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"^{13}C-NMR Determination of Simultaneous Xylose and Glucose Fermentation by a Newly Isolated Strain (G11) of *Klebsiella Planticola*"

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^{13}C-NMR DETERMINATION OF SIMULTANEOUS XYLOSE AND GLUCOSE FERMENTATION BY A NEWLY ISOLATED STRAIN (G11) OF *KLEBSIELLA PLANTICOLA*

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Abstract—*In vivo* NMR techniques and substrates selectively enriched with ^{13}C were used to follow the step-by-step metabolism of glucose and xylose, on their own or as mixed substrates in the ratio as they occur in hydrolysates from hemicellulose. The organism used was a newly isolated strain of *Klebsiella planticola* isolated from soil where maize has been cultivated for 30 years. Results suggest that glucose is converted to pyruvate via the Embden–Meyerhof pathway and then to lactate and ethanol. No evidence of 2,3-butandiol or formate metabolism was observed. This organism had a higher rate of uptake of xylose than previously studied microorganisms, resulting in ethanol, lactate, acetate succinate and formate as end products. Xylose metabolism in *K. planticola* G11, unlike that reported for many other organisms, was not inhibited by glucose. The addition of glucose, after 2 h of xylose fermentation, did not change the rate of xylose metabolism.

Keywords—*Klebsiella planticola*; ^{13}C-NMR; ^{13}C-enriched substrates; glucose; xylose; fermentation; hemicellulose; ethanol.

1. INTRODUCTION

Together with cellulose and lignin, hemicellulose, a plant cell-wall polysaccharide, is one of the fundamental constituents of lignified tissues.[1] Hemicellulose is an easily hydrolysable short-branched chain hetero-polysaccharide consisting of mixed hexosans and pentosans. The hydrolysis products of hemicellulose contain D-xylose and L-arabinose as the main pentoses, and D-glucose, D-mannose and D-galactose as the principal hexoses.[2] D-xylose and D-glucose constitute about 90% of the neutral carbohydrate content of the hemicellulose of plants and agricultural residues[3] and usually xylose occurs at about twice the abundance of glucose. Hexoses and pentoses obtained by hydrolysis of hemicellulose should form an excellent substrate for growing microorganisms to produce chemicals such as ethanol. Ethanol is currently produced from hexoses, but efficient biological processes in which pentoses are utilised have not yet been developed. The search for an appropriate microorganism for this process is currently directed towards yeasts and bacteria. Although yeast can be used to break down xylose, wild and modified bacteria can give the best yields in the transformation of pentoses.

In this paper we report studies on the process of ethanol production from xylose using a wild strain of bacteria identified as *Klebsiella planticola*, isolated from the soil of a maize field.

The main objectives of our research were:

(i) to elucidate the pathway of xylose metabolism by this non-pathogenic bacterium;

(ii) to compare the metabolic pathway for xylose and glucose metabolisation pathways as well as ethanol yields.

In vivo NMR techniques and selectively ^{13}C-enriched substrates were utilised to follow the step-by-step fermentation of glucose and xylose as single or mixed substrates, as they occur in hemicellulose hydrolysate.

2. EXPERIMENTAL SECTION

2.1. *Methods and sample preparation*

The culture medium consisted of 5.25 g/l KH_2PO_4, 6.85 g/l K_2HPO_4, 5 g/l $NaHCO_3$, 0.1 g/l

$MgSO_4$, 0.1 g/l NaCl, 0.2 g/l $(NH_4)_2SO_4$, 0.3 g/l urea, 0.02 g/l $CaCl_2$, 0.2 g/l yeast extract and 10 g/l xylose unless otherwise stated. The following trace elements were also present: Fe, Cu, Co, Mo, Mn. *Klebsiella planticola* G11 was grown in flasks at 35°C in a nitrogen atmosphere. The pH of the medium was adjusted to 7.5. Growth was followed by spectrophotometric optical density (O.D.) measurements at 660 nm. A unitary value of O.D. was verified corresponding to 0.53 g/l of dry weight of biomass. Inocula for *in vivo* microbatch ^{13}C-NMR experiments were prepared by growing a single agar colony overnight in the medium with 10 g/l of D-xylose. A fraction of this culture was diluted 2:100 in the medium for 2–3 further duplication cycles. The cells were then collected by centrifugation and used as the inoculum for NMR measurements. The initial O.D. value of the cell culture was 0.5. After complete xylose metabolism, an average O.D. value of 4.0 was obtained. ^{13}C-NMR spectra were collected on a Varian XL-200 spectrometer operating at 200.058 and 50.288 MHz for proton and carbon nuclei, respectively. Carbon spectra were recorded under continuous broad-band proton decoupling conditions. In order to avoid temperature change, decoupling was obtained using the Waltz-16 pulse sequence. The microbatch was realized in a coaxial tube containing 100% D_2O as NMR lock signal in the external section. All chemical shifts were expressed in relation to tetramethylsilane. Identification of the end-products of fermentation was based on carbon chemical shift considerations. Comparison with NMR spectra of pure compounds and experimental procedure such as the detection of proton-coupled carbon spectra and carbon spin–lattice relaxation rate measurements (R_{1C}) were also used for the final identification of the end-products. [1-^{13}C]-xylose and [2-^{13}C]-glucose were obtained from Cambridge Isotope Laboratories and used without any further purification. The Michaelis–Menten kinetic constant calculated had an error of $\pm 3\%$ due to the error in measured NMR parameters.

2.2. *Source isolation and identification of the microorganism*

Samples of soil were collected near Siena (Italy) in an area where corn has been cultivated for 30 years. Total recycling of the untreated vegetable biomass in the soil was practised for many years. Soil was inoculated at 35°C in 50 ml bottles containing a medium with 100 g/l xylose. Single colonies were obtained on deep agar plates by diluting the mixed population grown in bottles. Each colony was tested for ethanol production from xylose by gas chromatographic analysis.

Ethanol-yielding strains were then tested for their growth rates on xylose in anaerobic conditions. Strain G11, an immobile, Gram negative rod-like bacterium showed the highest growth rates as revealed by the O.D. value detected after the first, second, third and tenth hours of fermentation.

Several procedures were used for the identification of the G11 strain. These include the Enterobacteriaceae identification test, "rapid ID32E" (BioMerieux, Lyon—France) with API automatic detection system (BioMerieux, Lyon—France). Growth at 10°C, gas production at 44.5°C and melizitose fermentation tests were positive, negative and negative, respectively, leading to the final identification of the bacterium as a strain of *Klebsiella planticola* according to Bergey's classification.[4]

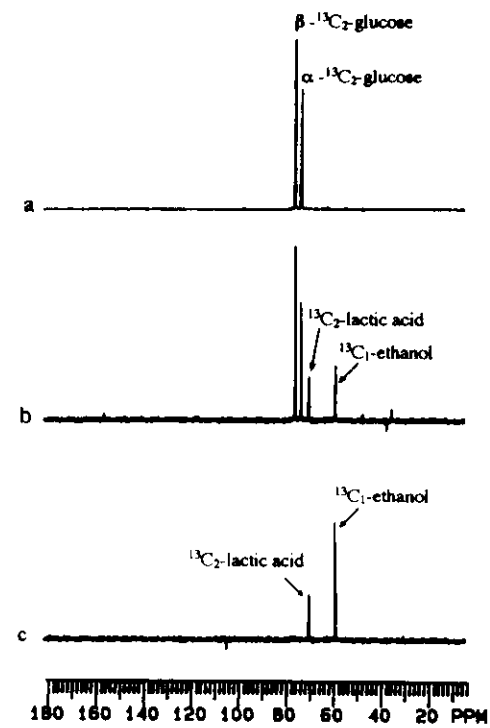

Fig. 1. ^{13}C-NMR spectra of anaerobic *Klebsiella planticola* G11 culture as a function of time after [2-^{13}C]-glucose addition: (a) before inoculation; (b) after 2 h of fermentation; (c) at the end of the fermentation process. The enriched end-products were ethanol and lactic acid.

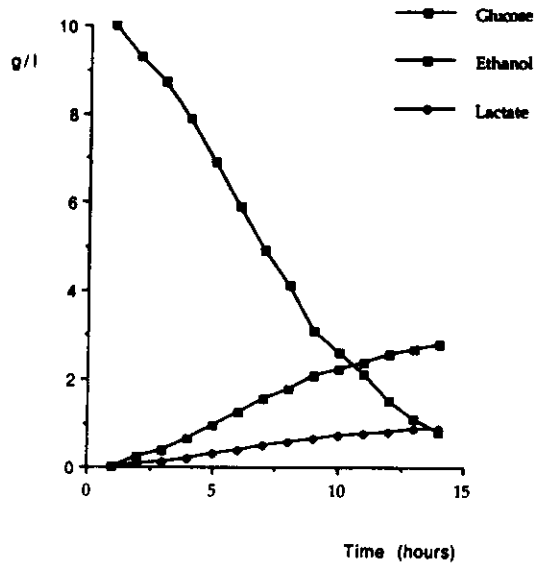

Fig. 2. Time course of [2-^{13}C]-glucose disappearance and [2-^{13}C]-lactate and [2-^{13}C]-ethanol formation during fermentation of glucose by *Klebsiella planticola* G11.

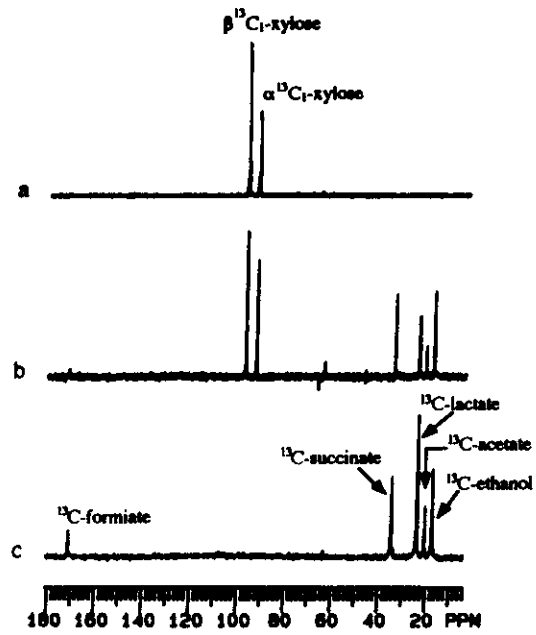

Fig. 3. ^{13}C-NMR spectra of anaerobic *Klebsiella planticola* G11 cell culture as a function of time after [1-^{13}C]-xylose addition: (a) before inoculation; (b) after 5 h of fermentation; (c) at the end of fermentation. The enriched end-products were ethanol, lactic acid, acetic acid, succinic acid and formic acid.

3. RESULTS AND DISCUSSION

3.1. *Anaerobic metabolism of glucose*

Carbon-13 selective enrichment of sugar substrates enabled us to follow the metabolic pathways by NMR spectroscopy.[5,6] Figure 1 shows the ^{13}C-NMR spectrum of the *K. planticola* G11 culture at two different stages of fermentation after addition of [2-^{13}C]-glucose. These spectra show the transfer of the [2-^{13}C]-glucose carbon from the sugar substrate to the end-products, which were [2-^{13}C]-lactic acid (68.43 ppm) and [1-^{13}C]-ethanol (57.4 ppm). Figure 2 shows the time course of the disappearance of the [2-^{13}C] signals from α and β glucose and the formation of [2-^{13}C]-lactate and [1-^{13}C]-ethanol. The curves were obtained from a set of spectra, recorded at 30 min

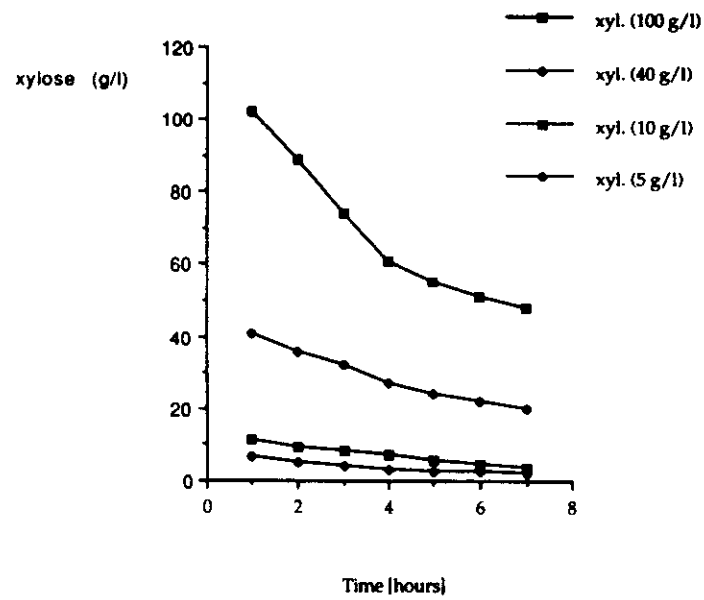

Fig. 4. Time course of [1-^{13}C]-xylose consumption by *Klebsiella planticola* G11. The xylose concentration was in the range 5–100 g/l.

intervals from the addition of 10 g/l [2-^{13}C]-glucose until complete sugar metabolisation. From the Lineweaver–Burk plot[7] of glucose fermentation by *K. planticola* G11, a Michaelis–Menten kinetic constant, K_m, of 33.6 mM was calculated. The K_m value refers to glucose disappearance from the culture medium and is therefore related to the glucose transport step.

In other experiments we analysed the end-products of bacterial fermentation obtained using [1-^{13}C]-glucose and [2-^{13}C]-glucose in the culture medium. After complete glucose metabolisation only ethanol and lactic acid were formed. In particular, from [1-^{13}C]-glucose the methyl signal of lactic acid (20.22 ppm) and the methyl of [2-^{13}C]-ethanol (16.96 ppm) were detectable, whereas [2-^{13}C]-glucose yielded [2-^{13}C]-lactic acid and [1-^{13}C]-ethanol.

These results suggest that glucose is converted to pyruvate via the Embden–Meyerhof pathway. Pyruvate is then metabolized to lactate and ethanol. No evidence of 2,3-butandiol or formate, typical of the *Klebsiella genera* glucose metabolism, were observed. A total of 3.02 g/l of ethanol and 0.95 g/l of lactate was produced from 10 g/l glucose fermentation. These values globally amount to a stoichiometric yield of about 70% in end-products. The theoretical metabolic yield of ethanol from 10 g/l of glucose is 5.0 g/l. This means that about 60% of the initial glucose was converted to ethanol.

3.2. *Anaerobic metabolism of xylose*

Figure 3 shows the carbon spectra of a *K. planticola* G11 cell suspension after the addition of [1-^{13}C]-xylose at three different stages of fermentation. Analysis of the chemical shifts revealed the following end-products of xylose metabolism: ethanol, lactate, acetate, succinate and formate. The ^{13}C signals of the end-products correspond to the methyls of ethanol, acetate and lactate, to the methylene of succinate and the carboxylic group of formate. The end-products of xylose fermentation are typical of the ethanol and mixed acid degradation pathways[8–13] previously described.

The time course of xylose consumption by the bacteria at different sugar concentrations in the range 5–100 g/l is shown in Fig. 4. From these results a Michaelis–Menten kinetic constant, K_m, of 111.5 mM for the xylose transport step through the bacterial cell membrane, was calculated. The concentration of the sugar substrate and the end-products (determined on the basis of ^{13}C-NMR peak intensities) as a function of time from the addition of *K. planticola* to a medium containing 10 g/l xylose is reported in Table 1. After complete fermentation, this quantity of xylose yields a total amount of 1.19 g/l of ethanol, 0.85 g/l of acetate, 0.53 g/l of lactate and 0.48 g/l of succinate. This is about 66% of the theoretical metabolic yield of end-products.

From analysis of the experimental results

Table 1. Time dependence of xylose degradation and end-product yield as a function of time from 10 g/l [1-^{13}C]-xylose addition. Each step refers to 30 min fermentation

Spectrum number	Xylose (g/l)	Succinate (g/l)	Lactate (g/l)	Acetate (g/l)	Ethanol (g/l)
1	10	—	—	—	—
2	9.49	0.01	0.01	0.02	0.08
3	8.79	0.04	0.05	0.12	0.39
4	8.04	0.09	0.10	0.21	0.67
5	7.24	0.13	0.15	0.28	0.82
6	6.55	0.16	0.19	0.36	0.92
7	5.98	0.20	0.23	0.41	0.99
8	5.31	0.22	0.26	0.46	1.06
9	4.68	0.25	0.29	0.48	1.11
10	4.30	0.27	0.30	0.53	1.16
11	3.94	0.29	0.33	0.54	1.19
12	3.65	0.31	0.34	0.56	1.24
13	3.28	0.33	0.36	0.57	1.26
14	3.14	0.33	0.38	0.59	1.30
15	2.97	0.35	0.38	0.61	1.34
16	2.75	0.35	0.39	0.62	1.38
17	2.58	0.36	0.38	0.63	1.39
18	2.45	0.36	0.40	0.64	1.42
19	2.35	0.37	0.40	0.65	1.44
20	2.21	0.37	0.41	0.67	1.47
21	2.17	0.38	0.41	0.67	1.48
22	2.11	0.38	0.42	0.68	1.51
23	1.95	0.39	0.43	0.68	1.52

shown in Fig. 4 and Table 1, and considering the sugar transport process as the limiting step of xylose metabolism, it emerges that *K. planticola* G11 has a higher xylose uptake rate than previously studied microorganisms.[14-19] The uptake and metabolisation rate increased in the first 3 h from 1 to 7 g/l h per gramme of biomass, when the xylose concentration in the culture medium rose from 5 to 100 g/l.

Lower uptake rates of 1.6 g/l h were obtained with *K. planticola* ATCC 33531 strain.[17] The high xylose uptake rate observed at high xylose concentrations (100 g/l) suggests the existence of a "low affinity" uptake mechanism unknown in the *Klebsiella* genus and in other bacteria, but probably similar to the transport mechanism described in *Candida shehatae*,[20] which has a "facilitated diffusion" transport process characterised by a K_m of 125 mM. The K_m of 125 mM reported[20] for the facilitated transport process in *Candida shehatae* and the K_m of 111.5 observed in *K. planticola* suggest that a similar transport process is responsible for the xylose uptake in both microorganisms.

3.3. *Non-diauxic growth*

Glucose is known[21] to inhibit the utilisation of different sugar substrates. Microorganisms growing in a mixture of glucose and other sugar sources generally metabolise the glucose first, after which enzymes for the metabolisation of the other sugar substrates are activated.[22] Investigation of simultaneous glucose and xylose metabolism in cell cultures of *K. planticola* G11 was performed using selectively ^{13}C-enriched sugar substrates and NMR spectroscopy. In these experiments [1-^{13}C]-xylose and [2-^{13}C]-glucose were used as energy sources. Isotopic enrichment in different positions of the sugar chain enabled us to separate the xylose from the glucose signals in the carbon spectrum and calculate the contribution of each sugar to end-products yield. This was possible because the metabolic pathway of [1-^{13}C]-xylose and [2-^{13}C]-glucose leads to end-product molecules with separate NMR chemical shift properties.

Figure 5 shows the ^{13}C-NMR spectra of cultures of *K. planticola* G11 during the fermentation of glucose and xylose. The first NMR spectrum (Fig. 5a) refers to ^{13}C-enriched sugar substrates before addition of the inoculum. The second spectrum (Fig. 5b) shows the end-products of the faster glucose fermentation, [1-^{13}C]-ethanol and [2-^{13}C]-lactic acid signals, with the end-products of the slower xylose metabolisation as less intense signals. The last spectrum (Fig. 5c), after complete sugar consumption, enabled us to analyse end-product formation and yields. From a concentration of 5 g/l of glucose, 1.84 g/l of ethanol and 0.22 g/l of lactic acid were produced; from 5 g/l of xylose, 0.95 g/l of ethanol, 0.32 g/l of lactic acid, 0.7 g/l of acetate, and 0.32 g/l of succinic acid were produced. From the fermentation of a total of 10 g/l of sugar mixture, a total concentration of ethanol of 2.79 g/l was produced, of which 34% was derived from the metabolisation of xylose and 66% from glucose.

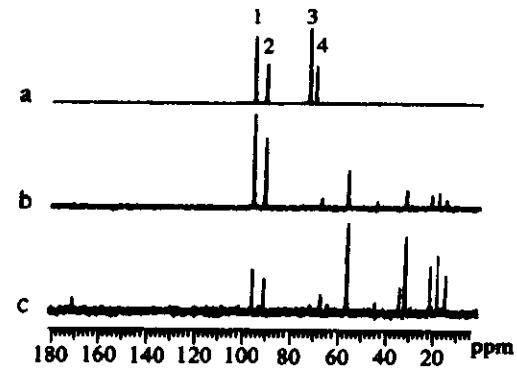

Fig. 5. ^{13}C-NMR spectra of anaerobic *Klebsiella planticola* G11 fermentation during the diauxic metabolism xylose and glucose substrates: (a) before inoculation; (b) after 3 h of fermentation; (c) at the end of fermentation. 1, 2, 3 and 4 refer to [1-$^{13}C_x$] and [1-$^{13}C_\beta$] xylose and [2-$^{13}C_x$] and [2-$^{13}C_\beta$] glucose respectively. The signals of the end-products of fermentation have the same NMR frequency as Figs 1 and 3.

The time course of the simultaneous fermentation of glucose and xylose by *K. planticola* G11 cells is reported in Fig. 6. The addition of 5 g/l of glucose to the culture medium after 2 h of xylose

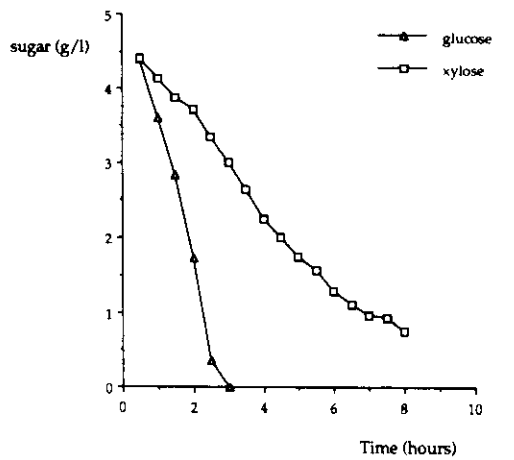

Fig. 6. Time course of glucose and xylose consumption by *Klebsiella planticola* G11 during anaerobic fermentation.

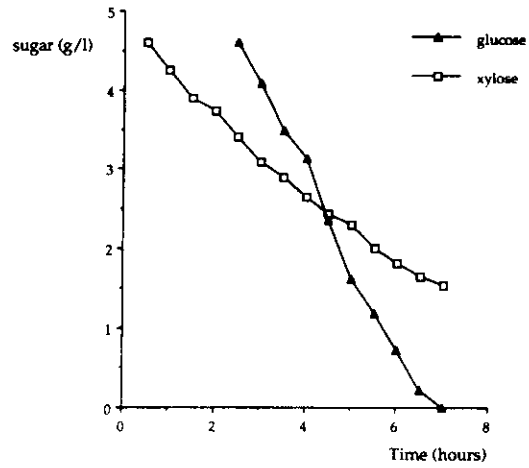

Fig. 7. Effect of glucose addition on anaerobic xylose fermentation by *Klebsiella planticola* G11.

fermentation did not change the rate of xylose metabolism, as shown in Fig. 7. The simultaneous use of glucose and xylose has been previously reported in yeast[14] in the presence of a great quantity of inoculum, under conditions that were very different from the ones used in our experiments. These results show that *K. planticola* G11 can be used for the metabolisation of sugar mixtures obtained from hemicellulose hydrolysis

They also suggest that glucose and xylose uptake occurs by two independent enzymatic routes in *K. planticola* G11. Moreover the faster glucose uptake and consumption do not alter the rate of xylose degradation. This is important because the bacterium shows a much higher xylose uptake than previously tested microorganisms and can be used to ferment sugar mixtures. These results, obtained with a wild strain may be improved by genetic selection and engineering.

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