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Thick Juice-Based Production of Amino Acids and Putrescine by *Corynebacterium glutamicum*

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Abstract

Thick juice (also regarded as syrup) is an intermediate product of sugar processing. It is cheaper than processed sugar and is mainly composed of sucrose. Sucrose is a preferred carbon source of *Corynebacterium glutamicum*, a workhorse of biotechnology used for million-ton-scale amino acid production. Here, it is shown for *C. glutamicum* that sugar beet thick juice led to higher growth rates and faster carbon source consumption than pure sucrose. Comparative DNA microarray analysis revealed differential expression of genes for butyrate and citrate catabolism and of NAD and biotin biosynthesis suggesting provision of these compounds by the thick juice. Thick juice was also shown to be superior to sucrose in production of the amino acids L-lysine, L-glutamate and L-arginine as well as of the diamine putrescine since higher volumetric productivities than with pure sucrose could be achieved. Taken together, sugar beet thick juice was shown to be a carbon source for growth and amino acid and diamine production of *C. glutamicum* superior to pure sucrose.

Keywords: Thick juice; L-lysine; L-glutamate; L-arginine; Putrescine; *Corynebacterium glutamicum*

Introduction

With resources of fossil oil becoming limiting, biotechnological production is getting more and more attractive [1,2]. Regarding the production of bulk chemicals the use of alternative carbon source for fermentation has become highly interesting to allow competing prices. One prospect is the use of thick juice, an intermediate product from sugar industry.

While production of table sugar involves many steps starting with e.g. sugar beet or sugar cane and ending in white crystalline sugar. First sugar beet is washed thoroughly and cut into chips. Out of these chips a crude juice is extracted by hot water treatment. This crude juice contains about 150g l⁻¹ sugar as well as several contaminants. These contaminants are removed by filtration resulting in light yellow colored thin juice. Using low temperature and high vacuum conditions water is reduced and thick juice is generated with a sugar content of 650–700 g l⁻¹. In order to get white crystalline sugar further concentration and cleaning steps like crystallization and centrifugation are required [3]. By using thick juice instead of fully processed sugar these steps are saved and thereby also the associated costs.

Until now thick juice has received only little attention as a carbon source in biotechnology. Conservation of thick juice and its microbial degradation have been studied [4-7]. Other sugar beet based substrates like beet sugar molasses [8] have been used to produce fructose and ethanol. In food biotechnology, sugar beet thick juice in combination with beet sugar molasses have been used for the production of fructo-oligosaccharides [9].

The Gram-positive soil bacterium *Corynebacterium glutamicum* is generally recognized as safe (GRAS) and used for the million-ton-scale production of amino acids. Besides being used for L-glutamate and L-lysine production, *C. glutamicum* has been engineered for the production of diamines like putrescine (1,4-diaminobutane) [10,11] and cadaverine (1,5-diaminopentane) [12-14], ketoacids such as pyruvate [15] and 2-ketoisovalerate [16], diacids such as succinate [17-20], the alcohols ethanol [21] and iso-butanol [22], and further amino acids, e.g. L-arginine, L-ornithine [23] and L-proline [24] or carotenoids [25] as well as gamma-amino butyric acid [26].

Amino acid production by *C. glutamicum* is typically based on glucose from starch hydrolysates and fructose and sucrose from molasses. These sugars are imported and phosphorylated by the phospho-transferase system (PTS) [27]. While fructose and sucrose are transported via the PTS only, PTS-independent glucose uptake has been reported and characterized [28,29]. For access to further carbon sources *C. glutamicum* has been metabolically engineered: e.g. for access to xylose and arabinose, which can be found in hydrolysates of agricultural wastes [23,30-33], starch [34,35], cellobiose [36], as well as lactose and galactose, which are present in whey [37,38] or the chitin monomer glucosamine [39]. To the best of our knowledge, the use of thick juices or syrups for biotechnological processes with *C. glutamicum* has not yet been reported. Therefore, the genetic response of *C. glutamicum* to thick juice has been analyzed by transcriptomics and the performance of thick juice-based production by *C. glutamicum* has been compared production with sucrose.

Methods

Strains and culturing conditions

All *C. glutamicum* strains used are listed in Table 1. For growth and production experiments CgXII medium [40] in 500 ml baffled shaking flasks was inoculated from LB overnight cultures and cultivated at 30°C and 120 rpm. Production of L-glutamate was triggered by 500 µg ml⁻¹ ethambutol [41]. Media for *C. glutamicum* ORN1 were supplemented with 500 µM L-arginine. Growth was followed by the determination of OD₆₀₀, which was measured using a UV-1650 PC photometer (Shimadzu, Duisburg, Germany). When appropriate 100

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Strain	Relevant characteristics	Reference
WT	Wild type; ATCC13032	[43]
DM1729	L-lysine overproducing strain, <i>pyc</i> ^{E458S} , <i>hom</i> ^{V59A} , <i>lysC</i> ^{T311I}	[44]
PUT21	Putrescine producing strain, ORN1 carrying pVWEx1- <i>speC</i> ^{S21} - <i>argF</i>	[11]
ARG1	L-arginine producing strain, Δ <i>argR</i> (pEKEx3- <i>argB</i> ^{A26V,M31V})	[23]

Table 1: *C. glutamicum* strains used.

Gene				relative mRNA levels thick juice/sucrose
designation	name	operon	function	
cg0095	<i>bioB</i>	OP_cg0095	biotinsynthase	2.6
cg0096	-	OP_cg0095	hypothetical protein	2.5
cg0566	<i>gabT</i>	OP_cg0566	4-aminobutyrate aminotransferase	5.3
cg0567	<i>gabD</i>	OP_cg0566	succinate-semialdehydedehydrogenase	8.3
cg0568	<i>gabP</i>	OP_cg0566	permease for amino acids	2.4
cg0793	-	-	secreted protein	2.2
cg1745	-	-	putative signal recognition particle GTPase	2.6
cg3105	-	OP_cg3106	hypothetical protein	2.2
cg3125	<i>tctA</i>	OP_cg3127	citrate uptake system, subunit A	3.0
cg3126	<i>tctB</i>	OP_cg3127	citrate uptake system, subunit B	5.1
cg3127	<i>tctC</i>	OP_cg3127	citrate uptake system, subunit C	7.6
cg3226	-	-	permease of the major facilitator superfamily	7.1
cg0156	<i>cysR</i>	-	Bacterial regulatory proteins, Crp family	0.4
cg0160	-	OP_cg0160	hypothetical protein	0.2
cg0654	<i>rpsD</i>	OP_cg0651	ribosomal protein S4	0.5
cg0759	<i>prpD2</i>	OP_cg0759	methylaconitase	0.4
cg0762	<i>prpC2</i>	OP_cg0759	methylcitrate synthase	0.5
cg0771	-	OP_cg0771	DtxR/iron regulated lipoprotein precursor	0.5
cg0924	-	-	ABC-type iron siderophore transport subunit	0.4
cg0926	-	OP_cg0926	ABC-type iron siderophore transport subunit	0.4
cg1214	<i>nadS</i>	OP_cg1218	cysteine sulfinate desulfinate	0.4
cg1215	<i>nadC</i>	OP_cg1218	nicotinate-nucleotidepyrophosphorylase	0.4
cg1216	<i>nadA</i>	OP_cg1218	quinolinate synthetase	0.3
cg1287	-	-	hypothetical protein	0.5
cg1765	<i>sufR</i>	OP_cg1765	predicted transcriptional regulator	0.4
cg1855	<i>hisS</i>	OP_cg1856	histidyl-tRNA synthetase	0.4
cg2055	-	-	putative membrane protein	0.4
cg3119	<i>fpr2</i>	-	probable sulfitereductase (flavoprotein)	0.4
cg3185	-	-	hypothetical protein	0.5

Table 2: DNA Microarray.

$\mu\text{g ml}^{-1}$ spectinomycin, $25 \mu\text{g ml}^{-1}$ kanamycin, and 1 mM isopropyl- β -D-thiogalactopyranosid (IPTG) were added to the medium to select and induce the plasmids listed in Table 1. Biomasses in g cell dry weight per liter (g CDW l⁻¹) were calculated from OD₆₀₀ as described previously by using a factor of 0.25 g CDW l⁻¹ per OD₆₀₀ [42].

Thick juice

Thick juice was obtained from Pfeifer and Langen Company, Jülich, Germany. Sucrose concentration in thick juice was 642 g l⁻¹.

Determination of amino acid, diamine, and sucrose concentrations

Amino acids, putrescine and sucrose concentrations were quantified by HPLC as described previously [10, 44,45].

Global gene expression analysis

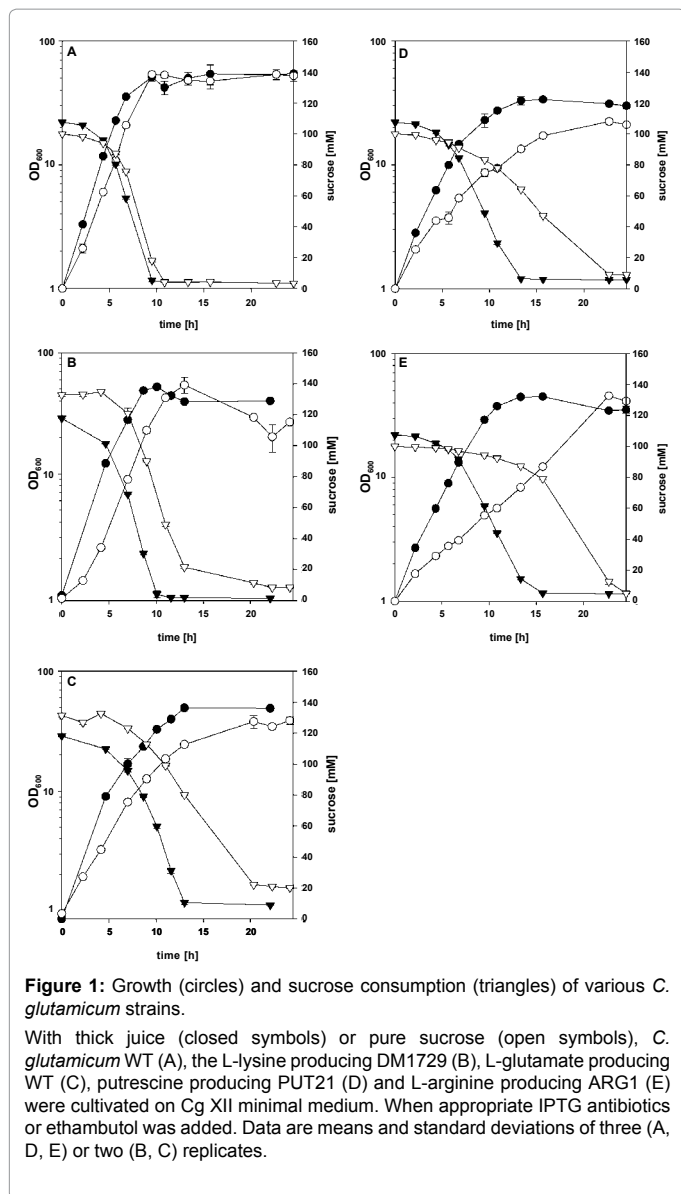
Preparation of total RNA, cDNA synthesis, DNA microarray hybridization, and gene expression analysis were performed as described before [46-48]. *C. glutamicum* WT strain ATCC 13032 was

used for this experiment. Growth conditions were CgXII minimal medium with 100 mM of either pure sucrose or the equivalent thick juice as carbon source.

Results

Utilization of thick juice by *C. glutamicum*

C. glutamicum WT which is able to utilize sucrose as sole carbon source via PTS_S was used for growth experiments with thick juice. The sucrose concentration in the thick juice used was determined by HPLC as 642 g l⁻¹. Growth experiments were carried out using CgXII minimal medium containing a final sucrose concentration of 34 g l⁻¹ either from thick juice or from pure sucrose. *C. glutamicum* WT was inoculated to a starting biomass of 0.25 g CDW l⁻¹ and growth was followed. As depicted in Figure 1A, growth with thick juice was slightly faster ($0.56 \pm 0.00 \text{ h}^{-1}$) than with pure sucrose ($0.49 \pm 0.02 \text{ h}^{-1}$) and the carbon source was depleted faster (after 9.5 h and 10.8 h, respectively). However, the final biomass concentrations were comparable ($13.6 \pm 0.5 \text{ g CDW l}^{-1}$ with thick juice and $13.1 \pm 1.3 \text{ g CDW l}^{-1}$ with pure sucrose).



DNA Microarray analysis of thick juice-specific gene expression changes

In order to characterize growth of *C. glutamicum* with thick juice on the gene expression level, *C. glutamicum* WT was grown in Cg XII minimal medium with pure sucrose or thick juice as carbon source. At a biomass of 1 g CDW l⁻¹ cells were harvested, total RNA isolated and cDNA synthesized using Cy5 Dyes to label the different pools of cDNA. After DNA microarray hybridization and scanning, different expression levels were analyzed using Genepix software.

Table 2 lists statistically significant expression differences for 29 genes in the transcriptome comparison of sucrose- and thick juice-grown *C. glutamicum* WT cells. Of the 17 genes that showed lower mRNA levels on thick juice as compared to pure sucrose, five genes code for proteins which are organized in two operon structures (*prpD2* and *prpC2* of OP_cg0759; *nadS*, *nadC* and *nadA* of OP_cg1218). Furthermore, two genes show lower mRNA levels which code for proteins involved in iron uptake (*cg0924*, *cg0926*).

Increased gene expression during growth on thick juice was observed for twelve genes. Among these were eight genes of three operons, namely *bioB* and *cg0096* (OP_cg0095; involved in biotin synthesis), *gabTDP* (OP_cg0566; involved in butyrate utilization) as well as *tctABC* (OP_cg3127; for citrate uptake).

Taken together, growth of *C. glutamicum* on thick juice differed from growth on pure sucrose by increased expression of genes for butyrate and citrate utilization and for biotin synthesis and by decreased expression of genes for iron uptake, NAD synthesis and propionate catabolism (Table 2).

Comparative global gene expression analysis of *C. glutamicum* WT grown in minimal medium with thick juice or pure sucrose as carbon source.

Amino acid and putrescine production on sucrose and thick juice

To characterize production of *C. glutamicum* with thick juice, various engineered *C. glutamicum* strains were used. Besides production of the amino acids L-lysine, L-glutamate and L-arginine, production of the diamine putrescine, which is synthesized from an intermediate of arginine biosynthesis, was done comparing pure sucrose and thick juice. All strains were inoculated to a final biomass concentration of 0.25 g CDW l⁻¹ into Cg XII minimal medium containing 34 g l⁻¹ sucrose either from thick juice or from pure sucrose. Figure 1 shows growth and carbon source depletion during the respective production experiments. The maximal amino acids or diamine concentrations are summarized in Table 3. Specific productivities are given in Figure 2.

Strains and the respective growth and carbon source consumption charts are given in Figure 1. Product concentrations are given as

substrate	product concentration [mM]			
	L-glutamate	L-lysine	L-arginine	putrescine
thick juice	56.5 ± 1.0	33.0 ± 0.2	43.9 ± 1.3	46.6 ± 1.3
sucrose	45.3 ± 2.3	14.4 ± 1.4	50.3 ± 1.4	49.4 ± 3.6

Table 3: Maximal titers of produced amino acids and putrescine with thick juice or sucrose.

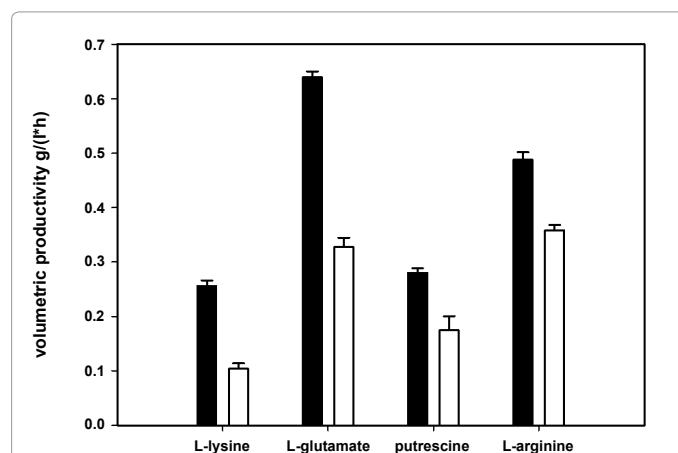


Figure 2: Volumetric productivities for L-lysine, L-glutamate, putrescine and L-arginine.

Production in Cg XII medium containing thick juice (black bars) or sucrose (white bars). Production conditions were as described in Methods and as in Figure 1. Data are means and standard deviations of three (putrescine, L-arginine) or two (L-lysine, L-glutamate) replicates.

means and standard deviations of three (putrescine, L-arginine) or two (L-lysine, L-glutamate) replicates are given after sucrose was depleted.

L-lysine production

L-lysine production experiments were carried out with *C. glutamicum* DM1729 [44]. Samples for the determination of L-lysine concentrations were taken when sucrose was depleted. L-lysine accumulated to a concentration of 14.4 ± 1.4 mM with pure sucrose. When thick juice was used the final L-lysine concentration doubled to 33.0 ± 0.2 mM (Figure 1B). Comparable growth rates were observed for both conditions (0.46 ± 0.01 h⁻¹ with thick juice and 0.46 ± 0.01 h⁻¹ with pure sucrose). Sucrose from thick juice was depleted after 10.1 h and after 20.3 h in the experiment with pure sucrose. The resulting volumetric productivity was almost three times higher with thick juice (0.26 ± 0.01 g l⁻¹ h⁻¹) than with pure sucrose (0.10 ± 0.01 g l⁻¹ h⁻¹; Figure 2).

L-glutamate production

L-glutamate production experiments were carried out with *C. glutamicum* WT [49]. Production of L-glutamate by *C. glutamicum* WT needs to be triggered e.g. by addition of ethambutol [50] which was added during inoculation to a concentration of 500 µg ml⁻¹. When sucrose was depleted, 45.3 ± 2.3 mM L-glutamate had accumulated with pure sucrose while around 25% more L-glutamate (56.5 ± 1.0 mM) accumulated with thick juice (Figure 1C). The use of thick juice resulted in an increased growth rate (0.44 ± 0.01 h⁻¹ as compared to 0.31 ± 0.01 h⁻¹ with pure sucrose) and faster depletion of the carbon source (after 13 h with thick juice as compared to 20.3 h with pure sucrose). An about twofold higher volumetric productivity with thick juice (0.64 ± 0.01 as compared to 0.33 ± 0.02 g l⁻¹ h⁻¹ with pure sucrose) entailed (Figure 2).

L-arginine production

C. glutamicum strain ARG1 [23] was used to demonstrate the use of thick juice for the production of L-arginine (Figure 1D). Growth with pure sucrose was twofold slower (0.18 ± 0.00 h⁻¹) than with thick juice (0.35 ± 0.00 h⁻¹). With pure sucrose 50.3 ± 1.4 mM L-arginine accumulated after sucrose depletion (24.5 h), which corresponds to a volumetric productivity of 0.36 ± 0.01 g l⁻¹ h⁻¹. With thick juice 43.9 ± 1.3 mM L-arginine accumulated, but sucrose was depleted already after 15.6 h. The corresponding volumetric productivity on thick juice was increased by about one third to 0.49 ± 0.01 g l⁻¹ h⁻¹ (Figure 2).

Putrescine production

Putrescine production by *C. glutamicum* [10,11] is based on over expression of the *E. coli* ornithine decarboxylase gene *speC* in the ornithine production strain ORN1 [23] via pVWEx1-*speC*-5'21-*argF*, which also renders the respective strain PUT21 prototrophic for L-arginine due to leaky expression of *argF* from the addiction plasmid [11]. When 34 g l⁻¹ pure sucrose were provided 49.4 ± 3.6 mM of putrescine were produced, which corresponds to a volumetric productivity of 0.17 ± 0.03 g l⁻¹ h⁻¹ since sucrose was depleted after 22.6 h. When thick juice was used putrescine accumulated to comparable concentrations (46.6 ± 1.3 mM), but productivity increased significantly to 0.28 ± 0.01 g l⁻¹ h⁻¹ as sucrose was depleted already after 13.3 h and growth with thick juice was clearly faster (0.36 ± 0.00 as compared to 0.19 ± 0.01 h⁻¹ with pure sucrose).

Discussion

Sugar beet thick juice-based production of amino acids L-glutamate, L-lysine and L-arginine and of the diamine putrescine by *C. glutamicum* is described here for the first time. While most crude substrates like technical grade glycerol [51] or hemi cellulosic hydrolysates [30,33] inhibit growth, thick juice was shown here to be beneficial for growth, carbon source utilization and amino acid and diamine production with *C. glutamicum*. Thick juice is an intermediate of the sugar production process with already high sucrose concentration, but there are still some sugar beet ingredients present, which can have advantageous effects.

Regarding the results obtained from global gene expression analysis higher expression levels for *bioB* and adjacent cg0096 were detected under thick juice conditions. *C. glutamicum* is biotin auxotroph due to an incomplete biotin synthesis pathway and therefore is dependent on biotin import via BioYMN [52]. Subsequently, biotin protein ligase BirA [53] catalyzes covalent attachment of biotin to the two biotin-dependent enzymes of *C. glutamicum*, acetyl-CoA carboxylase [54] and pyruvate carboxylase [55]. Despite being biotin auxotrophic, genes of the incomplete biotin pathway are transcriptionally repressed by BioQ in *C. glutamicum* [56]. Higher RNA-levels of *tctABC*, which code for the citrate uptake system of *C. glutamicum* [57] might be due to citrate present in thick juice as citrate can be used as carbon source by *C. glutamicum* [58]. Cg3226 is adjacent to *lldD*, which is essential for lactate utilization in *C. glutamicum* [41] and both are regulated by LldR [59,60]. Cg3226 is suggested to be a lactate transporter [41] and lactate can be found in small amounts in thick juice (data not shown).

Reduced expression levels on thick juice were found for NAD synthesis genes *nadACS*. NAD biosynthesis involves three enzymatic reactions starting with aspartate which is converted to nicotinate mononucleotide via iminoaspartate and quinolinate usually catalyzed by Nad-ABC. In *C. glutamicum* the enzyme catalyzing the reaction from aspartate to iminoaspartate is unknown as there is no *nadB* [61]. From nicotinate mononucleotide NAD is built and this can be further phosphorylated by PpnK [62] to the reduced NADP. Furthermore *prpCD* showed lower expression which are part of propionate metabolism consisting of *prpDBC* in *C. glutamicum* and is regulated by PrpR [63]. With cg0924 and cg0926 two genes involved in iron uptake show lower expression levels, suggesting good iron conditions in thick juice. Supporting this also SufR regulated *suf* genes, which are important for assembly of iron-sulfur cluster, and *cysR*, involved in regulation of sulfur metabolism [64], are found to be differentially expressed.

For the production of amino acids and diamines thick juice was superior to pure sucrose since in each case the volumetric production rate was higher (Figure 2). In the case of L-glutamate and L-lysine significantly higher concentrations accumulated with thick juice than with pure sucrose (Table 3). In addition, growth and sucrose utilization was faster (Figure 1). Thus, thick juice had a beneficial effect on both, product yields and the carbon source uptake rates. By contrast, production of L-arginine and putrescine, which is derived from L-ornithine, an intermediate of L-arginine biosynthesis, was characterized by comparable final product concentrations on pure sucrose and on thick juice (Table 3). Apparently, L-arginine and putrescine product yields were limited by factors other than by compounds provided by thick juice. These factors remain unknown at least for *C. glutamicum*, however in higher plants like tobacco it is known that high putrescine concentrations can decrease ATP synthesis [65]. For L-arginine and putrescine production, the increased volumetric productivities as compared to pure sucrose were only due to faster growth and carbon source uptake, and thus, increased by a lesser

extent as those for L-lysine and L-glutamate (Figure 2). It is interesting to note that growth of the L-arginine and putrescine producing strains was about twofold faster with thick juice than with pure sucrose, while the growth rates of the L-glutamate producing strain only increased by about one third and that of the L-lysine producer was comparable on thick juice and on pure sucrose.

Conclusion

In this study it was shown that sugar beet thick juice is a suitable carbon source for the industrial heavily used *C. glutamicum*. Microarray data revealed beneficial influence of some remaining ingredients in sugar beet thick juice. Growth as well as L-glutamate, L-lysine, L-arginine and putrescine production with thick juice was accelerated and/or increased. Therefore thick juice as carbon source for amino acid or diamine production is superior sucrose.

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