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L-Rhamnose metabolism in *Clostridium beijerinckii* DSM 6423

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Abstract (250 words)

Macroalgae (or seaweeds) are considered potential biomass feedstocks for the production of renewable fuels and chemicals. Their sugar composition is different from that of lignocellulosic biomasses, and in green species, including *Ulva lactuca*, the major sugars are L-rhamnose and D-glucose. *C. beijerinckii* DSM 6423 utilized these sugars in an *U. lactuca* hydrolysate to produce acetic acid, butyric acid, isopropanol, butanol and ethanol (IBE) and 1,2-propanediol. D-glucose was almost completely consumed in diluted hydrolysates, while L-rhamnose or D-xylose were only partially utilized. In this study, the metabolism of L-rhamnose by *C. beijerinckii* DSM 6423 was investigated to improve its utilization from natural resources. Fermentations on D-glucose, L-rhamnose and a mixture of D-glucose and L-rhamnose were performed. On L-rhamnose, the cultures showed low growth and sugar consumption, and produced 1,2-propanediol, propionic acid and n-propanol in addition to acetic and butyric acids, whereas on D-glucose, IBE was the major product. On a D-glucose/L-rhamnose mixture, both sugars were converted simultaneously and L-rhamnose consumption was higher, leading to high levels of 1,2-propanediol (78.4 mM), in addition to 59.4 mM of butanol and 31.9 mM isopropanol. Genome and transcriptomics analysis of D-glucose- and L-rhamnose-grown cells revealed the presence and transcription of genes involved in L-rhamnose utilization, but also in bacterial microcompartment (BMC) formation. These data provide useful insights into the metabolic pathways involved in L-rhamnose utilization and the effects on the general metabolism (glycolysis, early sporulation, stress-response) induced by growth on L-rhamnose.

Importance (150 words)

A pre-requisite for a successful biobased economy is the efficient conversion of biomass resources into useful products, such as biofuels, bulk- and specialty chemicals. In contrast to other industrial microorganisms, natural solvent-producing *Clostridia* utilize a wide range of sugars, including C5, C6 and deoxy-sugars, for production of long-chain alcohols (butanol, 2,3-butanediol), isopropanol, acetone, n-propanol and organic acids. Butanol production by *Clostridia* from first generation sugars is already a commercial process, but for the expansion and diversification of the A/IBE process to other substrates, more knowledge is needed on the regulation and physiology of fermentation of sugar mixtures. Green macroalgae, produced in aquaculture systems, harvested from the sea or from tides, can be processed into hydrolysates containing mixtures of D-glucose and L-rhamnose, which can be fermented. The knowledge generated in this study will contribute to the development of more efficient processes for macroalgae fermentation, and of mixed sugar fermentation in general.

Introduction

The increasing worldwide demand for fuels and chemicals contradicts the diminishing availability of fossil resources, which are currently the main source of these compounds. In past decades, the concept of biorefinery has been established as an alternative to petroleum-based refineries, in which multiple products (energy, fuels and (high-value) chemicals) are produced from one biomass source (1, 2). Nowadays, the most established biorefineries are based on lignocellulosic biomasses. However, a diversification of biomass resources is needed to ensure sufficient availability and flexibility of processes. Macroalgae have gained attention in recent years as feedstock for production of fuels and chemicals due to the advantages that they show with respect to traditional terrestrial feedstocks for biorefinery: i) higher productivity (biomass produced per unit of surface) than terrestrial crops, ii) no competition for arable land, iii) lower fresh water consumption during cultivation, and iv) no requirement for fertilizers (3). In addition, macroalgae show a distinctive chemical composition compared to lignocelluloses and terrestrial crops, as some species are rich in carbohydrates, proteins, fatty acids and/or bioactive components that make them very suitable for biorefinery as sources of multiple valuable products (4, 5). Current developments in sustainable large scale cultivation of macroalgae could result in increased availability of these biomasses at economic conditions (6).

In the green seaweed *Ulva lactuca*, D-glucose and L-rhamnose are the main carbohydrates present in the ulvan polysaccharide and it has been reported that these sugars could be extracted using mild pre-treatment conditions (7, 8). In fermentations performed with *Clostridium acetobutylicum* and *Clostridium beijerinckii,* using *U. lactuca* hydrolysates as substrate, the solvents acetone, butanol, and ethanol (ABE) were produced. Interestingly, *C.*

beijerinckii was also able to produce 1,2-propanediol when grown on L-rhamnose, but not on D-glucose (7). In contrast, *C. acetobutylicum* did not show any production of 1,2-propanediol, and was unable to grow solely on L-rhamnose. *C. beijerinckii* DSM 6423 (also NRRL B-593, formerly known as *C. butylicum* NRRL B-593), was able to grow on L-rhamnose as a sole carbon source, producing 1,2-propanediol, but also propanol and propionate, in addition to acetic- and butyric acids (9). L-Rhamnose utilization is well-studied for some microorganisms, such as *Escherichia coli* or *Salmonella typhimurium* (10, 11), and has been described for *C. phytofermentans* (12). In the latter species, L-rhamnose was shown to be converted along a phosphorylated pathway, involving rhamnulose, rhamnulose-P and lactaldehyde as key intermediates. Lactaldehyde is the precursor of the main end-product 1,2-propanediol (12, 13). 1,2 Propanediol is an interesting chemical. Its production has been studied in different micro-organisms, including fungi, bacteria and yeasts (14, 15). The involvement of bacterial microcompartments (BMC) in the catabolism of 1,2-propanediol into n-propanol and propionic acid has been described in *C. phytofermentans* and other organisms as an interesting feature (12, 15). L-Rhamnose metabolism by solventogenic *Clostridia*, however, is not well characterized. Production of 1,2-propanediol, propionate and propanol by *C. beijerinckii* suggests that in this solventogenic species, L-rhamnose is converted by a metabolic route similar to that reported for *C. phytofermentans*.

The aim of this study is to investigate the L-rhamnose metabolism in the solventogenic strain *C. beijerinckii* DSM 6423. Growth and product-formation on D-glucose or L-rhamnose were compared. The pathway for L-rhamnose conversion was reconstructed through analysis of the recently sequenced genome of this strain (16). The gene transcription profile in cultures grown on D-glucose and on L-rhamnose as sole carbon sources were determined using RNA sequencing, and the differences observed were analyzed with respect to sugar metabolism,

early sporulation and stress response. The results obtained contribute to enhance our knowledge about the unique capability of solventogenic *Clostridia* to ferment a variety of carbohydrates into a wide spectrum of products with commercial interest.

Results

Fermentation of *Ulva lactuca* hydrolysate by *C. beijerinckii*

The potential of *C. beijerinckii* for utilization of *U. lactuca* hydrolysate, containing D-glucose and L-rhamnose as major sugars was investigated using a hydrolysate prepared according to Bikker et al. (17). Cultures on hydrolysate and on control media containing D-glucose, L-rhamnose or a mixture of sugars as main carbon- and energy sources were grown in serum flasks. The hydrolysate was rich in D-glucose (115 mM) and L-rhamnose (86 mM), and in addition, contained 28 mM of D-xylose. Very low growth was observed in cultures of the pure hydrolysate, or hydrolysate supplemented with nutrients as in CM2 medium. Only a small amount of the D-glucose in the hydrolysate was consumed after 144h of incubation at 37°C, leaving the D-xylose and the L-rhamnose unused in the medium. When the hydrolysates, both pure or supplemented with nutrients, were diluted 1:1 with demineralized water, their fermentability improved. In the cultures on the diluted hydrolysates (DH), the D-glucose was consumed almost completely, whereas approximately 50% of the D-xylose, and approximately 20% of the L-rhamnose were consumed. The major products of these last fermentations were butyrate, acetate, isopropanol and butanol. Low levels of 1,2-propanediol were detected in the cultures on the diluted hydrolysate (DH) cultures (Table S1).

The major fermentation products in the L-rhamnose control cultures were butyrate and 1,2-propanediol, whereas an IBE fermentation took place on glucose cultures (Table S1). On all the cultures tested, L-rhamnose was only partially utilized. In the cultures with mixtures of D-glucose and L-rhamnose, D-glucose was completely utilized, and the consumption of L-rhamnose was higher compared to that observed on L-rhamnose-only cultures (Table S1).

129 The yields of IB(E) produced per D-glucose or D-glucose/D-xylose consumed in the different
130 cultures are shown in Table S1. In this table it can be observed that in the non-diluted
131 hydrolysate cultures the yields obtained are higher than the yields of IBE produced from D-
132 glucose in the control cultures (0.94 and 0.80 in H-*Ulva* and H-*Ulva*+N respectively, vs 0.72 in
133 D-glucose control cultures). This is also the case for DH-Ulva+N cultures, where the yield of
134 IB produced is 0.82 mM of IB per mM of D-glucose and D-xylose consumed. This indicates
135 that in the hydrolysates most probably other carbon sources, such as oligo- or di-
136 saccharides, are present that can be utilized to produce solvents by *C. beijerinckii*.
137
138 **Fermentation of L-rhamnose and L-rhamnose/D-glucose mixture**
139 To better characterize the fermentation of L-rhamnose, and to obtain cell material for
140 RNAseq analysis, cultures were grown in bioreactors with 400-mL of working volume without
141 pH control. Samples were taken at different time points for determination of metabolites and
142 for RNAseq analysis. Fermentations on D-glucose and D-glucose/L-rhamnose mixtures were
143 performed as reference. The results are shown in Figure 1 and Table 1. *C. beijerinckii* was
144 able to grow on L-rhamnose as a carbon- and energy source, albeit growth and L-rhamnose
145 consumption were lower than in D-glucose-grown cultures, with OD600 values of
146 approximately 3 and 11 for L-rhamnose and D-glucose, respectively (Fig. 1). Growth on L-
147 rhamnose ceased as soon as the pH of the culture dropped below 5, and in contrast to the
148 case of D-glucose-grown cultures, the pH did not increase anymore. To check if the growth
149 stopped due to the low pH, a second fermentation on L-rhamnose only was performed, in
150 which the pH was controlled to, or above, 5.2. The growth profile, L-rhamnose consumption,
151 and product formation were not significantly different to those of the non-pH-controlled
152 cultures (results not shown), indicating that the low pH was not the only cause of growth

cessation. Interestingly, L-rhamnose fermentation does not lead to the generation of typical solvents like acetone, butanol and ethanol. Also, re-assimilation of acids apparently does not occur, as 19.2 mM of acetate and 11.7 mM of butyrate were produced in the L-rhamnose culture (Fig. 1, Table 1).

Instead of producing IBE, as was seen in D-glucose-grown cultures, the strain produced 1,2-propanediol, propanol, and propionate in addition to acetate and butyrate, when L-rhamnose was provided as the carbon source. Propionate and propanol are known to be typical products of the catabolism of 1,2-propanediol in many microorganisms, including clostridial species (12).

For biomass determination from the L-rhamnose and on the L-rhamnose/D-glucose cultures, dry matter content was measured at the end of the fermentations. The calibration curve that relates biomass to OD_{600} values of the cultures obtained in D-glucose cultures was not applicable for L-rhamnose-grown cultures, as these show a very different cell morphology (Figure S1). Highest yields were found for cultures grown on D-glucose or the mixture, with yields of 0.12 and 0.059 mol biomass/mol of sugar consumed, respectively.

As L-rhamnose and D-glucose are both present in hydrolysates from green seaweeds, their co-metabolism was studied in cultures grown on a mixture of these sugars in a ratio 1:1. In Figure 1 and Table 1, it can be seen that in these cultures all D-glucose was consumed and that the consumption of L-rhamnose was significantly higher compared to the rhamnose only cultures (86.7 mM and 46.4 mM on D-glucose/L-rhamnose cultures and on L-rhamnose, respectively). Interestingly, both sugars in the medium were consumed simultaneously, although glucose was consumed at a higher rate (Figure 1). L-rhamnose was only partially consumed, as observed in the L-rhamnose-only cultures, remaining approximately 31% of the initial content in the medium.

177 The major fermentation products on the D-glucose/L-rhamnose mixture corresponded to
178 those observed for the D-glucose- and L-rhamnose-only fermentations, IBE and 1,2-
179 propanediol, respectively. The concentration of 1,2-propanediol reached 78 mM,
180 approximately four times higher than that was seen in the L-rhamnose-only cultures, as a
181 result of a higher sugar consumption.

182 As mentioned above, in the L-rhamnose-only cultures, low concentrations of propionic acid
183 and n-propanol were detected in the medium (Table 1, Figure 1). Remarkably, in cultures
184 grown on mixtures of D-glucose and L-rhamnose, no propanol or propionate were detected
185 (Table 1).

186 **L-Rhamnose pathway reconstruction**

187 To further investigate the pathways of L-rhamnose metabolism in *C. beijerinckii*, bioinformatic
188 analysis were performed on the genome sequence of the strain, as recently published (16,
189 18). Since the L-rhamnose catabolism was recently described for *C. phytofermentans* ISDg,
190 this strain was used as main source of genes for query for BLASTp searches (19), but data
191 on other organisms were used as well (15), as shown in Table 2. Genes encoding enzymes
192 involved in all steps of the transport and metabolism of L-rhamnose into 1,2-propanediol
193 could be identified (Table 2), with similarities ranging from 51 % to 83 %. As in *C.*
194 *phytofermentans*, most genes involved in the L-rhamnose catabolism were clustered within a
195 genomic region (Fig. 2). For two of the enzymes encoded in the cluster, the rhamnulose-
196 phosphate aldolase enzyme (CIBE_0615) and the 1,2-propanediol oxidoreductase
197 (CIBE_0617), gene duplications with high similarity were present (CIBE_3969 and
198 CIBE_2890, respectively) elsewhere in the genome.

199 Proteins putatively involved in L-rhamnose transport into the cell did not show similarity to
200 those of *C. phytofermentans* but were most similar to those of an ABC transporter found in
201 the soil bacterium *R. leguminosarum* and a transporter of the Major facilitator superfamily
202 (MFS) of the plant pathogen *Dickeya dadantii* (20).
203 A cluster of 21 genes contains the genes for the further conversion of 1,2-propanediol to
204 propionic acid and *n*-propanol (Fig.2). This cluster is almost identical to the clusters found in
205 other organisms, including *C. phytofermentans* (13). 1,2-Propanediol is expected to be
206 converted to propionaldehyde, by a propanediol dehydratase. Unlike, *S. typhimurium*, but
207 similar to *C.phytofermentans*, this is likely catalyzed by a B_{12}-independent type of
208 dehydratase (CIBE_4900; pduCDE). Propionaldehyde is further converted to propanol or
209 propionyl-CoA, catalyzed by a propanol dehydrogenase and a propionaldehyde
210 dehydrogenase, respectively. A homologue for the propanol dehydrogenase is present
211 (CIBE_4892), belonging to the Zn-dependent dehydrogenases. For the propionaldehyde
212 dehydrogenase 2 homologues are present in the cluster (CIBE_4884 and 4893); which is
213 unlike *C. phytofermentans*, that only contains one. Propionyl-CoA is converted to propionate
214 involving a phosphate propionyl transferase and a propionate kinase. The cluster contains a
215 gene encoding for the transferase (CIBE_4886), but not for the kinase. In *C.phytofermentans*,
216 the propionate dephosphorylating is catalyzed by a kinase that is not specific to proniate-P,
217 an acetate kinase which is encoded by a gene outside the L-rhamnose cluster. In
218 *C.beijerinckii,* the bioinformatics analysis does not provide enough proof to identify the gene
219 encoding the propionate kinase.,
220 The BMC cluster identified in our *C. beijerinckii* belongs to the Glycyl Radical Enzyme-
221 Containing Microcompartment type (GRM) like the one described in *C. phytofermentans* (13).
222 GRM are found mainly in *Firmicutes* and some Deltaproteobacteria and *Olsenella* (21). This

BMC locus type contains the metabolosome core enzymes and a glycyl radical enzyme which is the pyruvate lyase (CIBE_4900) in *C. beijerinckii*.

Bioinformatic studies (21, 22) showed that the GRM can be divided into subgroups dependenton the BMC shell proteins and the accessory genes that belong to the locus. The BMC cluster from *C. beijerinckii* belongs to the GRM.3 Group because it contains genes that encode a peptidase, a flavoprotein, a EutJ homolog, S-adenosylmethionine synthetase and signaling proteins. They are suspected to be involved in several metabolic pathways, such as vitamin B_{12} or S-adenosylmethionine synthesis.

Transcriptome analysis

Samples for mRNA isolation were taken from the fermentations on D-glucose and on L-rhamnose, as shown in Figure 1. Time points for sampling were chosen in such a way that the early exponential, acidogenic, and solventogenic growth phases were represented. For D-glucose-grown cultures, samples were taken after 3.0, 5.0, and 9.5 hours. For L-rhamnose-grown cultures, samples were taken after 3.0, 6.5, and 10 hours. After RNA isolation and sequencing, the data were mapped against the recently sequenced genome of this *C. beijerinckii* strain to quantify gene expression levels under each condition (16, 18, 23).

In summary, mapping of the RNAseq reads against the published genome of *C. beijerinckii* resulted in reliable reads in a range of 93.17 % to 98.60 % (Table S3) For analyzing differentially expressed genes, the TAMARA tool on the MicroScope platform was used (24). Thresholds were selected at $|\log2(\text{fold change})| > 3$ and adjusted p-value< 0.005, which resulted in a list of 671 significantly differentially expressed genes on L-rhamnose (11% of the genome). To see the impact of the L-rhamnose metabolism on selected functional clusters (Table 2, 3, S4, S5, S6) in all three time points, the log2(fold change) was decreased

to 0.5. Only fifty-nine of these significantly differentially expressed genes have a log2-fold change above 3.0 at the three time points and 115 genes at two time points. Out of these fifty-nine genes, 25 correspond to the genes involved in L-rhamnose uptake and conversion and BMC formation. Moreover, except for the rhamnose mutarotase, the corresponding genes of the putative L-rhamnose degrading enzymes were among the highest expressed during growth on rhamnose compared to glucose (Table 2). They were upregulated between 6.84 and 8.58 times on a log2-fold scale, depending on the enzyme and the sampling time point. For L-rhamnose transport two different transport systems were identified, an ABC and an MFS transporter. The transcript data show that both systems are indeed upregulated during growth on L-rhamnose. Especially, the transporter of the MFS-type was highly upregulated (8.8-fold). As mentioned, for the rhamnulose-phosphate aldolase two putative genes (*CIBE_0615* and *CIBE_3969*) were present. Of these only *CIBE_0615* was highly expressed on L-rhamnose, suggesting that *CIBE_0615* encodes for the rhamnulose-phosphate aldolase, which cleaves rhamnulose-phosphate to lactaldehyde and DHAP. Likewise, two putative genes were proposed to encode for the 1,2-propanediol oxidoreductase which converts lactaldehyde to 1,2-propanediol. However, only *CIBE_0617* was highly expressed on L-rhamnose indicating that this gene encodes for the functional protein. Also, most putative genes for the conversion of 1,2-propanediol to propanol and propionate were highly expressed, confirming their role in L-rhamnose fermentation. For the propionaldehyde dehydrogenase two genes were identified in the BMC-cluster and both were highly upregulated. As for the propionate kinase, since no gene encoding for this enzyme is present in the BMC-cluster, we looked at the differential expression of gene encoding for kinases outside of the BMC-cluster. We noticed the up regulated expression a putative butyrate kinase (2 fold on a log2 scale) , encoded by a gene present elsewhere in the

genome (CIBE_5515). We can assume that this putative butyrate kinase acted as a propionate kinase *C. beijerinckii* was grown on L-rhamnose.

All the genes from the BMC locus were among the highest expressed when *C. beijerinckii* was grown on L-rhamnose (Table 3). A very low expression of this cluster was detected in glucose-grown cultures from 4 to 55 reads per genes compared to 234 to 81,830 reads in L-rhamnose-grown cultures.

As the L-rhamnose cultures produced acetate and butyrate, but no IBE, the expression of the main genes associated with glycolysis, acidogenesis and solvent production were also analyzed. Most of the genes of the central metabolism to solvents were found to be less expressed in the L-rhamnose cultures (Fig. 3, Table S5). Genes predicted to code for enzymes involved in acidogenesis by Máté de Gerando et al. (16) were also less expressed in L-rhamnose cultures, suggesting that the reactions for acetate or butyrate production from L-rhamnose might be catalyzed by different enzymes. Genes involved in solvent formation (butanol and ethanol), however, were slightly upregulated during the early exponential growth phase of the L-rhamnose cultures. In Table S5, a list of differentially expressed genes encoding for enzymes or other proteins predicted to be involved in glycolysis, acidogenesis and solventogenesis is shown, including the fold-change in expression between L-rhamnose and D-glucose.

The gene predicted to encode Spo0A, the global regulator of the metabolism in solventogenic *Clostridia* (CIBE_2041), did not show significant difference in expression levels under growth on D-glucose or on L-rhamnose. This indicates that stationary phase processes, including sporulation and stress-response mechanisms, might not be differently regulated under both conditions. In Table S6, a list of predicted proteins related to sporulation and to stress

response, and their fold-change in expression during growth on L-rhamnose compared to D-glucose is shown. Most genes encoding sporulation related proteins or enzymes did not show important expression changes. However, data on the gene expression at stationary phase, after 20 hours of fermentation, is needed to have a better insight on differences in the regulation of stress-response mechanism between D-glucose or on L-rhamnose grown cultures.

Discussion

Next to lignocellulosic biomasses, aquatic biomasses such as seaweeds are a promising source for various industries (5, 17). It has been shown that clostridial species are able to grow on hydrolysates from the green seaweed *U. lactuca* and that the main products are acetone, butanol, and ethanol (ABE), which can be used in the biofuel industry (7). In addition, it was observed that 1,2-propanediol was produced as a result of L-rhamnose utilization. The metabolism of L-rhamnose was studied in more detail in various organisms, including *E. coli* and *Salmonella typhimurium* (11, 25). In addition, Forsberg *et al.* showed already in the 1980s that several clostridial species were able to ferment L-rhamnose and a fermentation pathway homologous to the one in *E. coli* and *Salmonella typhimurium* was suggested (9). Recently, the production of propionate and propanol from L-rhamnose was demonstrated in *C. phythofermentans* and a fermentation model was proposed, which also included a specific organelle, the BMC (12). In the frame of this work, the L-rhamnose metabolism in the solventogenic *C. beijerinckii* DSM 6423 was studied by genome analysis, fermentation studies and transcriptomics.

317 *C. beijerinckii* DSM 6423 was tested for growth on *U. lactuca* hydrolysate, in a similar
318 approach as earlier described by our laboratory for the strain *C. beijerinckii* NCIMB 8052
319 (17). In contrast to the latter strain, *C. beijerinckii* DSM 6423 did not grow well on the pure
320 hydrolysate. When the hydrolysate was diluted, the growth and sugar consumption improved,
321 indicating that this strain could be inhibited by components of the hydrolysate. The content of
322 elements that take part in salts, as potential inhibitors of growth, in the hydrolysate was
323 estimated based on the data from Bikker and co-workers (17). The calculated content of
324 elements the *U. lactuca* hydrolysate is shown in Table S2, being S the most abundant with a
325 concentration of 304 mM. The effect of salts on the growth of solventogenic *Clostridia* is not
326 well characterized yet, and only few reports can be found on this topic. Ezeji et al showed
327 that levels of S corresponding to 93 mM in the form of sodium sulfate resulted in inhibitory
328 effects in growth of *C. beijerinckii* on control media (26). The supplementation of microalgae-
329 derived hydrolysates with sodium chloride (NaCl) at 342 mM and higher resulted in inhibition
330 of growth in *C. pasteurianum* (27). In a different study, it was shown that the removal of S,
331 among other elements, from a wood hydrolysate increased fermentability by *C. beijerinckii*
332 (28). The strain used in this study showed higher sensitivity to inhibitors in the hydrolysate as
333 compared to strain NCIMB 8052, although they are both genetically very similar.
334 Interestingly, the hydrolysate was rich enough in nutrients, and supplementation with
335 nutrients was not required for growth.
336 On control medium, *C. beijerinckii* DSM 6423 was capable of growth on L-rhamnose as the
337 sole source of carbon and energy. L-rhamnose was converted into acetate, butyrate and the
338 typical L-rhamnose-derived products 1,2-propanediol, propanol and propionate. Remarkably,
339 typical solvents like isopropanol, butanol and ethanol were not produced. Possibly, solvent
340 production may not be necessary during L-rhamnose fermentation, as all reducing

341 equivalents are required for the production of 1,2-propanediol and propanol (Figure S2).
342 Indeed DHAP (46.4 mM) conversion to acetyl-CoA leads to the formation of 46.4 mM of
343 NADH and 46.4 mM of reduced ferredoxin. Reduced ferredoxin cannot directly donate
344 electrons for solvent production, but should first transfer its electrons to NAD. Assuming that
345 all ferredoxin is converted to NADH, this would yield 92.8 mM NADH in total. This NADH is
346 then used for the production of 46.4 mM 1,2 propanediol and 10,4 mM propanol (7.8 mM
347 NADH is derived from propionaldehyde conversion) in the lactaldehyde-branch, and the
348 production of 15.5 mM hydroxybutyryl-CoA and 15.5 mM butyryl-CoA in the DHAP branch
349 (Figure S2; Figure 3). Thus, this leaves no reducing equivalents for solvent formation, which
350 agrees with the absence of IBE production during L-rhamnose fermentation. Inside the BMC,
351 propionaldehyde is either oxidized or reduced to propanol and propionyl-CoA, respectively. If
352 both products would be produced in equimolar amounts, no net NADH would be
353 produced/consumed. However, the non-equal production of propanol (18.2 mM) and
354 propionate (7.8 mM) indicates that some NADH must be come from outside the BMC,
355 suggesting that reductant (NADH) is able to pass the BMC shell. It has been proposed before
356 that NAD(H) is able to cross the BMC via specific pores (29).
357 From the metabolic pathway shown in Figure 3, based on Mate de Gerando *et al.* (13), it can
358 be estimated that from 1 mol of L-rhamnose; 1 mol of 1,2-propanediol and 1 mol of DHAP
359 should be formed. The 1,2-propanediol is then further metabolized into n-propanol and
360 propionic acid. Table 1 shows that from 46.4 mM of L-rhamnose consumed, 43.2 mM of total
361 products derived from 1,2-propanediol are formed, *viz.* 17.2 mM 1,2 propanediol, 18.2 mM of
362 n-propanol and 7.8 mM of propionic acid. DHAP is converted along the EMP pathway to
363 pyruvate, which is further metabolized into acetic acid (19.2 mM) and butyric acid (11.7 mM).
364 These numbers are in agreement with the expected stoichiometry of this part of the pathway,

as 19.2 mM acetate and 11.7 mM butyrate are derived from 42,6 mM DHAP (19.2 mM + 2 x 11.7 mM). However, some carbon that is not included in these calculations should end up in biomass. Thus, from 46.4 mM of L-rhamnose (-2.1 mM of biomass), 44.3 mM of lactaldehyde and DHAP are produced. Based on the fermentation data, the following equations can be composed for both branches:

1 lactaldehyde → 0.39 1,2-propanediol + 0.41 propanol + 0.18 propionate

1 DHAP → 0.43 acetate + 0.26 butyrate (derived from 0.52 acetyl-CoA)

In accordance, the carbon- and electron recovery, both reached 96% for the L-rhamnose cultures. For the D-glucose and the D-glucose/L-rhamnose mixture the recoveries were also high, with 95% and 88%, respectively.

Growth on L-rhamnose, however, stops before all L-rhamnose is converted. The reason for this is not clear. Possibly, too much acids are produced, that may become toxic. Commonly, during growth on D-glucose, solvents are produced to prevent excessive production of (undissociated) weak acids. However, running the fermentation under pH-controlled conditions did not improve the L-rhamnose conversion. During co-fermentation of L-rhamnose and D-glucose, substantially more L-rhamnose is fermented, suggesting that there might be an energetic reason for the growth retardation on pure L-rhamnose. Theoretically, solventogenic D-glucose fermentation yields ~2 moles of ATP per mole of sugar (30), which is more than twice the amount that can be obtained on L-rhamnose (0.9 mole ATP/mole sugar), assuming that L-rhamnose uptake requires 1 ATP/mole sugar (Figure S2). The OD_{600} data show that growth is best on D-glucose (OD_{600} =11.1), followed by the sugar mixture (OD_{600}=6.8) and the L-rhamnose culture (OD_{600}=2.8). The lower growth yield on L-rhamnose correlates with the calculated ATP yield, which is approximately 38% of the yield on D-glucose (Table 1, Fig S2). Moreover, L-rhamnose specifically induces the formation of the

BMC (*vide infra*), whose protein shell may impose an extra biosynthetic energetic burden for the cell. The lower biomass yield on L-rhamnose compared to on D-glucose was observed earlier by Forsberg et al (9), but its origin was not further studied then. However, despite this apparent difference in growth yields, the reason for the premature growth stop on L-rhamnose remains obscure.

The genome analysis revealed the presence of all necessary genes that are specifically needed for the anticipated enzymes of the L-rhamnose pathway. These include genes for L-rhamnose uptake and subsequent conversion to 1,2-propanediol, propanol and propionate, and from which, many are clustered and probably organized in several operons. Also, various genes coding for shell proteins of the BMC were identified (Table 3). Sequence analysis of the different operons show that the organization of the genes involved in L-rhamnose metabolism is similar to what was found in *C. phytofermentans*. Indeed, the genes responsible for L-rhamnose uptake and conversion are located in a different region then the BMC cluster. However, we observed significant differences in the genes involved in rhamnose transport and the size of the BMC cluster. In our strain, a gene coding for a L-rhamnose-specific ABC-type transporter was present in the genome, but another L-rhamnose-specific transporter gene belonging to the L-rhamnose conversion cluster (CIBE_0612, Table 2) was highly upregulated during growth on L-rhamnose. Thus, the latter transporter is most likely responsible for L-rhamnose uptake. This transporter belongs to the Major Facilitator Superfamily type (MFS), which uses a H+ gradient to transport the sugar, described in *Rhizobium leguminosarum bv. Trifolii*. In solventogenic clostridia, this type of transporter has not been studied in detail yet.

The BMC cluster identified in *C. beijerinckii* DSM 6423 shows some differences compared to the one described in *C. phytofermentans* ISDg. It harbors 21 genes organized in nine

operons in one locus whereas a recent study shows that the *C. phytofermentans* genome harbors three BMC clusters, but only one was experimentally studied (12) . The BMC gene cluster found in *C. beijerinckii* DSM 6423 is more related to the cluster found in other Clostridia such as *C. saccharolyticum* K10 or *C. ljungdahlii* DSM 13528 and α-proteobacteria, such as *Rhodobacter capsulatus* SB 1003. Homologues of the L-rhamnose utilization clusters found in *C. beijerinckii* DSM 6423 were also found in the genome of *C. beijerinckii* strain NCIMB 8052, which utilizes L-rhamnose as well (17).

Transcriptome analysis confirmed the involvement of the predicted genes in L-rhamnose conversion. Most metabolic proteins were highly upregulated (up to eight-fold). The only exception is the propionate kinase. There was no specific propionate kinase gene identified by bioinformatic analysis. However the up regulation (2 fold) of one of the copies of the butyrate kinase gene CIBE_5515 in L-rhamnose-grown cells, suggests that this gene may have activity towards propionyl-P. The various BMC-shell proteins were also highly upregulated (9-12 fold compared to D-glucose-grown cells). Thus, the BMC is specifically induced during growth on L-rhamnose, as has also been described for L-rhamnose conversion in *C. phytofermentans* (13). On the L-rhamnose/D-glucose mixture we observed production of 1,2-propanediol but not of propanol and propionate. This suggests that D-glucose prevents induction of the BMC even when L-rhamnose is present.

It is assumed that the polyhedral shell prevents leakage of volatile metabolites or that it protects the cell against toxic intermediates; in this case propionaldehyde (31) or radicals of the 1,2-propanediol dehydratase reaction (13). As mentioned above, synthesis of the protein–shell may exert a heavy burden on the protein-synthesis machinery and may, therefore, also affect the growth rate and energetics of the cell.

Applied and Environmental Microbiology

In this study, we show that *C. beijerinckii* is able to ferment L-rhamnose as a sole carbon- and energy source, to produce acetic and butyric acids, 1,2-propanediol, propionic acid and n-propanol, which are products of commercial interest. The metabolism of L-rhamnose in this strain shows similarities to pathways described in other Clostridia, but also presents interesting novelties, such as the presence of an MFS transporter for L-rhamnose. Co-fermentation of L-rhamnose with D-glucose leads to higher L-rhamnose utlization, with shows potential for the use of this strain for fermentation of *U. lactuca* hydrolysates, or other L-rhamnose-containing streams, provided that salt toxicity can be reduced. The results in this study serve as a basis for further developments towards efficient biomass utilization for production of chemicals.

Materials and Methods

Bacterial strains and culture conditions

C. beijerinckii DSM 6423 was stored at -20 °C as spore suspension in 20% glycerol. The spore suspension was heat-shocked for 1 min at 95 °C before inoculation. Fermentations were performed in CM2 medium containing (in g L^{-1}): yeast extract, 1.00; KH_2PO_4, 1.00; K_2HPO_4, 0.61; $MgSO_4 \times 7\ H_2O$, 1.00; $FeSO_4 \times 7\ H_2O$, 0.0066; *para*-aminobenzoic acid, 0.10; and ammonium acetate, 2.90. Stock solutions of D-glucose and L-rhamnose were autoclaved separately and added after autoclaving of the medium to a final concentration of 40 g L^{-1}. All liquid media were made anaerobic by flushing with nitrogen gas. Fermentations in 400-mL working volume were performed in Infors HT Multifors bioreactors at 37 °C and a stirrer speed of 150 rpm. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}).

459

460 **Product analysis**

461 Fermentation substrates and products were measured by HPLC. Glucose, rhamnose,
462 acetate, butyrate, lactate, acetone, ethanol, butanol, propanol, and isopropanol were
463 measured in a Waters HPLC system equipped with a refractive index detector (Waters model
464 2414) and a Shodex KC-811 300 × 8 mm column at 80 °C with 3 mM H_2SO_4 as mobile phase
465 and a flow rate of 1.00 mL min^{-1}. As internal standard, 3 mM valeric acid in 1 M H_2SO_4 was
466 used. Propionate and 1,2-propanediol were measured in a Dionex UltiMate3000 HPLC
467 system equipped with a refractive index detector (Waters model 2414) and a Biorad Aminex
468 HPX 87H 300 × 8 mm column at 30 °C with 3.7 mM H_3PO_4 as mobile phase and a flow rate
469 of 0.60 mL min^{-1}. As internal standard, 2.5 mM phthalic acid in water was used.

470

471 **Carbon recovery**

472 For the calculation of the carbon recovery, the total number of moles of carbon present in the
473 products and biomass was divided by the total number of C-moles of the substrates. Since
474 acetate was present at the start and end of the fermentation, it was considered as substrate
475 and product. For simplicity, acetate was considered as substrate when its final concentration
476 was lower than at the start of the fermentation, whereas it was considered as product when
477 its final concentration was higher than at the start. For D-glucose-grown cultures, the cell dry
478 weight (cdw) was calculated from the optical density at 600 nm using the following formula:

$$cdw\ [g\ L^{-1}] = OD600 * 0.28 + 0.13$$

479 For the cultures grown on L-rhamnose and on D-glucose/L-rhamnose mix, the cell dry weight
480 was determined by filtration of 10 mL of culture on a 0.22 µm porous filter, drying the biomass
481 on the filter in an oven at 50°C overnight and weighing.

The carbon content of biomass was calculated using the standard elemental biomass formula ($CH_{1.8}O_{0.5}N_{0.2}$) given by (32). CO_2 production during fermentation was taken into account. It was assumed that for the production of one mole of acetate or ethanol, butyrate or butanol, and acetone or isopropanol, one, two, and three moles of CO_2 are produced, respectively.

Electron recovery

The electron recovery was determined by calculating the degree of reduction per mole of all compounds produced divided by all compounds produced. For simplicity, acetate was again considered as substrate when its final concentration was lower than at the start of the fermentation, whereas it was considered as product when its final concentration was higher than at the start. Since the production of H_2 could not be accurately quantified during the fermentation, it was calculated from the stoichiometry of the reactions. The degree of reduction per mole of substrate or product are (in brackets): D-glucose (24), L-rhamnose (26), acetate (8), butyrate (20), lactate (12), acetone (16), isopropanol (18), butanol (24), ethanol (12), 1,2-propanediol (16), propanol (18), propionate (14), and H_2 (2) The degree of reduction of biomass was calculated from the standard elemental biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$, which corresponds to a degree of reduction of biomass of 21 electrons per mole.

RNA sequencing

Total RNA was isolated from *C. beijerinckii* DSM 6423 for transcriptome studies. Samples were taken from duplicate 400-mL fermentations from cells in early exponential, acetogenic, and solventogenic phase. Cells were pelleted for 15 min at 3000 × g and 4 °C and stored at -

80 °C until further use. RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) and the PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. In short, the cell pellet was thawed on ice and resuspended in 5 mL TRIzol reagent for cell lysis. Next, 1 mL chloroform was added and after centrifugation for 15 min at 13,000 × g, the upper aqueous phase was mixed with an equal volume of 70 % ethanol. The solution was loaded on a spin cartridge, washed once and treated with 30 U DNase I. After two additional washing steps, the RNA was eluted in RNase-free water. Quality and quantity of the isolated RNA was checked by gel electrophoresis and NanoDrop, respectively. Afterwards, the samples were stored at -80 °C before being sent for sequencing. Library construction and sequencing was performed by Novogene Co. Ltd. Messenger-RNA was depleted with the Ribo-Zero Magnetic Kit and a 250-300 bp insert cDNA library was constructed. Pair-ended 150 bp fragments were sequenced using the Illumina HiSeq platform. After sequencing, data were uploaded and analyzed with the MicroScope platform (24). Reads were mapped against the recently sequenced *C. beijerinckii* DSM 6423 genome (16).

Nucleotide sequences

The DSM 6423 full genome sequence is available on the European Nucleotide Archive (ENA) under the accession number PRJEB11626. Link:

https://www.ebi.ac.uk/ena/data/view/PRJEB11626

The DSM 6423 RNAseq data described by Mate de Gerando et al. (16) were deposited on the NCBI BioProject Database under the Accession Number GSE100024. Link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100024 (23)

The DSM 6423 RNAseq data described in this study have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress)under accession number E-MTAB-7487.

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Table 3 Composition and differential expression of the BMC locus in *C. beijerinckii* DSM 6423.

673 **Table 1 Fermentation data of cultures of *C. beijerinckii* grown on D-glucose, L-**
674 **rhamnose and on a D-glucose/L-rhamnose mixture.**

	D-Glucose (t=56h)	***L-Rhamnose (t=56h)***	***D-Glucose/L-Rhamnose (t=72h)***
Substrates at t = 0 h (mM)			
D-Glucose	224.8		111.2
L-Rhamnose		243.4	129.3
Acetate	39.5	38.3	37.3
Substrates consumed at t = end (mM)			
D-Glucose	215.3	nd	111.2
L-Rhamnose		46.4	86.7
Acetate*	30.7	0	1.2
Products at t = end (mM)			
Acetate*	8.8	57.5 (*19.2**)	36.2
Lactate	7.5	nd	2.4
Butyrate	2.1	11.7	25.9
Acetone	3.2	nd	7.9
Isopropanol	68.5	nd	31.9
Ethanol	6	nd	1.1
Butanol	111.8	nd	59.4
1,2-Propanediol	nd	17.2	78.4
Propanol	nd	18.2	nd
Propionate	nd	7.8	nd
Biomass	26.6	2.1	11.7
Yields and recovery			
Biomass (mol/mol sugar)	0.12	0.045	0.059
Yield Butanol (mol/mol D-glucose)	0.52		0.53
Yield 1,2-PD (mol/mol L-rhamnose)		0.37	0.904
Yield propanol (mol/mol L-rhamnose)		0.39	
Yield propionate (mol/mol L-rhamnose)		0.17	
Carbon recovery** (%)	95	96	88
Electron recovery (%)	98	96	88

675 nd: not detected, * Acetate was produced in L-rhamnose cultures, ** The release of CO_2 was
676 estimated and included in the calculations

Table 2 Relative expression values from RNAseq of *C. beijerinckii* DSM 6423 proteins putatively involved in L-rhamnose uptake and conversion.

C. beijerinckii protein	Proposed protein function	Closest homolog with experimental evidence [a]		Log2 fold change in expression relative to D-glucose cultures		
		Organism	Protein (% similarity)	3 h	6.5 h	10 h
L-Rhamnose transport into the cell						
CIBE_5333	rhamnose ABC transporter, permease subunit	*Rhizobium leguminosarum bv. trifolii*	RhaP (51)	n.d	4.18	3.96
CIBE_5334	rhamnose ABC transporter, ATPase subunit	*R. leguminosarum bv. trifolii*	RhaT (62)	1.26	4.47	4.11
CIBE_5335	rhamnose ABC transporter, periplasmic solute binding subunit	-	-	3.14	5.9	4.47
CIBE_0612	MFS rhamnose cation symporter	*Dickeya dadantii (Erwinia chrysanthemi)*	TogT (63 %)	8.83	8.38	6.91
L-Rhamnose conversion to 1,2-propanediol						
CIBE_0605	rhamnulokinase	*C. phytofermentans*	Cphy_1146 (73)	7.78	7.79	7.09
CIBE_0606	L-rhamnose isomerase	*C. phytofermentans*	Cphy_1147 (78)	8.46	8.58	7.36
CIBE_0613	L-rhamnose mutarotase	*C. phytofermentans*	Cphy_1149 (83)	n.d	1.44	1.34
CIBE_0614	Transcriptional regulator	*C. phytofermentans*	Cphy_1187 (66)	1.8	1.46	1.55
CIBE_0615	rhamnulose-1-phosphate aldolase	Escherichia coli (strain K12)	RhaD (69)	6.99	7.37	6.84
CIBE_0617	L-1,2-propanediol oxidoreductase	*Escherichia coli (strain K12)*	FucO (79)	8.35	7.69	6.67

[a] All percent similarity values were determined using global alignments of protein sequences using the gapped BLAST algorithm (19).

References for the characterized functional equivalent are as follows: *Rhizobium leguminosarum bv. trifolii* rhamnose transporters (15) , *C. phytofermentans* rhamnose dissimilation enzymes (12), *Escherichia coli* (strain K12) rhamnulose-1-phosphate aldolase and L-1,2-propanediol oxidoreductase (33, 34), rhamnose MFS transporter (15, 35, 36)

685 **Table 3 Composition and differential expression of the BMC locus in *C. beijerinckii* DSM 6423.**

C. beijerinckii protein	Proposed protein function	Closest homolog with experimental evidence[a]		Log2 fold change in expression relative to D-glucose cultures		
		Organism	Protein (% similarity)	3 h	6.5 h	10 h
CIBE_4883	Propanediol oxidoreductase	*Salmonella typhimurium*	PduS (68)	5.79	5.42	3.83
CIBE_4884	propionaldehyde dehydrogenase	*Clostridum phytofermentans*	Cphy_1178 (70)	9.49	8.32	6.24
CIBE_4885	eutJ , putative chaperonin, ethanolamine utilization protein	*S. typhimurium*	EutJ (63)	8.62	7.61	6.57
CIBE_4886	Phosphate propanoyl transferase	*C. phytofermentans*	Cphy_1183 (67)	9.11	7.87	6.06
CIBE_4887	BMC-H shell protein	*C. phytofermentans*	Cphy_1182 (88)	8.73	7.91	5.63
CIBE_4888	conserved membrane protein of unknown function	-	-	8.75	7.81	5.87
CIBE_4889	metK, S-adenosylmethionine synthetase	-	-	9.12	8.08	6.58
CIBE_4890	Response regulator receiver protein	-	-	9.16	8.34	7.15
CIBE_4891	Signal transduction histidine kinase, LytS	-	-	9.42	8.49	6.93
CIBE_4892	Propanol dehydrogenase	*Klebsiella pneumoniae*	Dhat (43)	9.99	10	7.57
CIBE_4893	propionaldehyde dehydrogenase	*C. phytofermentans*	Cphy_1178 (63)	9.54	9.56	7.27
CIBE_4894	BMC-P shell protein	*C. phytofermentans*	Cphy_1184 (71)	9.47	9.43	7.45
CIBE_4895	BMC-H shell protein	*C. phytofermentans*	Cphy_1186 (61)	10	10	7.8
CIBE_4896	conserved protein of unknown function	-	-	11	10	8.15
CIBE_4897	BMC-H shell protein	*C. phytofermentans*	Cphy_1182 (89)	11	10	7.54
CIBE_4898	BMC-T shell protein	*S. typhimurium*	PduB (67)	10	8.99	7.04
CIBE_4899	Propanediol dehydratase activator	*Clostridium butyricum*	DhaB2 (56)	11	11	7.69
CIBE_4900	Propanediol dehydratase	*C. butyricum*	DhaB1 (58)	12	10	6.94

C. beijerinckii protein	Proposed protein function	Closest homolog with experimental evidence[a]		Log2 fold change in expression relative to D-glucose cultures		
		Organism	Protein (% similarity)	3 h	6.5 h	10 h
CIBE_4901	Glutamine amidotransferase	-	-	6.3	5.94	5.44
CIBE_4902	Propanediol utilization protein	*S. typhimurium*	PduV (62)	6.56	6.31	5.62
CIBE_4903	BMC-H shell protein	*C. phytofermentans*	Cphy_1176 (76)	7.67	6.72	5.73
CIBE_4904	Propanediol utilization protein	*S. typhimurium*	PduO (62)	7.11	6.14	5.01
CIBE _4905	hypothetical protein	-	-	2.15	n.d[b]	n.d
CIBE _4906	xanthine/uracil/vitamin C permease	-	-	-2.26	-3.31	1.65

686 [a] All percent similarity values were determined using global alignments of protein sequences using the gapped BLAST algorithm (19) [b] n.d for not
687 detected, References for the characterized functional equivalent are as follows: *Salmonella typhimurium* (11), *C. phytofermentans* (12), *Klebsiella*
688 *pneumoniae* (37), *C. butyricum* (38), for the BMC shell protein types (39).

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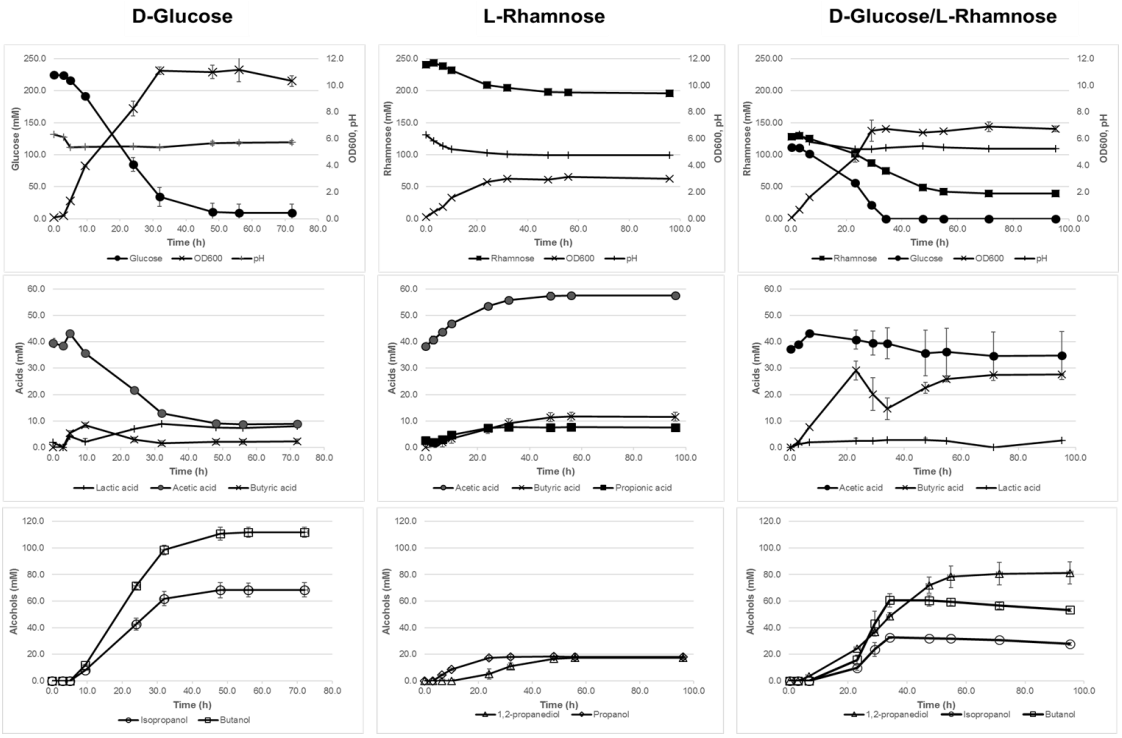
691 **List of Figures**

692 **FIG 1 Fermentation profiles of *C. beijerinckii* grown on D-glucose (left), L-rhamnose**
693 **(center) and D-glucose/L-rhamnose mixture (right).** Fermentations were performed in
694 duplicate in CM2 medium supplemented with the indicated sugars. Only products present at
695 a concentration >1mM are shown. Standard deviation is shown with error bars, indicating one
696 standard deviation of the mean (n=2).

697 **FIG 2 Schemes of the rhamnose utilization (A) and Bacterial Microcompartments**
698 **(BMC) (B) clusters *in C. beijerinckii* DSM 6423.** 2A) The genes predicted to encode
699 enzymes involved in the L-rhamnose metabolism are shown in blue, the genes in green
700 encode for putative L-rhamnose transporters and the genes in grey are not reported to be
701 involved in L-rhamnose metabolism. The functional homologue in *Rhizobium leguminosarum*
702 *bv. Trifolii* is indicated for each gene, * Homologs involved in rhamno-galacturonan
703 catabolism were identified in *Dickeya dadantii* 3937. 2B) BMC superlocus, as compared to
704 GRM3 (21). The functional equivalent in *Salmonella typhimurium* of the genes predicted to be
705 involved in the BMC are indicated below each gene. The genes are shown in different colors
706 according to the function of the protein encoded: in blue, enzymes involved in the conversion
707 of propanediol into propionate and propanol, in red, genes predicted to encode for BMC-H
708 shell proteins, in purple for BMC-T shell proteins, in orange for BMC-P shell proteins and the
709 genes in grey have an unknown function.

710 **FIG 3 Model for the metabolic pathway of L-rhamnose by *C. beijerinckii***. The gene
711 functions are based on sequence homology as shown in Table 2 and 3 for the L-rhamnose-
712 metabolizing pathway, and on Mate de Gerando et al (2018) for the other routes. Genes

shown in blue are over-expressed on L-rhamnose when compared to glucose. Genes in red indicate lower expression in L-rhamnose cultures compared to glucose cultures.



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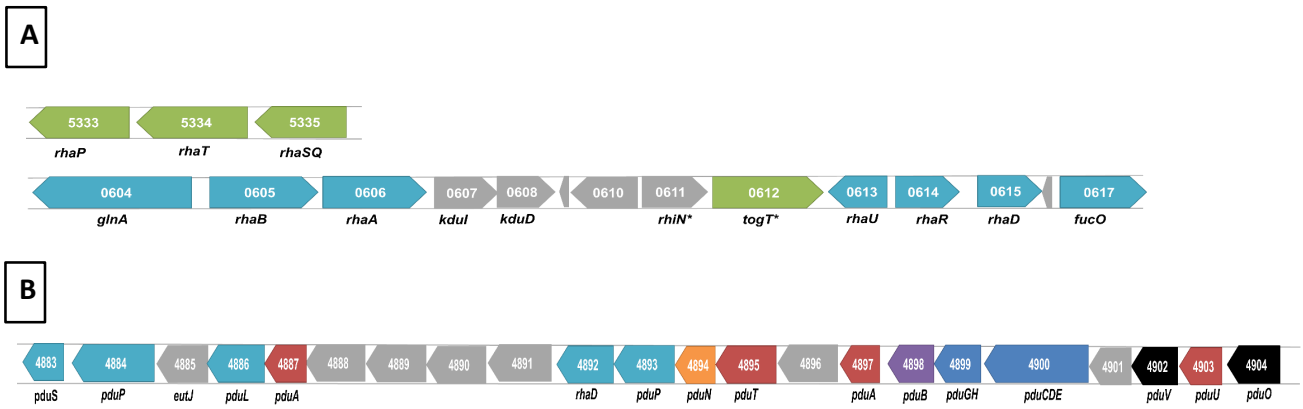

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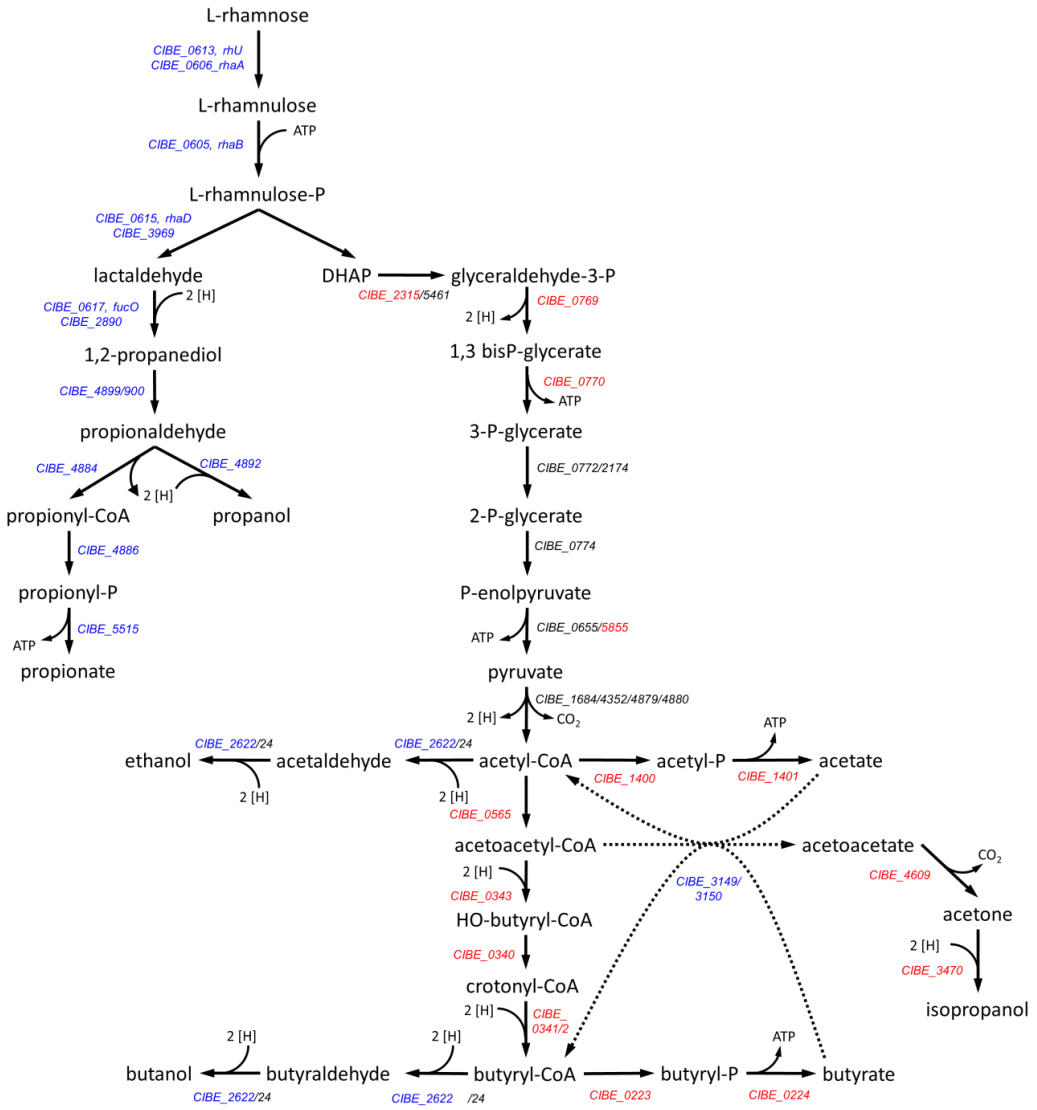

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