Journal of Chromatography, 507 (1990) 321–326
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1817

Monitoring and purification of gluconic and galactonic acids produced during fermentation of whey hydrolysate by *Gluconobacter oxydans*

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SUMMARY

Because the sugar concentration of whey is low, the substrate conversion must lead to products with a high added value and with the highest possible yields. Deproteinised and hydrolysed whey was found to be an excellent medium for acidic fermentations and both gluconic and galactonic acids were produced in high yields during the growth of *Gluconobacter oxydans* on hydrolysed whey.

The production, the identification by ionic high-performance liquid chromatography and the purification of both acids on ion-exchange columns will be described here.

INTRODUCTION

Usually the whey that is available in large amounts in most European countries is ultrafiltered and the lyophilized proteins are used for cattle feed. The retentate contains up to 5% of lactose and low-molecular-weight peptides and it has been found to be an excellent growth medium for bacteria involved in acidic fermentations[1–3].

The fermentation of sugars to alcohols or acids is well known and is very reliable but, in contrast to studies where sugar had been used at high concentrations, the lactose is present at a maximum level of only 5%. Accordingly, the conversion must lead to products with the highest possible yields. The products must also be easily isolated with a high added value and a high marketing potential. In order to meet all these requirements, acid fermentations leading to gluconic acid and galactonic acid have been assessed.

The use of whey as a culture medium is not very common because many microorganisms are not able to metabolize lactose. For example, less than 12% of yeast strains can assimilate this sugar[4]. This is the reason why hydrolysed whey was used for *Gluconobacter oxydans* fermentations[5].

The products that are obtained are so closely related that different chromatographic methods have been needed to identify and quantify them. The selected

0021-9673/90/$03.50

analytical methods have been high-performance liquid chromatography (HPLC) on a cation-exchange column or on a strong anion-exchange column[6] and capillary gas chromatography (GC) after derivatization of the acids that are produced[7]. The results of the assays are presented here.

EXPERIMENTAL

Instrumentation

The HPLC system consisted of a Model 6000A pump, a WISP intelligent automated injector, an R-401 refractometer and a UV-441 detector (set at 214 nm) (all from Waters Assoc., Milford, MA, U.S.A.). The results were recorded with a Trilab 2500 from Trivector (Sandy, Bedfordshire, U.K.). Two columns were used: a Bio-Rad Aminex HPX-87-H with the corresponding precolumn (elution with $5 \cdot 10^{-3}$ *M* sulphuric acid at 0.7 ml/min and 70°C) and a Dionex HPIC-AS5 fitted with an HPIC-AG5 precolumn (elution with 0.01 *M* sodium hydroxide solution at 0.7 ml/min and 25°C).

Capillary GC was performed with a Carlo-Erba Vega instrument equipped with an OV-17 column (20 m × 0.32 mm I.D.). The column temperature was programmed at 10°C/min from 60 tot 150°C and at 5°C/min from 150 to 300°C.

The sugar derivatization procedure has been described previously[6].

The ultrafiltration system consisted of a Minipuls-2 peristaltic pump (Gilson) and a tangential ultrafiltration module (Amicon) with an effective surface area of 43 cm^2 and a molecular-weight cut-off of 10^4.

The Model C16/100 glass purification columns (Pharmacia, Uppsala, Sweden) with adaptor, having a volume of 175 ml were filled with Amberlite IRC-120 (Rohm and Haas, Philadelphia, PA, U.S.A.) as a cation-exchange resin and Bio-Rad AGMP-1 (Bio-Rad Labs., Richmond, CA, U.S.A.) as an anion-exchange resin.

The Biolafitte(F) 2-L fermenter was thermostated at 30°C and equipped with a automatic pH regulator (Consort, Turnhout, Belgium).

The *Kluyveromyces bulgaricus* IRC 101 used for whey hydrolysis has been described previously[5]. *Gluconobacter oxydans* ATCC 621H was grown on the hydrolysed whey.

RESULTS

The kinetics of both sugar conversion and acid production by the bacteria can be monitored in a single chromatographic run when a strong cation-exchange column is used. The rare infections that occurred during long fermentations can be detected at an early stage by the production of polar acidic or alcoholic metabolites, mainly acetic acid and butyric acid and ethanol, respectively. Typical chromatograms of the standards used for monitoring the whey fermentation are presented in Fig. 1.

As shown in Fig. 2, the gluconic acid production, occurring from hydrolysed whey as described[3], can give ambiguous results because of the overlapping of the glucose and gluconic acid peaks. To overcome this problem, the gluconic acid formation was then monitored without any sugar interferences by UV detection at 214 nm.

Before using the whey hydrolysate to produce gluconic acid, preliminary fermentations were conducted on either glucose or galactose medium. The glucose was

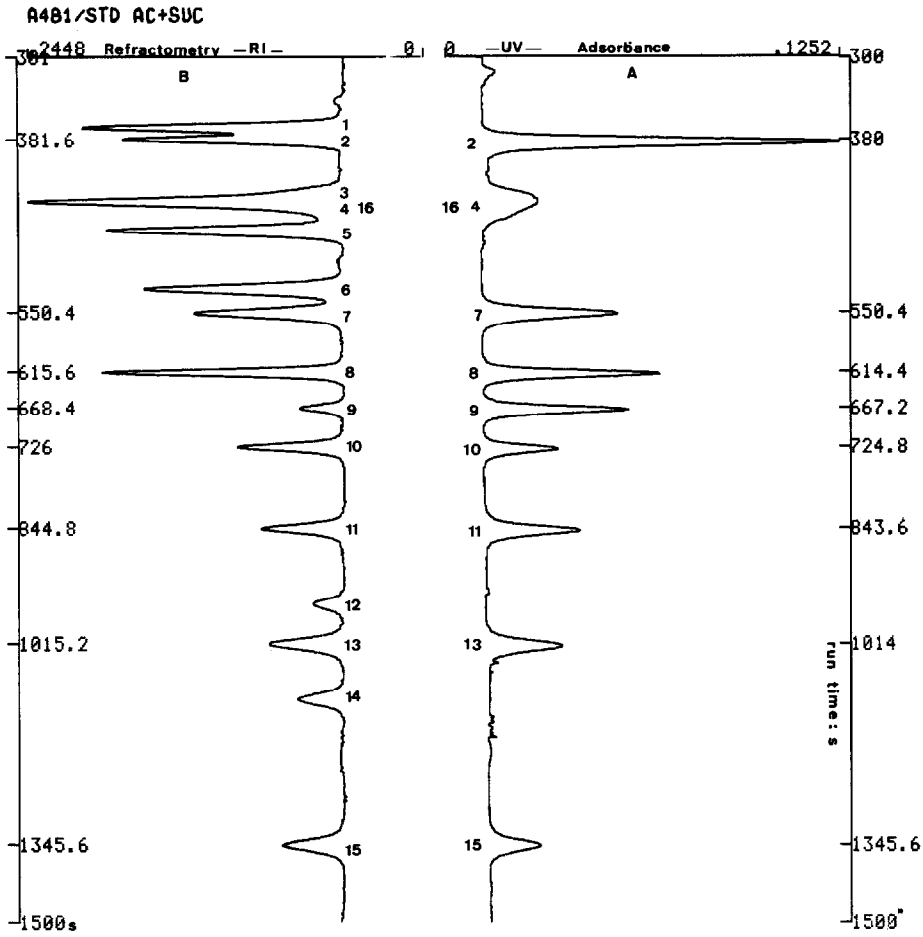

Fig. 1. (A) UV and (B) refractometric determinations of the standards used for the identification of the most important compounds present in whey. Peaks: 1 = citric acid; 2 = lactose; 3 = glucose; 4 = gluconic acid; 5 = galactose; 6 = ribose; 7 = succinic acid; 8 = lactic acid; 9 = formic acid; 10 = acetic acid; 11 = propionic acid; 12 = methanol; 13 = butyric acid; 14 = ethanol; 15 = valeric acid.

completely oxidized to gluconic acid within 24 h and if the reaction was stopped at this stage no ketogluconic acid could be found[9]. Galactose was metabolized at an obviously slower rate. Up to 72 h were needed for its complete conversion into the corresponding acidic molecule that had the same retention time as that of gluconic acid on the cation-exchange column.

In order to try to separate the acids produced, an anion-exchange column was also used. No differences in retention time was observed in either the isocratic mode or the gradient mode using a gradient of sodium hydroxide concentration. While the search for an HPLC method for the separation of these products is being continued and will be published elsewhere, capillary GC was used to identify both metabolites. As shown in Fig. 3, two peaks eluting at 167 and 172°C were observed. After comparison with standards of both acids, it was clearly established that *Gluconobacter ox-*

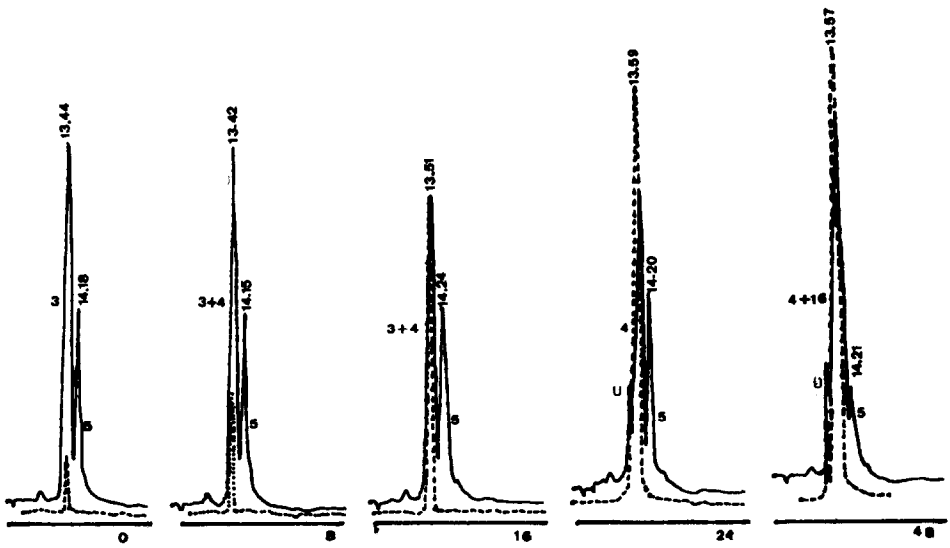

Fig. 2. On-line monitoring of gluconic acid fermentation at 0, 8, 16, 24 and 48 h, as indicated. Peaks: 3 = glucose; 4 = gluconic acid; 5 = galactose; U = unknown; 16 = galactonic acid. Retention time given in min. UV, 214 nm (---) and RI (——) detection.

ydans produces gluconic acid and galactonic acid from glucose and galactose, respectively.

The use of the whey hydrolysate leads rapidly to a broth containing mainly gluconic acid and galactose as sugar. Subsequently, the broth was ultrafiltered and saved. The biomass was concentrated for a further run while the ultrafiltered gluconic acid and galactose were separated by using ion-exchange column. The resulting galactose solution, sterilized by ultrafiltration, was directly oxidized to galactonic acid by *Gluconobacter oxydans* that had grown previously on a galactose medium. Following this, a two-step fermentation was conducted with no cross-contamination of the two acids.

In order to elute the acids produced (gluconic or galactonic acid) from the anion-exchange column, dilute hydrochloric acid was used first. The eluted organic acid contained all the other acids present in the broth. A rapid darkening of the solution was also observed. Moreover, hydrochloric acid could not be removed even after concentration under reduced pressure and the crystallization yields were low.

Better results were obtained by using formic acid, which could also be easily removed by evaporation under reduced pressure while concentrating the eluted acid. These solutions were lightly coloured and pure enough to be used for the crystallization process without any further purification. As shown in Table I, under the conditions used during this study, more than 75% of the total galactonic acid can be eluted with a maximum of 5% (w/w) contamination by formic acid. The fractions that follow the desired acid can be combined and exchanged again on a regenerated column.

Table II shows the different yields of galactonic acid obtained during the fermentation process and from the first crystallization. Further improvements in crystallization are under development.

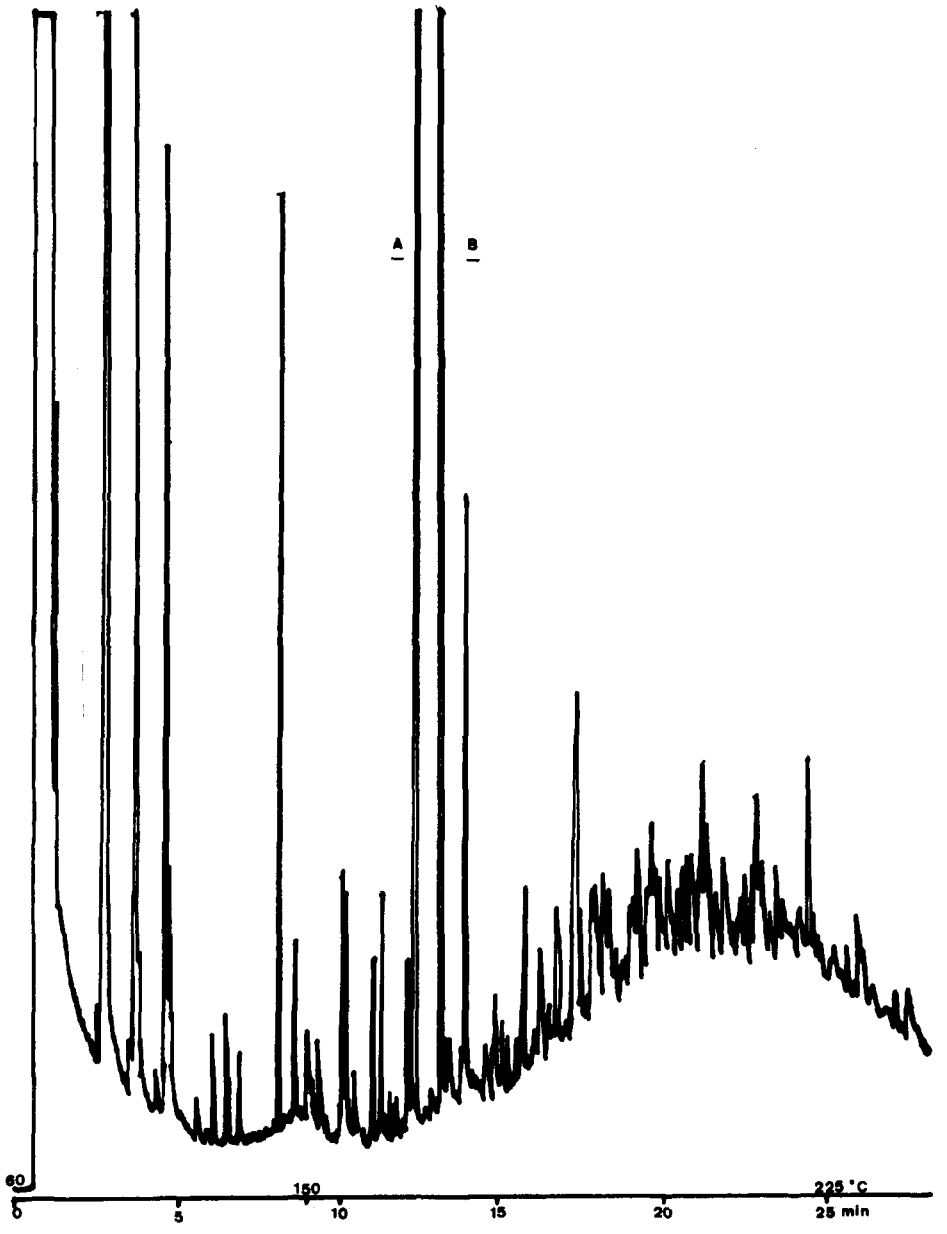

Fig. 3. Capillary GC of the derivatized acids. Peaks: A = gluconic acid; B = galactonic acid.

While galactonic acid is obtained as white crystals, gluconic acid is only concentrated up to a 50% concentrated solution. This solution is comparable to the already commercially available product and it is light yellow, turning to light brown on standing.

In conclusion, the HPLC method with a UV detector set at 214 nm may be used

TABLE I

ELUTION OF GALACTONIC ACID FROM THE ANION-EXCHANGE COLUMN WITH 1 *M* FORMIC ACID

Elution volume (ml)	*Galactonic acid (mg/ml)*	*Formic acid (mg/ml)*
0	0	0
10	0	0
20	0	0
30	0	0
40	0	0
50	5	0
60	26.9	0
70	61.8	1.3
80	60.1	6.7
90	30.5	21.6
100	11.4	32.9
110	4.2	38.3
120	1.3	41.2
130	0.5	45
140	0	45

TABLE II

YIELDS FROM VARIOUS GALACTONIC ACID FERMENTATION

Assay No.	*Yield (%) fermentation*	*Yield (%) purification*	*Total yield after crystallization (%)*
1[a]	90	98	36
2[a]	73	97	32
3[a]	89	99	40
4[b]	57	98	46
5[b]	80	99	69

[a] Hydrochloric acid elution of the galactonic acid produced by fermentation.
[b] Formic acid elution of the galactonic acid produced during fermentation

to monitor the fermentation of the whey hydrolysate, but the simultaneous determination of both gluconic and galactonic acid needs a derivatization of the acidic compounds followed by a gas GC analysis. Highly reproducible yields of both acids were obtained both from the fermentation process and from the purification steps.

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