# 读书报告

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# One-step process for production of *N*-methylated amino acids from sugars and methylamine using recombinant *Corynebacterium glutamicum* as biocatalyst

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Melanie Mindt[1], Joe Max Risse[2], Hendrik Gruß[3], Norbert Sewald[3], Bernhard J. Eikmanns[4] & Volker F. Wendisch[1]

使用重组谷氨酸棒杆菌作为生物催化剂从糖和甲胺生产N-甲基化氨基酸的一步法

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# Introduction

# 氨基酸的N-甲基化

氨基酸的N-甲基化发生在细菌和真核生物中。

In green tea leaves, the $N^5$-ethylated L-glutamine derivative theanine was shown to be responsible for their umami taste(Cartwright,Sakato).

N-methylated amino acids are also found in depsipeptides that are used as drugs e.g. vancomycin, actinomycin D and cyclosporine.

与非甲基化肽相比，含有N-甲基氨基酸的肽在蛋白水解和增加膜通透性方面具有更高的稳定性。

# Introduction

In certain bacteria, utilization of mono-methylamine (MMA) may lead to N-methylated amino acids.
Some bacteria that can grow with reduced carbon substrates without carbon-carbon bonds such as methane or methanol can utilize MMA as sole source of carbon.

A

NADPH NADP$^+$

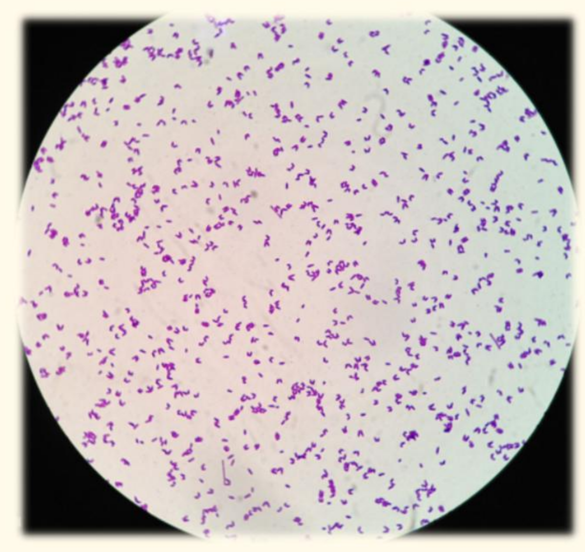
DpkA

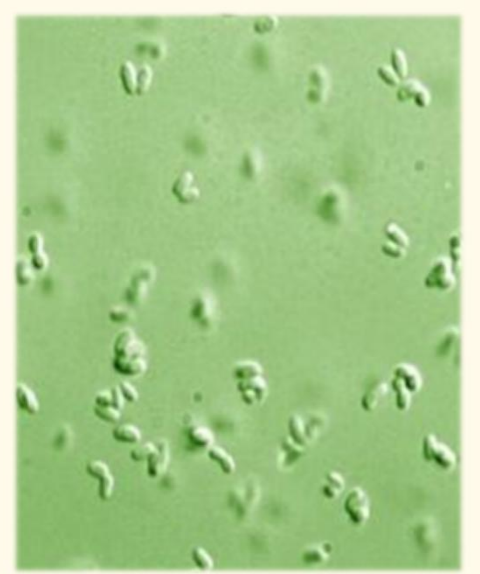
pyruvate
丙酮酸

methylamine
甲基丙氨酸

NMeAla
N-甲基丙氨酸

# 谷氨酸棒杆菌

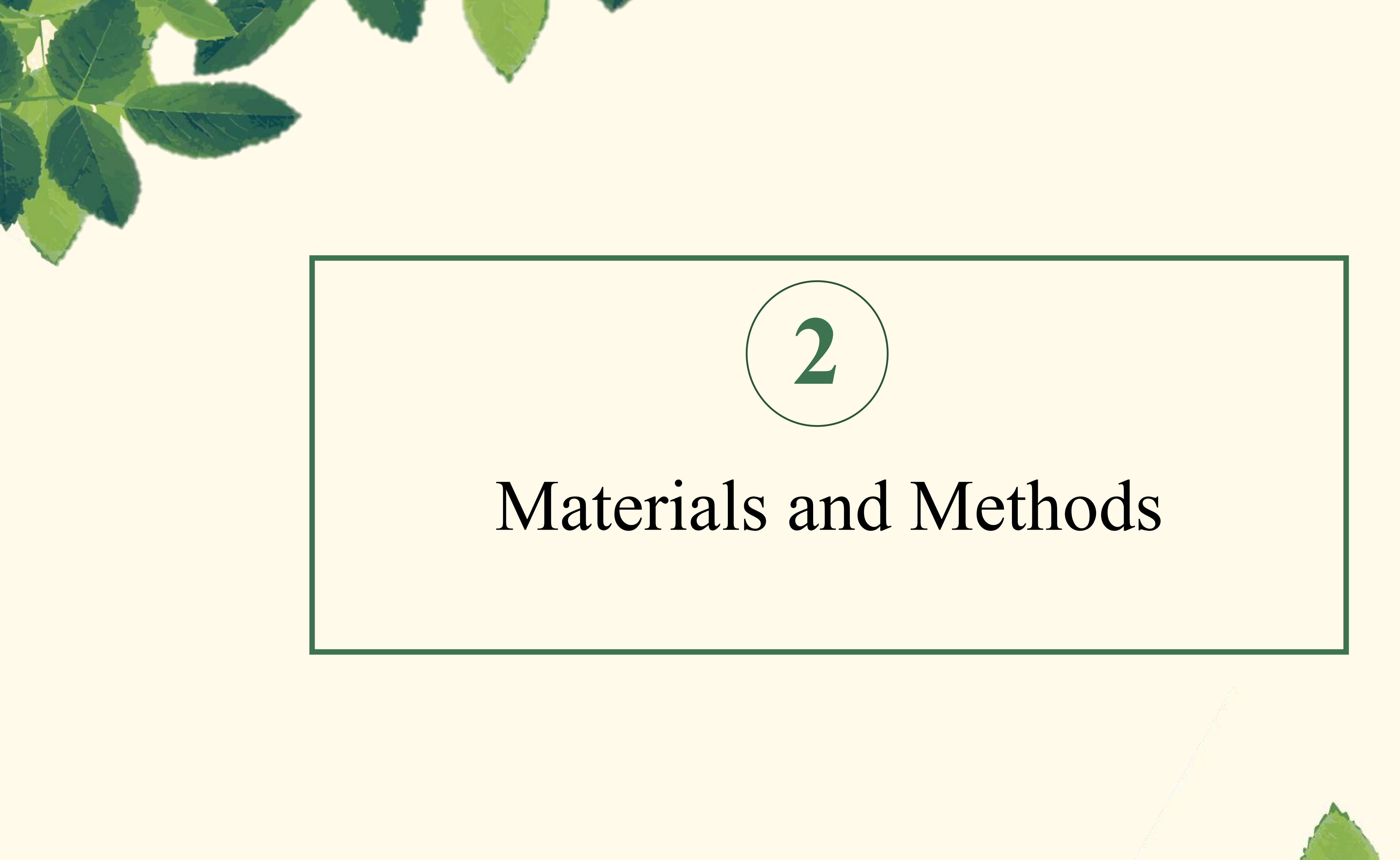




For more than fifty years, *C. glutamicum* has been used for the safe production of food and feed amino acids (Taylor & Francis, Boca Raton, 2005).

Further amino acids and related compounds can effciently be produced by glucose- and ammonium-based fermentation using recombinant *C. glutamicum* strains.

Metabolic engineering of C. glutamicum has not been restricted to amino acids but also production of the α-keto acids pyruvate, ketoisovalerate and ketoisocaproate were established.



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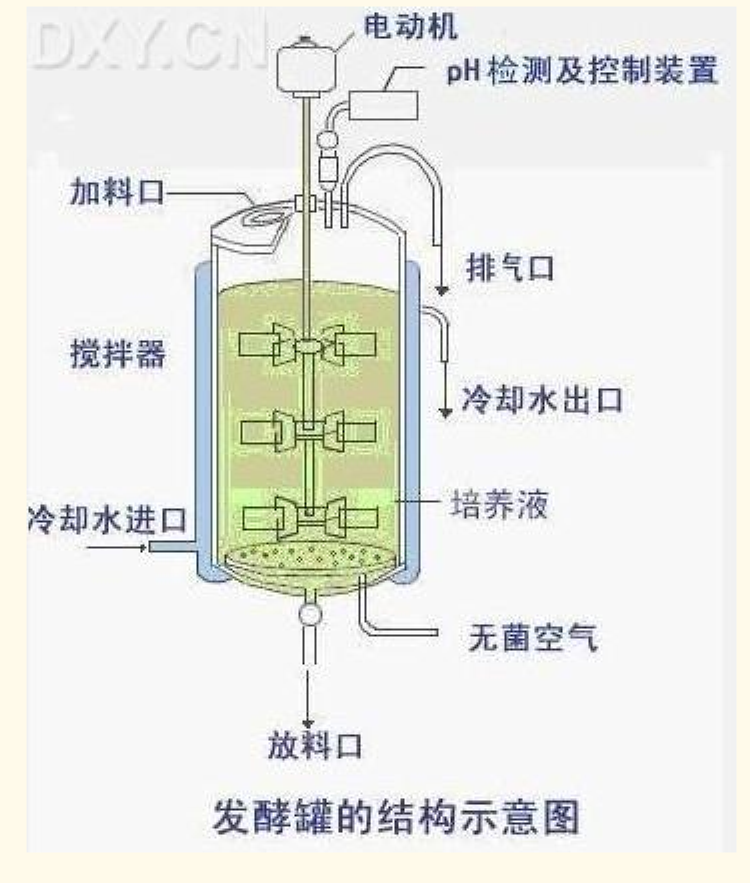
# Materials and Methods

# Bacterial strains and growth conditions

Bacterial trains and vectors used in this study.

| Strains and vectors | Description | Source |
|---|---|---|
| **Strains** | | |
| WT | *C. glutamicum* wild type, ATCC13032 | American Type Culture Collection |
| ELB-P | WT carrying deletions *ΔaceE Δpqo ΔldhA ΔC-T ilvN ΔalaT ΔavtA* | 31 |
| NMeAla1 | WT carrying deletions *ΔaceE Δpqo ΔldhA ΔC-T ilvN ΔalaT ΔavtA* and vector pVWEx1-*dpkA* | This work |
| **Plasmids** | | |
| pVWEx1 | $Kan^R$, *C. glutamicum/E. coli* shuttle vector ($P_{tac}$, *lacI*, pHM1519 $oriV_{C.g.}$) | 85 |
| pEKEx3 | $Spec^R$, *C. glutamicum/E. coli* shuttle vector ($P_{tac}$, *lacI*, pBL1 $OriV_{C.g.}$) | 86 |
| pECXT99A | $Tet^R$, *C. glutamicum/E. coli* shuttle vector ($P_{trc}$, *lacI*, pGA1 $OriV_{C.g.}$) | 87 |
| pVWEx1-*dpkA* | $Kan^R$, pVWEx1 overexpressing *dpkA* from *P. putida* KT2440 and change of start codon GTG to ATG | This work |
| pEKEx3-$xylA_{Xc}$-$XylB_{Cg}$ | $Spec^R$, pEKEX3 overexpressing *xylA* from *Xanthomonas campestris* SCC1758 and *xylB* from *C. glutamicum* ATCC 13032 | 45 |
| pECXT99A-*araBAD* | $Tet^R$, pECXT99A overexpressing *araBAD* from *E. coli* MG1655 | This work |
| pECXT99A-*amyA* | $Tet^R$, pECXT99A overexpressing *amyA* from *Streptomyces griseus* IMRU3570 | 42 |

# Fed-Batch cultivation



发酵罐的结构示意图

## 第一次进料阶段

26.7 g $L^{-1}$乙酸钾溶液（总体积：500mL）
取决于相对溶解氧饱和度，当rDOS信号升至60％以上时激活，当rDOS感觉低于60％时停止。

## 第二次进料阶段（22h后）

初次加入164g $L^{-1}$葡萄糖和84g $L^{-1}$ MMA（总体积：1000mL）然后以12.3mL $h^{-1}$进行线性进料。
在最初的24小时内每隔2小时自动取样，然后每8小时取样，并冷却至4℃直至分析。

# 3

# Results and Discussion

# *Corynebacterium glutamicum* as suitable host for NMeAla production

minimal medium with:

carbon source
- 50 mM MMA
- 50 mM NMeAla
- glucose

nitrogen source
- 50 mM MMA
- 50 mM NMeAla
- 30 mM ammonium sulfate and 17 mM urea

This growth experiment revealed that C. glutamicum could neither use MMA nor NMeAla as sole carbon or nitrogen source (data not shown).

# Corynebacterium glutamicum as suitable host for NMeAla production

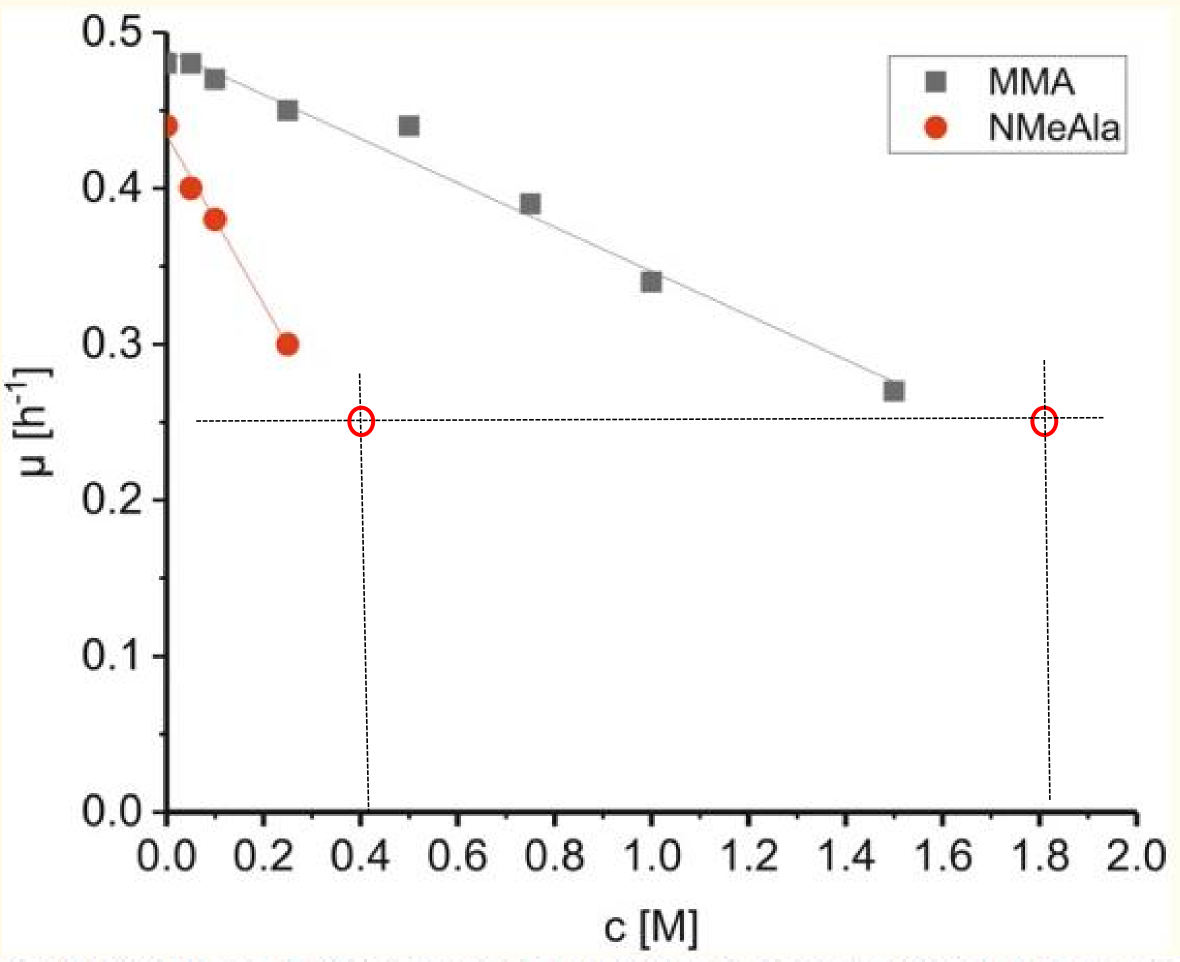



**Figure 2.** Growth rates of *C. glutamicum* wild type in the presence of varying concentrations of MMA or NMeAla. *C. glutamicum* wild type was grown in presence of increasing MMA (0.05 M to 1.5 M) or NMeAla (0.05 M to 0.25 M) concentrations and specific growth rates were determined. Half maximal growth rates were obtained by extrapolation.

The growth rate was diminished at higher concentrations to about half-maximal rates at 1.8 $_M$ MMA and 0.4 $_M$ NMeAla, respectively.

# Corynebacterium glutamicum as suitable host for NMeAla production

**Supplementary Table: Differential gene expression of *C. glutamicum* wild type grown in glucose containing CGXII minimal medium supplemented with 250 mM MMA or 125 mM $(NH_4)_2SO_4$.**

| Gene ID[ab] | Gene name[b] | Gene annotation[b] | mRNA level $(MMA/(NH_4)_2SO_4)$[c] |
|---|---|---|---|
| cg0759 | *prpD2* | 2-Methylcitrate dehydratase, involved in propionate catabolism | 5.0 |
| cg0762 | *prpC2* | 2-Methylcitrate synthase, involved in propionate catabolism | 2.7 |
| cg0801 | - | Hypothetical protein | 2.7 |
| cg2566 | - | Putative secreted protein | 0.3 |
| cg3402 | - | Putative $Hg_2^+$ permease, MerTP-family | 2.7 |

[a] Genes shown are sorted to their identifiers.

[b] Gene ID, name and annotation are according to BX927147

[c] Differential gene expression as calculated for two biological replicates. Values listed were selected for $P < 0.05$ and at least twofold change of the RNA level.

# Metabolic engineering of C. glutamicum for fermentative production of NMeAla.

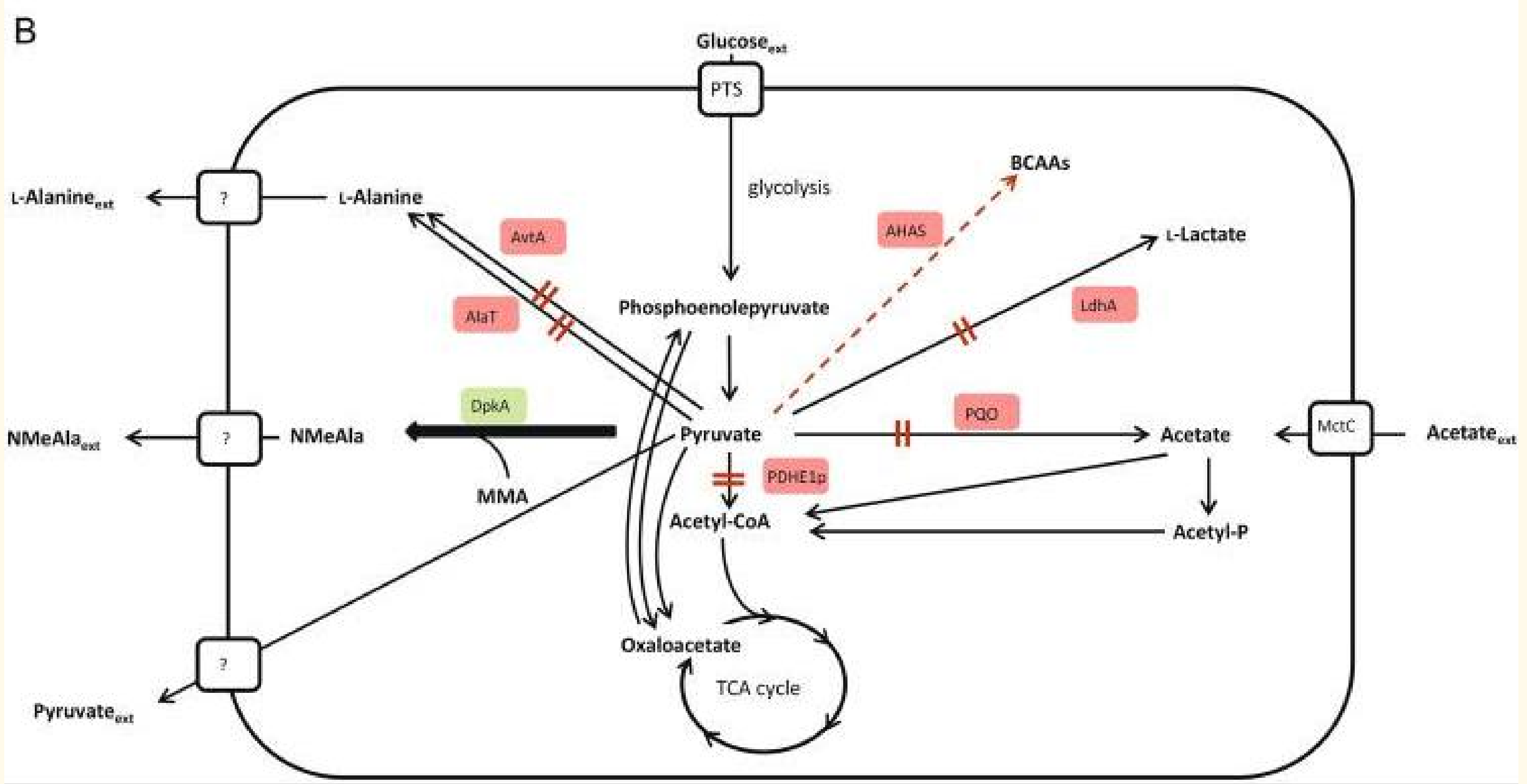
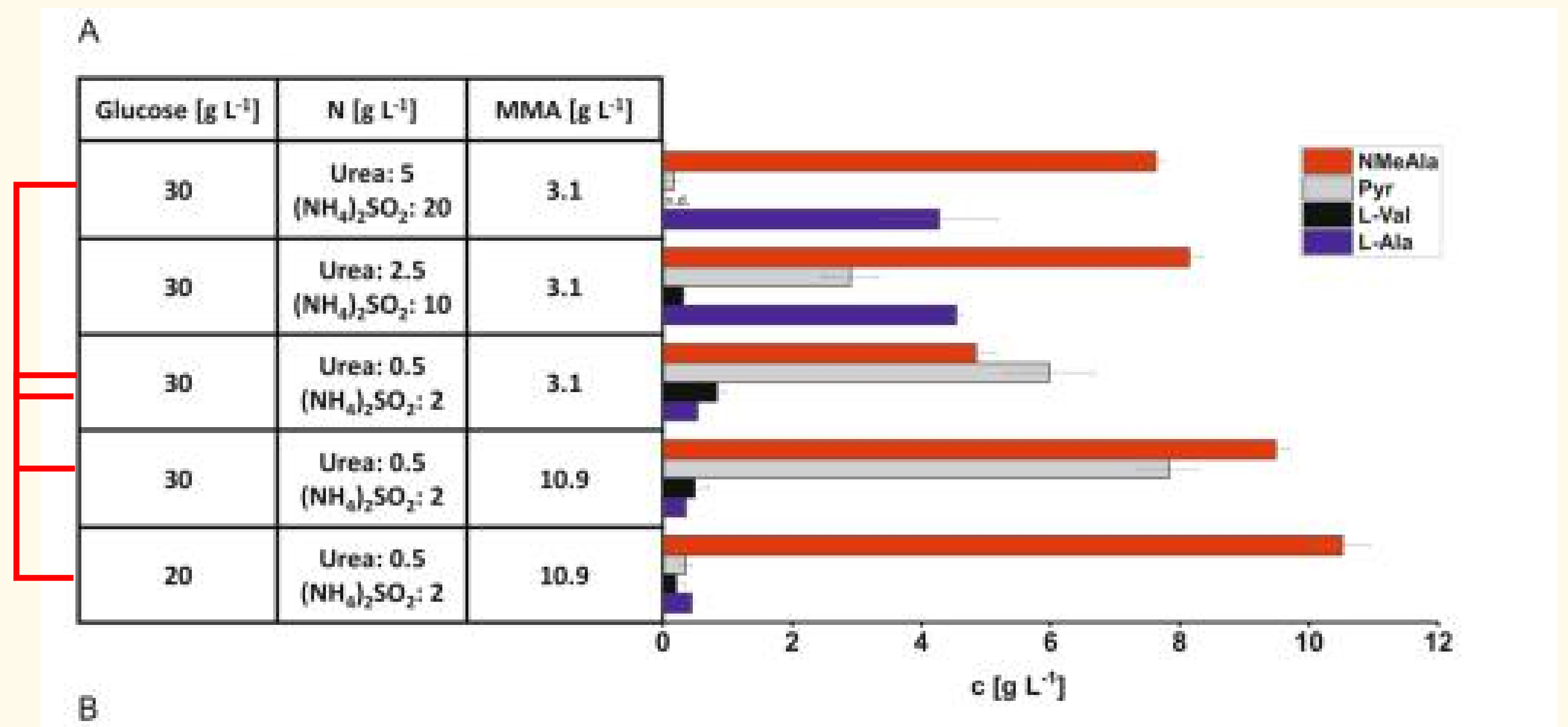


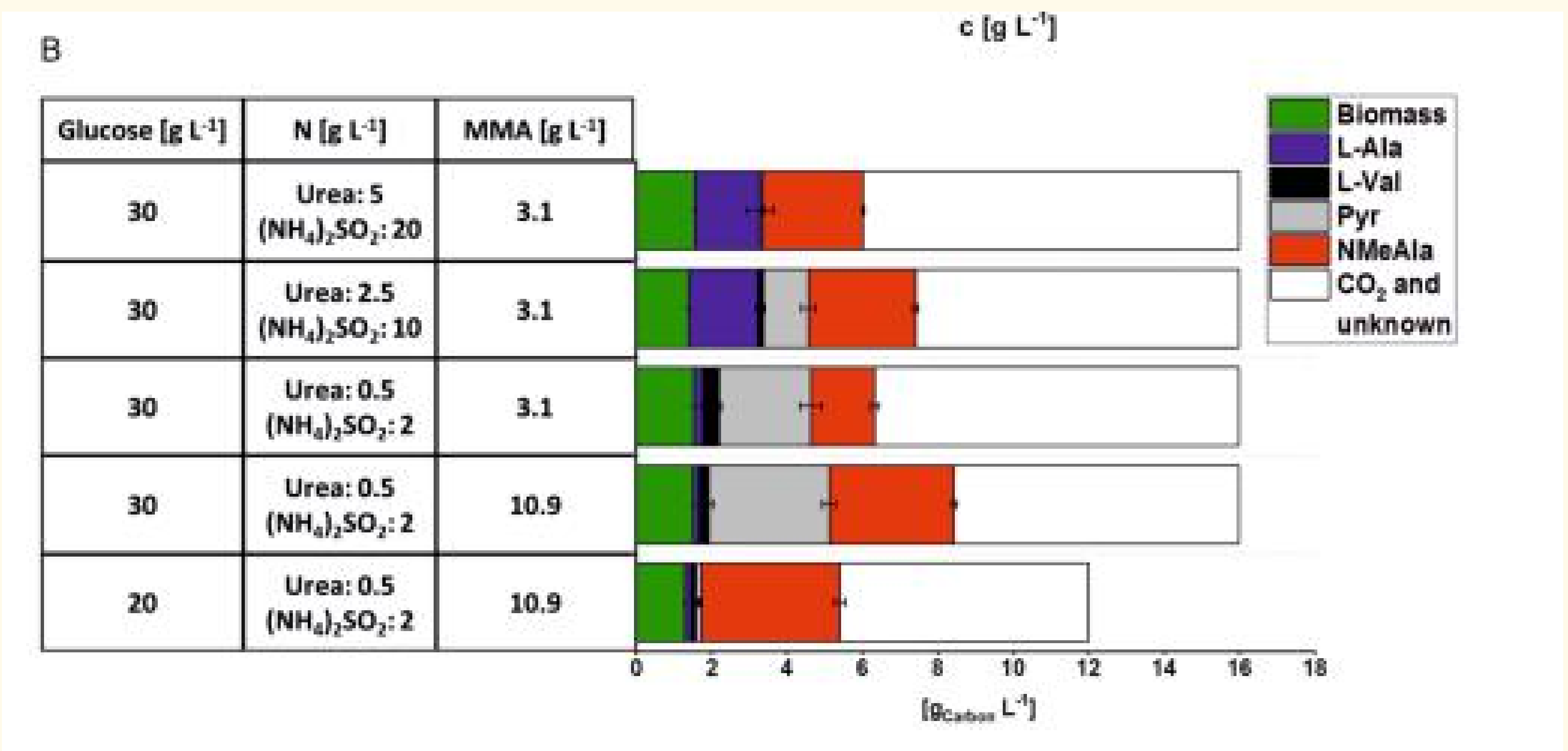

**Figure 1.** Schematic overview of the reaction catalyzed by DpkA (**A**) and its integration into the central carbon metabolism in *C. glutamicum* NMeAla1 (**B**). The gene deletions for improved pyruvate production are shown by black arrows with red double bars: deletion of *aceE* (encoding PDHE1p, the E1p subunit of the PDHC) and *pqo* (encoding pyruvate-quinone oxidoreductase, PQO) and both genes coding both major enzymes for L-alanine supply by pyruvate aminotransferases (*alaT* and *avtA*, encoding the alanine aminotransferase AlaT and the valine-pyruvate aminotransferase AvtA, respectively) were deleted. In addition, the acetohydroxyacid synthase (AHAS) activity was downregulated by deletion of the C-terminal part of *ilvN* (small subunit of AHAS) shown by red dashed arrow. Enzymes highlighted by red background indicate missing or down regulated enzymes. The thick arrow displays the NMeAla formation by heterologous expressed *dpkA* from *P. putida* KT2440 coding for the *N*-methylated amino acid dehydrogenase DpkA (green shadowed Enzyme).

# Improvement of precursor conversion and reduction of by-product formation




Increased pyruvate concentrations and decreased NMeAla concentrations in the supernatants indicated incomplete reductive N-methylamination of pyruvate to NMeAla.

# Improvement of precursor conversion and reduction of by-product formation.




As expected for aerobic processes, about 50% of carbon from the growth substrates will end up in $CO_2$.

# Fed-Batch bioreactor process of NMeAla production

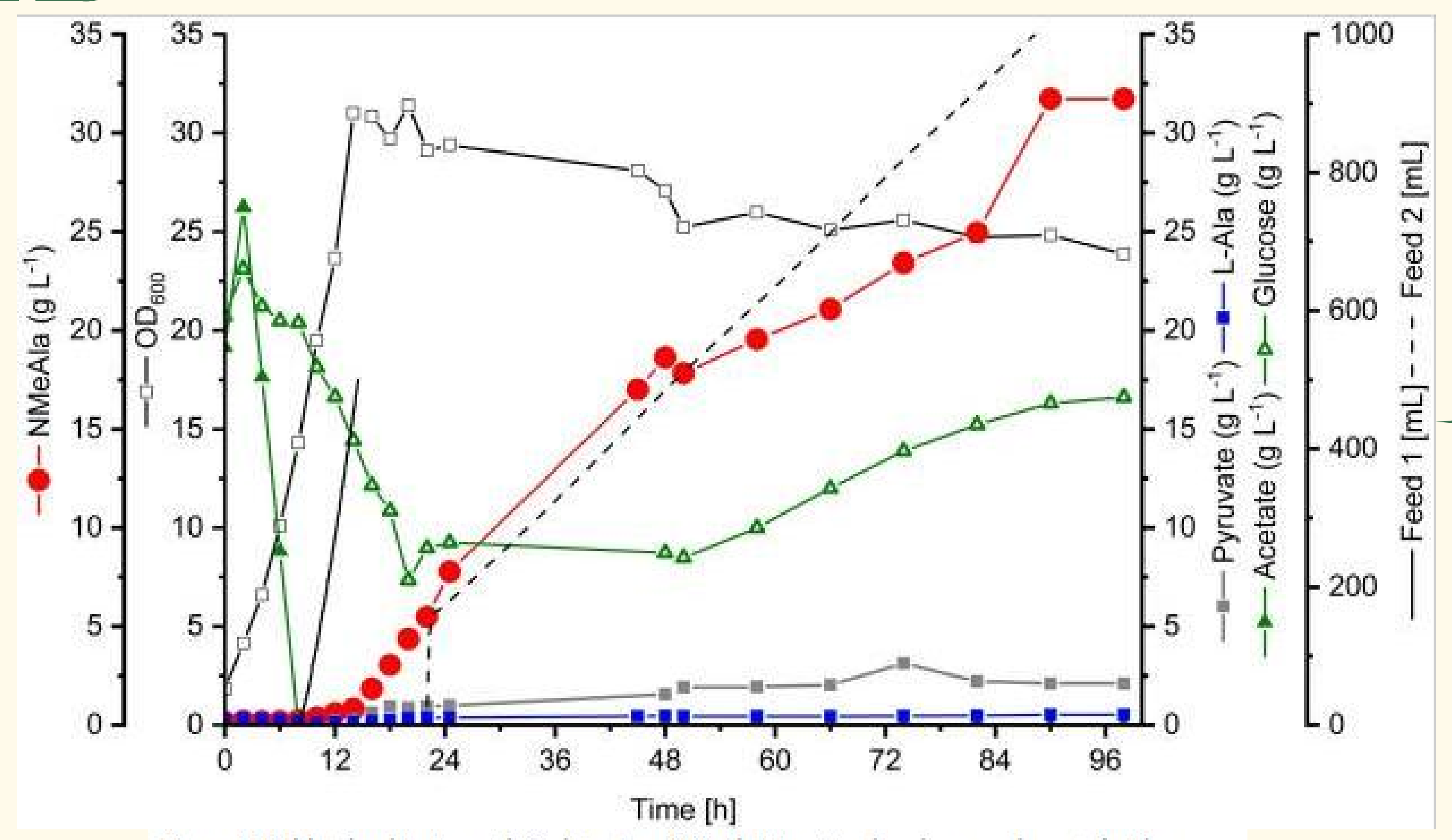



**Figure 4.** Fed-batch cultivation with *C. glutamicum* NMeAla1 in minimal medium supplemented with potassium acetate and glucose as carbon and energy sources. A fermenter with an initial start volume of 4 L was used. First feed phase (potassium acetate) was coupled to the rDOS value. After 22 h the second feed phase was started by the initial addition of 162 mL of a glucose/MMA solution followed by a linear feed of 12.3 mL $h^{-1}$. The biomass formation (black open squares), concentrations of NMeAla (red circles), L-alanine (blue squares), pyruvate (grey squares), acetate (green filled triangles) and glucose (green open triangles) were depicted. The volume of both feeds is shown as black lines. All depicted concentrations and the biomass formation was related to the initial volume.

final NMeAla titer : 31.7 g $L^{-1}$

volumetric productivity: 0.35 g $L^{-1}$ $h^{-1}$

# Establishing production of NMeAla from alternative feedstocks

Sustainable production from sugars that have competing uses in human and animal nutrition have to be succeeded by production processes based on second generation feedstocks such as lignocellulosic hydrolysates.
Fermentative production of amino acids is typically based on glucose present in molasses or obtained from starch by hydrolysis.

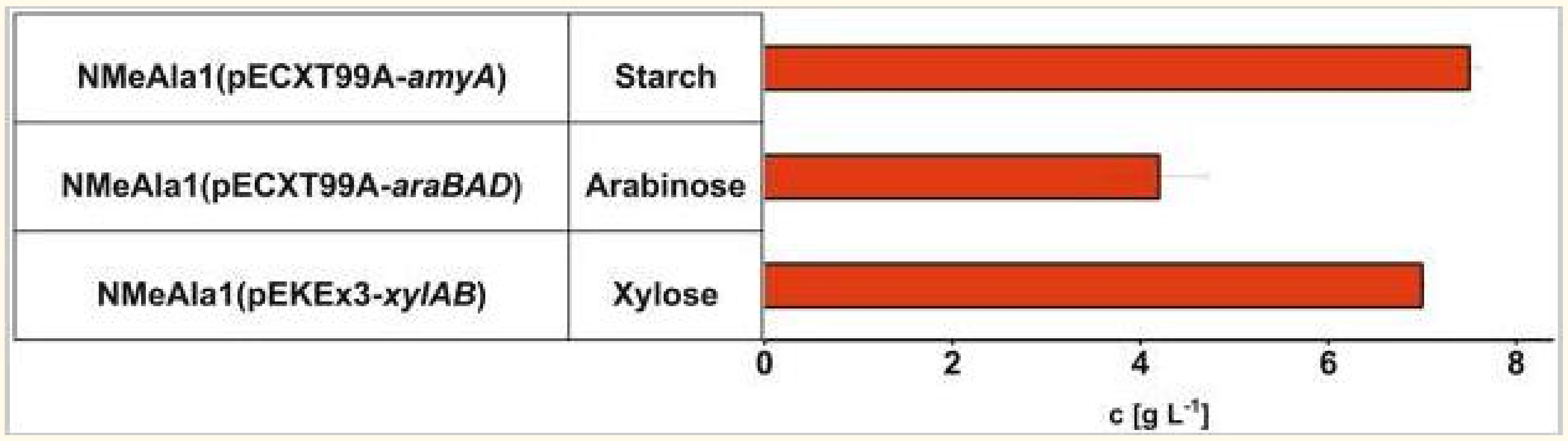



**Figure 5.** Production of NMeAla from alternative carbon sources. The CGXII minimal medium with 16.6 g $L^{-1}$ potassium acetate contained 30 g $L^{-1}$ starch for cultivation production experiments using *C. glutamicum* strain NMeAla1(pECXT99A-*amyA*), 30 g $L^{-1}$ arabinose using *C. glutamicum* strain NMeAla1(pECXT99A-*araBAD*) and 30 g $L^{-1}$ xylose using *C. glutamicum* strain NMeAla1(pEKEx3-*xylAB*). Concentrations were determined after 72 h and are given as means with standard deviations of three replicates.

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# Conclusion

# Conclusion

本研究开发了N-甲基化氨基酸NMeAla的发酵途径。

在平衡主要底物葡萄糖和MMA的比例之后，在补料分批发酵中实现31.7g $L^{-1}$的最终NMeAla滴度。

产生NMeAla的谷氨酸棒杆菌菌株进一步改造可以将该绿色化学途径延伸至替代原料如淀粉或木质纤维素糖、木糖和阿拉伯糖生产NMeAla。

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