Calculation of Maximum Theoretical Yield of TMP

To assess the higher limit of production that could be expected from the engineered strain in a defined medium, the maximum theoretical yield (MTY) was calculated. The MTY of TMP was calculated by using a genome-scale metabolic model (GSMM) of *C. glutamicum*, iCGB21FR (Feierabend et al., 2021). Reactions (given in Equations 1–3) for diacetyl, acetoin, TMP formation, and TMP exchange were added to the GSMM for facilitating *in silico* TMP production.

$$\alpha - \text{Acetolactate} + 0.5\,O_2 \leftrightarrow \text{Diacetyl} + CO_2 + H_2O \quad (1)$$

$$\text{Diacetyl} + \text{NADH} + H^+ \leftrightarrow \text{Acetoin} + \text{NAD}^+ \quad (2)$$

$$2 - \text{Acetoin} + 2\text{NH}_4 \rightarrow \text{TMP} + 4H_2O + H^+ \quad (3)$$

Using flux balance analysis (Orth et al., 2010) and a fixed constraint of 10 mmol g (dry cell weight)$^{-1}$ hr^{-1} for the glucose uptake, the TMP formation was set as the objective to be maximized in order to calculate the MTY of TMP for *C. glutamicum*. Flux balance analysis was conducted using the COBRA toolbox (Schellenberger et al., 2011) for MATLAB (version R2020a, http://www.mathworks.com) and the solver Gurobi (version 6.0, http://www.gurobi.com).

Selection of Significant Medium Components Using Statistical Design of Experiments

One main objective of this study was to interrogate components of the production medium that could influence TMP levels. High-throughput assays using the flower plate format and design of experiments were used for testing the effect of CGXII minimal medium components on growth and TMP production. The statistical design and analysis were carried out using the Design-Expert software (Stat-Ease, USA). For the initial fractional factorial design (FFD) to screen significant components in the CGXII minimal medium, 10 of the components were evaluated. Each component was examined at two levels, a high (+) and a low (−) level of concentration (Table 2) in a resolution IV screening design leading to 32 runs (Supplementary Table S3). It is to be noted that in the resolution IV designs, typically the main effects are not confounded with other main effects or two-factor interactions but aliased with three-factor interactions. Also, certain two-factor interactions could be confounded with other two- or three-factor interactions. The experimental responses measured as OD$_{600}$ and TMP titers (g L^{-1}) in each of these 32 experiments were subjected to statistical analysis. The Design-Expert software was used to assess the significance of each component based on the first-order model assumption using Equation 4,

$$Y = \beta_0 + \Sigma\beta_i X_i, \quad (4)$$

where Y is the response predicted, β_0 is the intercept, and β_i is the linear coefficient or the slope indicating the magnitude of change expected in Y when there is one-unit change in the medium component X_i. Medium components with a low p value ($p < .05$) indicating a significant effect on the responses were selected for subsequent optimization.

Response surface methodology (RSM) was then used to determine the optimum concentrations of the significant components, while the rest of the medium components were kept constant. The Design-Expert software was used to generate a 13-run central composite design (CCD) for this purpose from the two significant factors obtained from FFD screening with 2k, that is four

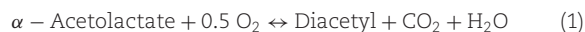## Production of TMP From Engineered *C. glutamicum*

A single colony was picked from Luria-Bertini (LB) agar plates containing kanamycin (50 mg L^{-1}) following standard laboratory procedures. Colonies were inoculated and grown in 5 ml LB medium (with kanamycin) at 30 °C on a rotary shaker at 200 rpm overnight. Cultures were then adapted by back diluting saturated cultures in the 5 ml CGXII minimal medium (Unthan et al., 2014) for 24 hr twice prior to starting production runs as described previously (Eng et al., 2020). For production runs in 24-deep well plates, 2 ml of the culture medium (CGXII with 40 g L^{-1} glucose) was used per well with a starting OD$_{600}$ (optical density at 600 nm) of 0.1. Deep well plates were incubated in a Multitron incubator (Infors HT, Switzerland) with a 3-mm orbital shaking platform shaken at 999 rpm (Bottmingen, Switzerland). The production pathway was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) after inoculation. The runs were monitored for periodic OD$_{600}$ measurements, residual sugars, and TMP accumulation at 24, 48, and 72 hr.

Production of TMP From Sorghum Hydrolysates

Before proceeding with further optimization of production, we examined whether this strain could tolerate and produce TMP in medium containing real-world plant biomass hydrolysates. The CGXII minimal medium was supplemented with hydrolysates obtained from sorghum to test the ability of the engineered high TMP producing strain to utilize real-world carbon streams treated with ionic liquid. Sorghum biomass (stems and leaves without panicles) from field-grown WT and genetically engineered plants that accumulate high amounts of 4-hydroxybenzoic acid (4-HBA) was obtained as previously described (Lin et al., 2022). Moreover, ensiled sorghum biomass was obtained from a commercial silage pit on a dairy farm in the southern part of the San Joaquin Valley, CA, USA. The hydrolysates were generated as described previously (Magurudeniya et al., 2021); briefly, sorghum biomass pretreatment was carried out at a solid loading of 15 wt% in a one-pot configuration using a 1-L Parr reactor (Parr Instrument Company, model: 4555–58, Moline, IL, USA), 10 wt% cholinium lysinate [Ch][Lys], and 75 wt% water. Typically, 60 g of the plant biomass was mixed thoroughly with 40 g of [Ch][Lys] and 300 g of water followed by heating at 140 °C for 3 hr. Post-pretreatment, 10 M HCl was added to adjust the pH of the slurry formed to 5. Subsequently, a commercial enzyme mixture, Cellic CTec3 and HTec3 (9:1 v/v) (Novozymes North America, Franklinton, NC, USA), was added to the slurry at a concentration of 10 mg enzyme/g sorghum biomass to carry out saccharification at 50 °C for 72 hr at 48 rpm in the same Parr vessel. Resulting hydrolysate was centrifuged at 8,000 rpm for 20 min and the supernatant was filtered using 0.45 and 0.22 µM rapid flow filters (Nalgene, USA). The hydrolysate pH was adjusted to 7.4 with 1 N NaOH. Hydrolysates were then added making up to 20% v v^{-1} or 40% v v^{-1} of the total sterile 1X CGXII media (to a total of 40 g L^{-1} glucose by supplementation of pure glucose). The production runs were carried out on 24-deep well plates as described earlier with the supplemented hydrolysates. OD$_{600}$, TMP (g L^{-1}), and residual sugars were measured at 24, 48, and 72 hr.

Table 2. Concentrations of the CGXII Minimal Medium Components Used for Fractional Factorial Design and Central Composite Design

| | | Fractional factorial design | |
| | | Levels | |
Factor	Component	−1	+1
A	Glucose	20	60
B	Ammonium sulfate ($NH_4)_2SO_4$	10	40
C	Urea	2.5	10
D	Potassium phosphate monobasic (KH_2PO_4)	0.5	2.0
E	Potassium phosphate dibasic (K_2HPO_4)	0.5	2.0
F	Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.125	0.5
G	Trace elements	0.5X	2X
H	Biotin	0.0001	0.0004
I	Protocatechuate (PCA)	0.015	0.06
J	Calcium chloride ($CaCl_2$)	0.005	0.02

| | | Central composite design | | | | |
| | | Levels | | | | |
Factor	Component	−1	0	+1	−α	+α
A	Glucose	60	70	80	55.86	84.14
B	Urea	10	15	20	7.93	22.07

Note. All concentrations in g L^{-1} except for trace elements, which is depicted in fold (X).

full-factorial points (where k is the number of components), five center points (replicates), and two axial points for each component (Table 2). Replication of design points provides an estimation of pure error in the design. Experiments were carried out on 48-well flower plates in a microbioreactor (BioLector Pro, M2P labs, Germany). For runs in the microbioreactor format, cells (inoculated to $OD_{600} = 0.1$ and induced with 0.5 mM IPTG) were grown in a 48-well flower plate (M2P labs, Germany) containing 1 ml medium in each well with antibiotic, sealed with gas permeable membrane (M2P labs, Germany) and shaken at 1,200 rpm at 30 °C. The runs were then analyzed for cell density, final residual sugar, and TMP at 48 hr. A final production run in 250 ml baffled shake flasks was carried out with 50 ml of the optimized medium shaken at 200 rpm, with cells inoculated to $OD_{600} = 0.1$ and induced with 0.5 mM IPTG and grown at 30 °C. Cells were harvested at 24 and 48 hr and monitored for OD_{600} and TMP.

Analytical Methods for Metabolite Quantitation

For metabolite quantification, 100 μl of the cell culture medium was combined with 100 μl of ethyl acetate containing *n*-butanol (30 mg L^{-1}) as an internal standard and analyzed as described previously (Eng et al., 2020). Briefly, post-extraction with ethyl acetate, 100 μl of the ethyl acetate phase was transferred into a glass vial with insert and 1 μl was analyzed using gas chromatography (GC) 8890 (Agilent Technologies, USA) equipped with a flame ionization detector (FID) and a DB-WAX capillary column (Agilent Technologies, USA) for quantification. The temperatures of the injector and detector were 250 and 300 °C, respectively. Helium was used as the carrier gas (2.2 ml min^{-1}) and the injection volume was 1 μl in splitless mode. The data were collected and analyzed using OpenLab software (Agilent Technologies, USA). Analytical grade standards were purchased from Sigma-Aldrich (St Louis, MO) and used to calculate analyte concentrations and confirm peaks of TMP and acetoin. Residual sugars and organic acids were measured using an Agilent 1260 high performance liquid chromatography (HPLC) (Agilent Technologies, USA) equipped with an Aminex 87H column with 4 mM sulfuric acid as the mobile phase

at 0.6 ml min^{-1} and analyzed using a refractive index detector. 4-Hydroxybenzoate (4HBA) and *p*-coumarate (pCA) from sorghum hydrolysates were quantified as reported previously (Garcia et al., 2023). The samples were appropriately diluted and analyzed using a 1260 Infinity II (Agilent Technologies) equipped with an Eclipse Plus Phenyl-Hexyl column (250-mm length, 2.6-mm diameter, and 5 μm particle, Agilent Technologies, 95990-912) at 50 °C and a UV detector. The mobile phases included 10 mM ammonium acetate with 0.07% formic acid in water (mobile phase A) and 10 mM ammonium acetate with 0.07% formic acid in 90% acetonitrile (mobile phase B) set at the following gradient: 30% solvent B, 0.5 ml min^{-1} for 12 min, 80% solvent B, 0.5 ml min^{-1} for 0.1 min, 100% solvent B, 0.5 ml min^{-1} for 0.5 min, 100% solvent B, 1.0 ml min^{-1} for 0.2 min, and 30% solvent B, 1.0 ml min^{-1} for 2.8 min. pCA concentrations and 4HBA concentrations were quantified using their respective analytical standard curves at 254 and 280 nm.

Results and Discussion
Overexpression of *alsS* and *budA* From *L. lactis* Enhances TMP Production in Engineered *C. glutamicum*

The proposed pathway for TMP biosynthesis is depicted in Fig. 1A. TMP is formed from condensation of two molecules of acetoin with ammonia. BioCyc shows production of (S)- or (R)-acetoin from acetolactate via pyruvate decarboxylation in *C. glutamicum* (de Man, 1959; Karp et al., 2019). Acetolactate either undergoes spontaneous decarboxylation to diacetyl, which is either reduced to acetoin as observed in *C. glutamicum* (Demain et al., 1967; Eng et al., 2020), or can be decarboxylated enzymatically via α-acetolactate decarboxylase (*budA*) as reported in *B. subtilis, B. licheniformis*, and *L. lactis* (Kumar Verma et al., 2022). Acetoin can also be converted to 2,3-butanediol in these microbes. Previous reports have shown that overexpression of the pathway enzymes and/or gene deletions blocking the competing pathways can enhance the carbon flux to TMP (Cui et al., 2020; Eng et al., 2020; Meng et al., 2020; Xu et al., 2018).
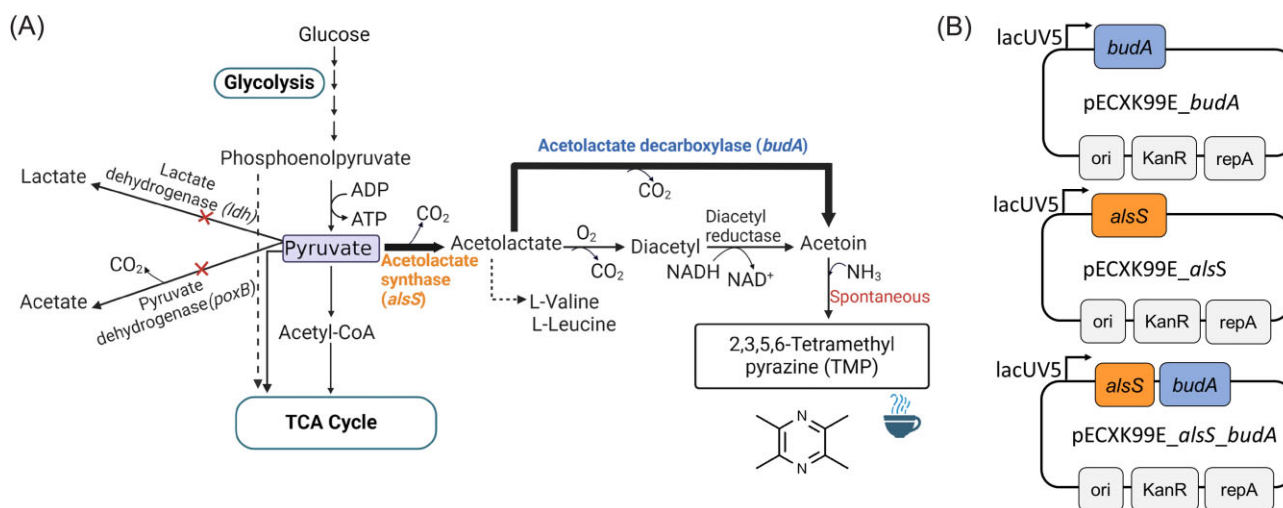
Fig. 1. The proposed 2,3,5,6-Tetramethylpyrazine (TMP) biosynthesis pathway in *Corynebacterium glutamicum* and the overexpression constructs employed in this study. (A) Reactions denoted by bold arrows are catalyzed by heterologously expressed proteins (in brackets are the genes encoding them) and crosses denote deletions. The spontaneous reaction from acetoin to TMP is marked in red. (B) The genes *alsS* and *budA* were amplified from either *Bacillus subtilis* (*Bs*) or *Lactococcus lactis* (*Ll*) genome and expressed under the control of the IPTG-inducible LacUV5 promoter using the pEC-XK99E shuttle vector with the kanamycin resistance marker.

We overexpressed TMP pathway enzymes (encoded by *alsS* and *budA*) from either *B. subtilis* and *L. lactis* under the control of the IPTG-inducible promoter using the shuttle expression vector pEC-XK99E in *C. glutamicum* ATCC13032 and tested their effect (individually and in combination) on growth and TMP production (Fig. 1B). The engineered strain overexpressing both the enzymes from *L. lactis* resulted in a high TMP titer of 898 ± 22 mg L^{-1} at 48 hr (Fig. 2A). GC-FID analysis indicated that, in the highest TMP producer, acetoin accumulates minimally (<100 mg L^{-1} at 24 and 48 hr) while TMP titers increase concomitantly (Fig. 2B). On the other hand, >0.7 g L^{-1} acetoin accumulation was observed at 48 hr in the strain(s) overexpressing *budA* from either *B. subtilis* or *L. lactis*—the highest titer (3.81 g L^{-1}) observed in the strain overexpressing *budA*$_{Bs}$ (Fig. 2B). Factors that may have affected spontaneous conversion to TMP from the accumulated acetoin in these strains could be pH dependency in the sampled time points, concentration of ammonia under the conditions, or rerouting of acetoin to other metabolites (Kim, 1991). Glucose was completely consumed in 48 hr across all the engineered strains, and considering the accumulation of TMP and its precursors in the late exponential/stationary phase, the production does not seem to impact growth (Fig. 2C) and glucose consumption significantly. We also did not observe any significant accumulation of overflow metabolites such as lactate or acetate in these transformants or the byproduct 2,3-butanediol. Since the TMP titer was maximum at 48 hr, in the remaining experiments we focused on harvesting the cells and reporting titers at this time point. Preliminary evaluation showed that overexpression of *alsS*$_{Ll}$ alone also led to higher levels of TMP (Supplementary Fig. S1). Nevertheless, we chose the engineered strain with the *alsS*$_{Ll}$–*budA*$_{Ll}$ overexpression, which we hypothesized to be a more reliable producer of the acetoin precursor.

Since pyruvate is the main precursor for TMP, we generated deletions (Δ*ldh* and Δ*ldh* Δ*poxB*) in the parent strain (ATCC13032) to reduce flux diversion to competing metabolites, lactate and acetate as demonstrated (Kou et al., 2022; Li et al., 2021) via allelic gene exchange. However, we did not see any significant differences in TMP titers upon deletion of *ldh* or the combined deletion of *ldh* and *poxB* (Supplementary Fig. S2). This observation could be specific to the tested media and culture conditions, as the flux redirection to overflow (lactate and acetate formation) in the parent strain was much less (11 and 23 mg L^{-1}, respectively) to start with.

The Engineered *C. glutamicum*-Type Strain Produced TMP More Efficiently Than the Engineered Strain BRC-JBEI 1.1.2

In a previous study, we observed serendipitous production of TMP in the *C. glutamicum* strain BRC-JBEI 1.1.2 engineered for isoprenol production upon exogenous addition of ionic liquids and under fed-batch bioreactor cultivation (Eng et al., 2020). The BRC-JBEI 1.1.2 strain is 99.9987% identical to *C. glutamicum* industrial strains SCgG1/SCgG2 and 89% similar to the type strain ATCC13032. It harbors ~320 unique genes when compared with the type strain that could be advantageous for production (Banerjee et al., 2021). This strain has been reported as a suitable production chassis for acetyl-CoA-derived terpenoid production at high titers (Luckie et al., 2024; Sasaki et al., 2019). In contrast, for the pyruvate-derived TMP production, we noted approximately fivefold lower TMP titers when transformed with the plasmid overexpressing *alsS*$_{Ll}$–*budA*$_{Ll}$ (JBEI-234439) (Fig. 3A). The acetoin levels for the JBEI-234439 strain in this production culture was higher than the acetoin titers in Fig. 2B (~80 mg L^{-1}). We attribute this variation observed to the spontaneous nature of the conversion from acetoin to TMP specifically in the 24-deep well plate format. We also observed acetate accumulating up to 1 g L^{-1} in the same strain at 48 hr. Since the last step in the proposed TMP formation in *C. glutamicum* is spontaneous and dependent on ammonia, we hypothesize that the residual carbon (including the excessive acetate overflow as seen in Fig. 3B) and nitrogen may affect the formation differentially in these two different *C. glutamicum* parent strains engineered to make TMP. Therefore, we focused on the engineered type strain (ATCC13032 overexpressing *alsS*$_{Ll}$–*budA*$_{Ll}$) for further optimization due to its superior performance.

Engineered *C. glutamicum* Was Able to Grow and Produce TMP on Lignocellulosic Carbon Streams

While many microorganisms engineered with non-native pathways demonstrate high production of final compounds on rich
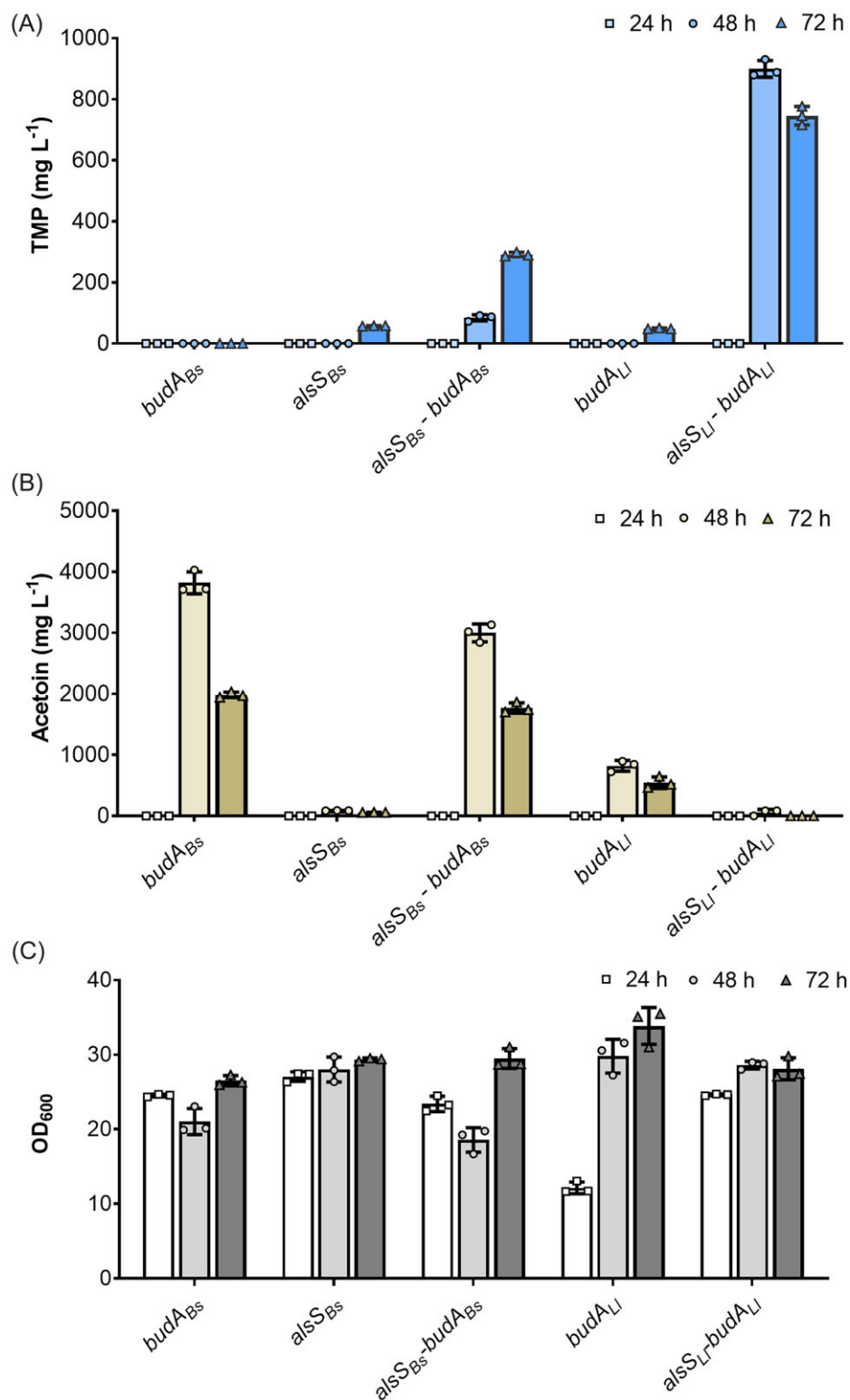
Fig. 2. 2,3,5,6-Tetramethylpyrazine, acetoin titers (mg L^{-1}), and cell density (OD_{600}) profiles in the various engineered strains. *Corynebacterium glutamicum* ATCC13032 was transformed with pEC-XK99E overexpressing the respective single- ($alsS_{Bs}$, $budA_{Bs}$, and $budA_{Ll}$) and double-gene ($alsS_{Bs}$–$budA_{Bs}$ and $alsS_{Ll}$–$budA_{Ll}$) cassettes derived from either *Bacillus subtilis* (*Bs*) or *Lactococcus lactis* (*Ll*). Strains were grown in the 2 ml CGXII minimal medium with 50 mg L^{-1} kanamycin and 4% glucose in 24-deep well plates. Samples were harvested at 24, 48, and 72 hr. Data represented as average ± SD (n = 3 replicates).
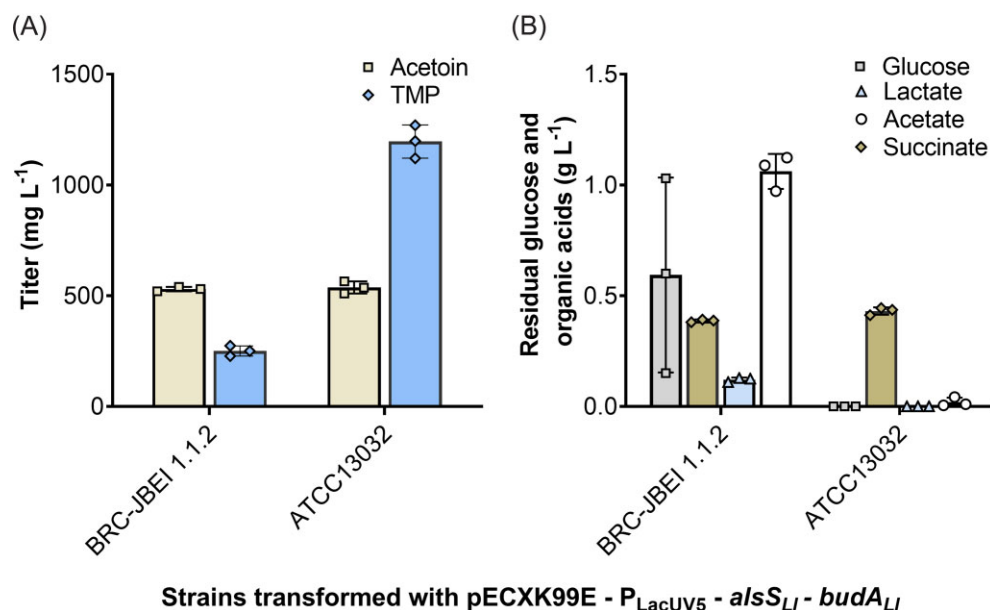
Fig. 3. 2,3,5,6-Tetramethylpyrazine (TMP) production in the engineered *Corynebacterium glutamicum* BRC-JBEI 1.1.2 and ATCC13032 strains transformed with the plasmid overexpressing *alsS_LI–budA_LI* (JBEI-234439). Production of TMP (mg L^{-1}) and acetoin (mg L^{-1}) (A). Residual glucose and organic acid profiles (g L^{-1}) in the spent medium of the two engineered strains analyzed using HPLC after 48 hr (B). Strains were grown in 24-deep well plates containing the 2 ml CGXII minimal medium with 4% glucose, 50 mg L^{-1} kanamycin, and induced with 0.5 mM IPTG. Data represented as average ± SD ($n = 3$ replicates).

or defined synthetic medium, sustainable production requires the use of complex real-world carbon streams such as those derived from lignocellulose (Lin et al., 2022). *Corynebacterium glutamicum* is known to be a good host for mixed carbon growth and production and has also been examined for bioproduction on plant biomass-derived hydrolysate-based growth medium (Banerjee et al., 2021; Eng et al., 2020; Mhatre et al., 2022; Sasaki et al., 2019). In this study, we used hydrolysates from different sorghum lines—WT, ensiled, and engineered to overproduce 4-HBA that represent other aspects of bioconversion, such as altered aromatics profile, co-utilization with sugars, and optimal pretreatment methods for one-pot feedstock to fuel conversion (Baral et al., 2024; Lin et al., 2022; Magurudeniya et al., 2021). Ensilage specifically is an important feature of biomass storage and pretreatment reducing biofuel production cost and carbon footprint (Magurudeniya et al., 2021). Our results confirm that the engineered *C. glutamicum* strain retained the capability for TMP production in these hydrolysates. Specifically, the engineered strain showed no growth defects when grown with up to 40% (v v^{-1}) hydrolysate supplemented medium and produced TMP in the range of 500–660 mg L^{-1} at 48 hr upon supplementation with 20% (v v^{-1}) sorghum biomass hydrolysates, which is slightly lower than the pure glucose supplemented minimal medium (Fig. 4A and B). Hydrolysate derived from ensiled sorghum could support growth and production to comparable levels even when the supplementation volume was increased to 40% from 20%, whereas the hydrolysate derived from WT (no ensiling) and engineered sorghum exhibited a further decrease in TMP titer at 40% (v v^{-1}) supplementation. On the other hand, increased accumulation of the TMP precursor acetoin (up to 2 g L^{-1}) was observed upon supplementation with 20% (v v^{-1}) WT (including ensiled sorghum) hydrolysate compared to glucose minimal medium and decreased with a further increase in supplementation volume (Supplementary Fig. S3). The lower TMP titers and significant acetoin accumulation under

these conditions could be due to the presence of various other plant biomass-derived components, which affect the carbon to nitrogen ratio (Supplementary Table S2) and pretreatment (of the sorghum biomass) residues altering the metabolic flux to TMP. This preliminary assessment of cultivability and production in hydrolysates was critical for us to ensure that further optimization with this strain in the defined medium was justified.

Glucose and Urea Are Significant Minimal Medium Components Affecting TMP Production in Engineered *C. glutamicum*

One of the pivotal challenges with engineered microbial strains is reliable performance upon scale-up. This is because the growth and performance are governed by numerous interdependent process variables. It therefore becomes imperative to understand how these factors impact the host physiology and product yields. One such process variable impacting production is the medium composition. A defined medium ensures reproducibility and minimizes interference with downstream purification. A commonly used synthetic minimal medium for *C. glutamicum* is CGXII (Keilhauer et al., 1993) and it is known to influence growth and/or production (Becker et al., 2018; Freier et al., 2016; Sasaki et al., 2019; Unthan et al., 2014; Wolf et al., 2021; Yang et al., 2021). We therefore explored medium optimization strategy to assess parameters that could influence the production of TMP. The MTY was calculated to be 0.58 mol TMP per mol of glucose (i.e. 0.44g TMP g^{-1} glucose or 17.66 g L^{-1} TMP from 40 g L^{-1} of glucose) using the GSMM, iCGB21FR. Experimentally, in the CGXII minimal medium, the highest TMP producing strain resulted in 0.03 mol TMP mol^{-1} glucose (i.e. only 5.2 % of the MTY), indicating considerable room for improvement.

The CGXII medium consists of >12 components, which could generate a larger search space for unanticipated determinants of
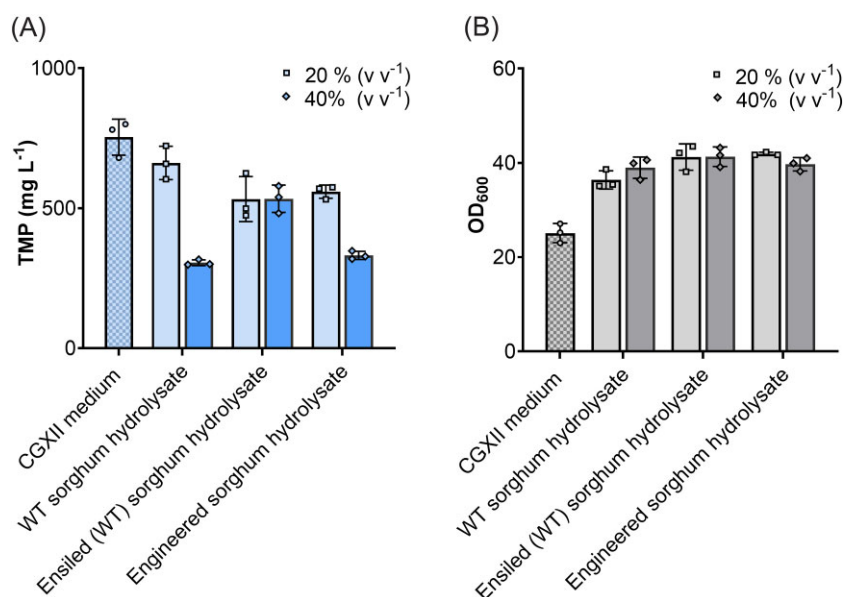
Fig. 4. Growth and production of 2,3,5,6-Tetramethylpyrazine (TMP) with sorghum hydrolysate supplementation. OD_{600} (A) and TMP titer (mg L^{-1}) (B) in the TMP producer strain overexpressing $alsS_{Ll}$–$budA_{Ll}$ on the CGXII minimal medium supplemented with 20% (v v^{-1}) and 40% (v v^{-1}) hydrolysates derived from wild-type, ensiled, and engineered sorghum. The strain was grown in 24-deep well plates and induced with 0.5 mM IPTG and samples harvested at 48 hr. Data represented as average ± SD (n = 3 replicates).

high TMP titer. Optimizing this during large-scale fermentations is expensive, time-consuming, and laborious. Therefore, to identify significant medium components affecting production systematically, we used a design of experiments approach (Yang et al., 2021). Given the large number of components in the CGXII medium, we chose to screen the main components affecting growth and/or TMP production using FFD that helps to identify the significant components utilizing limited experiment runs and resources. To prevent medium and product evaporation during tests, ensure high mass transfer by effective mixing at high cell densities, which is important due to the last spontaneous step from acetoin to TMP, and ensure scalability, the cells were grown in a high-throughput microbioreactor format (Fink et al., 2021; Kensy et al., 2009; Wagh et al., 2022). We retained usage of the standard 0.5 mM IPTG for induction in the 48-flower well microtiter plates of the Biolector microbioreactor as it did not significantly affect titers in the range of 0.125–1.5 mM and a fill volume of 1 ml for the flower plates for further experiments based on preliminary optimizations (Supplementary Fig. S4 and Fig. S5).

The biomass and TMP titers obtained after 48 hr in each of the experiment runs (Supplementary Table S3) were subjected to analysis by the Design-Expert software (Stat-Ease, USA). Our results indicated that glucose (A, the major carbon source) and its possible interaction (AC) with urea (C, one of the nitrogen sources) affected cell density (represented by OD_{600}) whereas glucose (A), urea (C), and their possible interaction (AC) significantly affected TMP production. The statistical significance was confirmed by an analysis of variance (ANOVA) (Supplementary Table S4). Other medium components were not significant in their respective tested range. It is to be noted that in this resolution IV screening design used, the interactive effect (AC) is confounded by another two-factor interaction (biotin with calcium chloride as well as other higher order interactions). We assume that the AC interaction effect is likely significant since the main effects (A and C) involved are also significant according to the heredity principle

(Hamada and Wu, 1992). High TMP titers (2–3 g L^{-1}) with high cell densities were obtained in runs 3, 11, 15, 17, 24, and 27, with 60-g L^{-1} glucose and 10-g L^{-1} urea (highest in the range tested). Similarly, cell density and TMP accumulation were relatively low when these medium components were low (where mostly TMP was not detected). Their significance is clear from the p-value in the ANOVA for the selected factorial model indicating these components as significant ($p < .05$) (Supplementary Table S4). Tolerance to and utilization of high glucose concentrations up to 140 g L^{-1} has been demonstrated in batch cultivations of *C. glutamicum*. Given the positive impact on TMP production with increasing glucose concentration, we explored higher glucose ranges for the second level of optimization (up to 80 g L^{-1} glucose) in the CGXII medium. Our results also concur with established studies (Yang et al., 2021) that urea is preferred to ammonium sulfate and affects growth and product formation significantly.

Optimizing the Significant Minimal Medium Components Using RSM

Optimum concentrations of glucose and urea were determined using RSM, while the rest of the medium components were kept at constant levels (reduced to half the concentrations in the CGXII medium except for 3-morpholinopropane-1-sulfonic acid (MOPS)), that is, at the lowest levels tested in the FFD where they did not significantly affect growth and TMP production. Reducing the rest of the medium components (including biotin, protocatechuate, minor salts, and trace elements) except MOPS to these low levels could offer an economic advantage or help to offset to some extent, the cost of increasing the other significant medium components while using the final optimized medium. A 13-run CCD, developed using the Design-Expert software (Stat-Ease, USA), was used to optimize the concentrations of the two factors. The experimental recipe and responses in CCD for individual runs is given in Table 3.

Table 3. Central Composite Design and Responses Obtained

| Run	Factor 1: Glucose (g L^{-1})	Factor 2: Urea (g L^{-1})	Cell density (OD$_{600}$)	TMP (mg L^{-1})
1	70	7.92	31.7	2,402.68
2	70	15	35.6	2,519.07
3	60	10	30.25	2,161.95
4	80	10	32.5	3,220.72
5	70	15	36.5	2,326.03
6	55.86	15	39.8	573.99
7	70	22.07	43.9	19.02
8	80	20	32.2	1,813.54
9	70	15	38.5	2,275.24
10	70	15	30	2,624.17
11	60	20	40.9	35.44
12	70	15	34.5	2,355.59
13	84.14	15	34.1	2,880.99

Note. TMP = 2,3,5,6-Tetramethylpyrazine.

The data on cell density (OD$_{600}$) and TMP production from RSM were subjected to an ANOVA to assess the statistical significance. The cell density varied in a narrow range of 30–43.9 across the runs. Statistical significance fitting for cell density response was poor in all the model types (linear, quadratic, 2-factor interaction, and cubic) in the tested range of glucose and urea and hence was not characterized further. On the other hand, we detected a wide range of variation in TMP concentrations from 19 to 3.2 g L^{-1} with different values of glucose and urea tested, indicating the importance of this specific carbon to nitrogen ratio on TMP production and the importance of design of experiments as demonstrated previously (Xu et al., 2018). We were able to fit the TMP production (mg L^{-1}) response at 48 hr using a quadratic model (the highest order polynomial where the additional terms are significant, and the model was not aliased) to quantify the individual and interactive effects of glucose (A) and urea (B) as follows:

$$\text{TMP} = +2,420.02 + 762.43\,A - 863.09\,B + 179.83\,AB$$
$$- 261.58\,A^2 - 519.9\,B^2 \tag{5}$$

The contour plot (2D) generated by the software was used to visualize the interactive effect of glucose and urea on TMP production and to find the optimized concentrations of the same (Fig. 5). The statistical significance of the model equation was confirmed by the ANOVA (Supplementary Table S4).

Based on the model, an experiment was run using the optimum values for both glucose and urea as given by the response optimization to confirm that the predicted values of TMP were obtained. The model predicted optimum (80-g L^{-1} glucose and 11.9-g L^{-1} urea) resulted in 3.56-g L^{-1} TMP (Fig. 6) reproducibly in two different growth formats—microbioreactor and batch shake flasks. In the final optimized medium, all the other CGXII medium components (except for the MOPS buffering component) were reduced to half their initial value (Table 4). Interestingly, there was also an increased accumulation of acetoin (up to 2.3 g L^{-1}) under optimized conditions (Supplementary Fig. S6). Glucose exhaustion in the supernatant at 48 hr in both optimized and control media was confirmed by HPLC.

In the shake flask format, a drop in cell density compared to the control medium was observed. This might be due to more glucose being siphoned off for production of other compounds
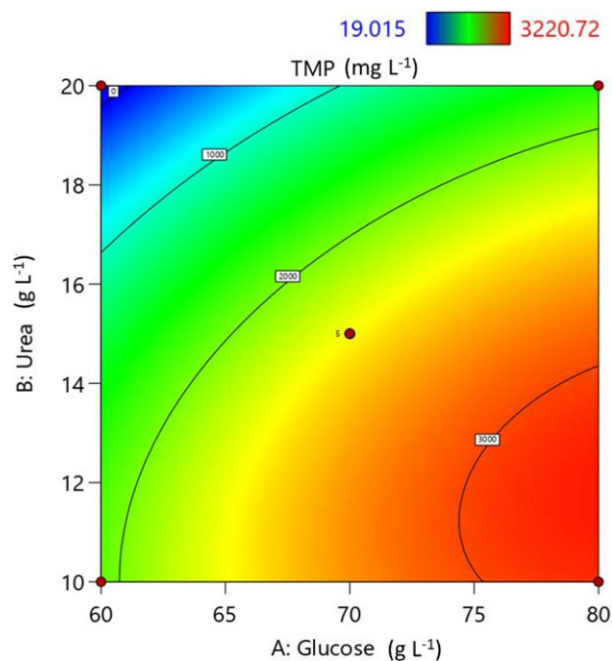
Fig. 5. Contour plot (2D) showing the significance and interaction of glucose and urea on 2,3,5,6-Tetramethylpyrazine (TMP) production. Glucose (Component A), the main carbon source and the significant nitrogen source, urea (Component B) and their effect on the response, TMP (mg L^{-1}). The values within the boxes are experimentally observed values of TMP at these design levels. A total of four design points (circles), four corner points, and one center point (replicated five times) were used in the design.

such as carotenoids (observed qualitatively as yellow cell pellets, data shown or not shown) in addition to TMP production compared to the control. In contrast to a published study (Yang et al., 2021), which shows optimal growth and production using a modified minimal medium with elimination of ammonium sulfate, we observed a significant drop in TMP titers (1,650 mg L^{-1}) when no ammonium sulfate was used in the optimized medium, reiterating the fact that ammonia is essential for the last spontaneous step in TMP production.
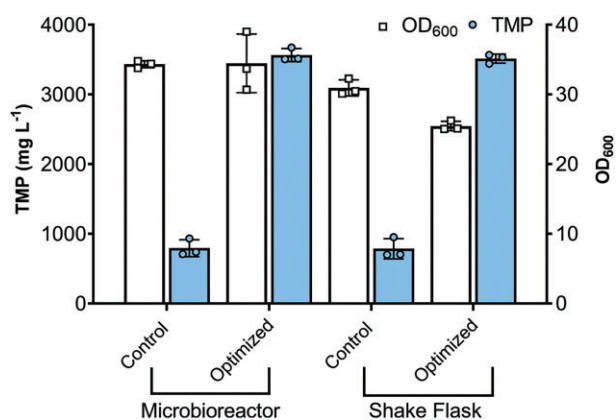
Fig. 6. 2,3,5,6-Tetramethylpyrazine (mg L^{-1}) production in the engineered *Corynebacterium glutamicum* strain grown in the control and the optimized CGXII minimal medium. The strain overexpressing $alsS_{Ll}$–$budA_{Ll}$ was grown in 48-well flower plates in a microbioreactor as well as in 50 ml of medium in 250 ml baffled shake flasks and induced with 0.5 mM IPTG. Samples were harvested at 48 hr. Data represented as average $\pm$ SD ($n = 3$ replicates).

Table 4. Concentrations of the Components Used in the Optimized and Standard CGXII Medium

Component	CGXII medium[a]	
	Optimized	Standard
Glucose	80	40
Ammonium sulfate ($(NH_4)_2SO_4$)	10	20
Urea	11.9	5.0
Potassium phosphate monobasic (KH_2PO_4)	0.5	1.0
Potassium phosphate dibasic (K_2HPO_4)	0.5	1.0
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.125	0.25
$FeSO_4 \cdot 7H_2O$	0.005	0.01
$MnSO_4 \cdot H_2O$	0.005	0.01
$ZnSO_4 \cdot 7H_2O$	0.0005	0.001
$CuSO_4 \cdot 5H_2O$	0.0001	0.0002
$NiCl_2 \cdot 6H_2O$	0.00001	0.00002
Biotin	0.0001	0.0002
Protocatechuate (PCA)	0.015	0.03
Calcium chloride ($CaCl_2$)	0.005	0.01
MOPS	21	21

[a]All concentrations given are in g L^{-1}.

Conclusion

In this study, we have successfully engineered *C. glutamicum* to be a high-titer production platform for TMP and established its robustness in real-world carbon streams. Metabolic engineering and the optimization of the biosynthetic pathway allowed reliable production of TMP in both defined and complex media. To quantitatively understand the role of medium components in production, we further examined the canonical glucose-defined medium and conditions used for *C. glutamicum* in a high-throughput plate format to recapitulate bioreactor performance. We established that a high glucose concentration of 80 g L^{-1} and urea 11.9 g L^{-1} had the highest benefit on productivity leading to up to 3.56 g L^{-1} TMP in 48 hr of cultivation, while the other medium components were reduced by half their original concentration in the CGXII medium. Functional genomics analysis would further help to shed light on competing pathways and candidates for targeted engineering, and further conversion of accumulated acetoin to TMP using a two-stage process would also improve production. *Corynebacterium*

glutamicum is an important industrial host, and this study further indicates it as a robust and stable production host for pyrazines and its precursors, which are commodity chemicals for a wide range of applications. The results from the conventional medium optimization strategy used in this study could also serve as test datasets for high-throughput omics and machine learning-guided optimization strategies for robust and sustainable production of this target chemical. Further, *C. glutamicum* has been known to simultaneously utilize multiple carbon sources (Sasaki et al., 2009). Together with the demonstration of its growth on ionic liquid-pretreated sorghum hydrolysate in the current study, a useful next step would be to extend the best producer strain's catabolic capabilities for mixed carbon utilization for TMP production.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

Conflict of Interest

A.M. and T.E. are inventors on the U.S. patent application No. 20210261511A1 assigned to the Regents of the University of California.

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