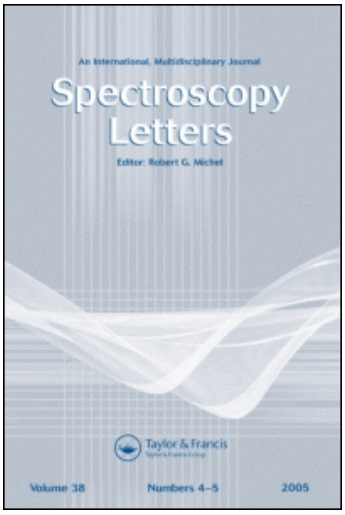

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ABSTRACT The demand for lactic acid as a raw material for the chemical industry is on the rise. Various lactic acid bacteria (LAB) have been tested for the fermentation of sugar-cane juice and molasses into lactic acid. Industrially, the most commonly used producer strains are from the *Lactobacillus* genus. We report here on the fermentation of sugar-cane juice and syrup into L-lactic acid using *Lactobacillus delbrueckii* subsp *delbrueckii* (NCIM 2365). We present a reliable method to quantitatively assess the amount of L-lactic acid produced during fermentation using ^{1}H NMR spectroscopy. This analytical method offers various advantages over existing ones and can be extended to the on-line monitoring of fermentation of sugars into lactic acid in industry.

KEYWORDS ^{1}H NMR spectroscopy, lactic acid, quantitative assessment

INTRODUCTION

The green bio-refinery concept presents itself as an interesting industrial opportunity and long-term sustainability for many countries worldwide, including Mauritius. A bio-refinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass. The bio-refinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum. Instead of refining petroleum to make hydrocarbon derivatives, the technology refines biomass mainly into sugars, fibers, fuel, and energy. The sugars are raw materials that can be used for making many further products.

The sugar industry the world over is presently faced with major challenges on account of the liberalization of the world market and abolition of established sugar protocols, resulting in a 36% cut in the price of sugar. Given this perspective, sugar may no longer be the end product but rather be considered as a raw material for the local manufacture of value-added chemicals and products.

One such niche product is lactic acid, which can be commercialized as such or after conversion into other products. Present global demand of lactic acid as a raw material for the chemical industry is estimated at 90,000 MT p.a. Various studies point to a major boost in demand of lactic acid and

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related products such as polylactic acid (bioplastics) and lactate esters.

Biofermentation Process

Lactic acid is mainly produced via industrial fermentation of sugars from sugar cane and molasses, corn, and so on. Microorganisms that can produce lactic acid can be divided into two groups: bacteria and fungi. However, most investigations have used lactic acid bacteria (LAB) for fermentation. In industry, the most commonly used producer strains come from the genus *Lactobacillus*, of which the most widely used strain for producing lactic acid is *Lactobacillus delbrueckii* NCIB 8130. Alternative producers of lactic acid include strains of the *Bacillus* family.[1–3] Only a few lactic acid bacteria, such as *Lactobacillus brevis, helveticus*, and *delbrueckii* can produce optically pure lactic acid.[4]

Homo-fermentative strains generally lead to higher yields of lactic acid as compared to the hetero-fermentative ones.[5] Indeed, with the latter strains considerable amounts of by-products such as ethanol, acetic acid, and carbon dioxide are also formed. Temperature and pH are reported to be important factors influencing LAB growth and lactic acid production. In general, the desirable characteristics for industrial LAB are their abilities to rapidly and completely convert cheap raw materials into lactic acid with minimal nutritional requirements and to provide high yields of preferred stereoisomer without by-product formation. For instance, molasses is a waste product from the sugar manufacturing process and usually contains a large amount of sucrose. Shukla et al.[6] previously engineered *Escherichia coli* W3110 derivatives, strains SZ63 and SZ85, to produce optically pure D(−) and L(+)-lactate from hexose and pentose sugars.

Timbuntam et al.[7] used a newly isolated *Lactobacillus* sp. strain FCP2 for the production of lactic acid from sugar-cane juice with good yield (96%) and productivity (2.8 $gl^{-1}h^{-1}$). The *Lactobacillus* strain FCP2 can use both disaccharides and monosaccharides in the sugar-cane juice as carbon sources for lactic acid fermentation. Kadam et al.[8] have used a mutant strain of *Lactobacillus delbrueckii* NCIM 2365 for the fermentation of hydrolyzed cane sugar and have obtained 90% lactic acid yield with 150 g/l of cane sugar in batch fermentation and a lactic acid concentration of 135 g/l.

Table 1 summarizes the experimental conditions used and the productivity of lactic acid obtained

TABLE 1 Lactic Acid Production by Fermentation of Glucose and Molasses

LAB	Temp (°C)	pH	Glucose (gl^{-1})	Productivity of lactic acid ($gl^{-1}h^{-1}$)
Glucose, Batch fermentation				
Lactococcus lactis subsp lactis[9]	32	6.0	20	1.00
			60	0.67
		No control	20	0.29
			60	0.21
Sporolactobacillus cellulosolvens[10]	40	6.5	5% (w/v)	0.34
Lactobacillus Mon4+[10]	37	6.5	4% gl^{-1}	30 gl^{-1}
Lactobacillus amylophilus[11]	30	6.0	20	1.56
Lactobacillus casei LA-04-1[12]	42	6.25	140	1.34
Lactobacillus delbrueckii NRRL B445[6]	49	5.8–6.0	150	0.94–1.25
Enterococcus Faecalis[13]	38	7.0	30	5–6
Lactobacillus[14]	—	6.5	—	3.00
Glucose, Continuous fermentation				
Lactobaccilus casei–rhamnosus 1828[15]	39		0.07 (Residual sugar)	38.2
Lactobacillus delbrueckii NRRL B445[6]	42	6.0	50	8.93
Molasses, Batch fermentation				
Sporolactobacillus cellulosolvens (NCIMB 12173)[10]	40	6.5	5	24.2

from fermentation of glucose and molasses using various bacteria. Batch, fed-batch, repeated batch, and continuous fermentations are the most frequently used methods for lactic acid production.

Analytical Determination of Lactic Acid Concentration

The classical analysis of lactic acid is a combined enzymatic-colorimetric method which makes use of an enzymatic test kit.[16,17] The enzyme L(+) - or D(−)-lactate dehydrogenase catalyze the oxidation of L(+)- or D(−)-lactate in the presence of nicotinamide adenine dinucleotide (NAD^+) followed by colorimetric analysis of the lactic acid formed. L-lactic acid has also been analyzed using an amperometric graphite-Teflon biosensor.[18] The correlation between the bienzyme sensor method and classical colorimetric enzymatic method was quite good, of the order of 0.95.

The quantitative analysis and separation of LA optical isomers by liquid chromatography has also been reported.[19]

In this article, we demonstrate the feasibility of the fermentation process of sugar-cane juice from Mauritian sugar factories into L-lactic acid using one specific *Lactobacillus* strain, namely *Lactobacillus delbrueckii* subsp *delbrueckii* NCIM 2365. To our knowledge, we report here for the first time the on-line quantitative determination of the amount of lactic acid produced during biofermentation using ^{1}H NMR spectroscopy as a green analytical chemistry alternative to existing methods.

MATERIALS AND METHODS

Apparatus

A Berthold Hermle AG (Germany), type "Z420" centrifuge with a maximum speed of 10,000 rpm +/ − 5% was used.

A Portaclave size 4 model AAJ 040/6 (ASTELL SCIENTIFIC) with digital temperature control was used.

^{1}H-NMR spectra were recorded in D_2O at room temperature using a Bruker FT Spectrometer at 250 MHz.

Materials and Reagents

Sucrose was purchased from Saarchem. Anhydrous D-(+) Glucose was purchased from Labosi. D-Fructose was obtained from Himedia Laboratories Pvt. Ltd.

Sugar-cane mixed-juice and syrup were obtained from Beau Champ Sugar Estate (Mauritius).

Lactobacillus delbrueckii subsp *delbrueckii* (NCIM 2365) was purchased from National Chemical Laboratories (Pune, India). Yeast extract was from Sigma. MRS Broth Agar (CM0361) and MRS Broth (CM0359) were obtained from Oxoid.

Microorganisms and Culture Conditions

Microorganisms were maintained in liquid MRS medium in screwed cap test tubes at 15°C in a refrigerator and every three weeks the cultures were transferred to fresh liquid MRS medium to maintain live bacteria. These bacteria were used as stock cultures for preparation of inoculum for fermentation.

Sub-Culture Preparation

MRS Broth (5.22 g) was placed in distilled water (100 ml). The mixture was stirred to give a homogeneous solution. Aliquots of solution (30 ml) were transferred to multiple test tubes, which were sterilized at 121°C for 15 min. After sterilization, the solutions were cooled to room temperature and a loopful of the culture medium was transferred to the MRS media near a flame to avoid contamination by atmospheric bacteria.

Inoculum Preparation

Cells from stock cultures (1 ml) were transferred to 100 ml sterile growth medium in 250 ml screw cap conical flask containing carbohydrates (10 g), calcium carbonate (5 g), and yeast extract (1 g). Flasks were incubated at 41°C for 24 hr under stationary conditions. After 24 hr, these cultures (2 ml) were transferred to another sterile growth medium of the same composition. The flask was incubated at 41°C for another 24 hr, under stationary conditions. This culture was used as the inoculum to be transferred to the fermentation medium.

Fermentation of Glucose, Fructose, and Sucrose

Glucose or fructose or sucrose (1 g or 10 g) was measured and transferred to separate conical flasks

to which yeast extract (1 g), calcium carbonate (5 g), and distilled water (100 ml) were added. The solutions were sterilized before the addition of bacteria from the inoculum medium (5 ml). The flasks were screwed and fermentation was carried out at 41°C in a shaking orbital with shaking speed of 150 rpm over a period of 5 days.

The fermentation of sucrose was also studied after hydrolysis. Sucrose (1 g or 10 g) was measured and transferred to a conical flask and distilled water (100 ml) added. Sulfuric acid (20%, 1 ml) was then added. The acidified sugar solution was heated in boiling water bath for 20 min before the addition of yeast extract (1 g) and calcium carbonate (5 g). The medium was sterilized and bacteria added. Fermentation was performed under the same conditions as above.

In the case of sugar-cane juice and syrup, 10% (w/v) sucrose solutions were first prepared. Calcium carbonate (5%) and yeast extract (5%) were added and fermentation allowed to proceed under similar conditions.

Treatment of Fermented Solutions

After fermentation the solutions were centrifuged or filtered to separate excess calcium carbonate and were boiled to stop fermentation. Then the solutions were acidified with sulfuric acid (1 M) to pH 1.6. The precipitate from the solutions was filtered and concentrated before further analysis.

Purification of Fermented Solution

Purification of the fermented solution was performed by extraction with diisopropyl ether (n vols) and re-extraction of the solution with an equal volume of distilled water.

Enumeration of Microorganisms after Fermentation

After fermentation of sugar-cane juice and syrup, a sample of the fermented solution (1.0 ml) was transferred to a clean test tube. Distilled water (9.0 ml) was added and the solution homogenized. This ten-fold dilution was repeated a further six times in separate test tubes. Then, an aliquot from the seven different test tubes (1 ml) was transferred to agar plates. The plates were placed in an oven (41°C) for 2 days to allow bacterial growth. After 2 days, the number of colonies formed was counted. Plates having 30–300 colonies were chosen, as this range is considered statistically significant.

^{1}H NMR Determination of the Amount of Lactic Acid in the Fermented Solutions Using Mandelic Acid as Internal Standard

Model Studies

NMR spectra were recorded for varying concentrations of commercial L-lactic acid solutions in the presence of a fixed amount of mandelic acid. Calibration curve of experimental ratios of lactic acid to mandelic acid as determined by ^{1}H NMR were plotted against the theoretical ratios in the range 0.5 to 2.5. NMR spectra of solutions containing mixtures of lactic acid, mandelic acid, and a fixed concentration of sucrose were analyzed under conditions preliminarily established and the corresponding calibration curve plotted.

NMR analysis was run in the presence of DL-Mandelic acid as an internal standard. A known amount of the latter was added to the solutions after fermentation had been stopped.

Determination of Lactic Acid

A known mass of DL-mandelic acid and a known volume of fermented solution were dissolved in D_2O and mixed in a 5 mm NMR tube. The ^{1}H-NMR spectra of the fermented solutions were used to calculate the amount of lactic acid present in the concentrated solutions.

RESULTS AND DISCUSSION

Preliminary Fermentation Studies on D(+)-glucose, D-fructose, Sucrose, and Hydrolyzed Sucrose

Initial fermentation studies were conducted on model compounds D(+)-glucose, D-fructose, sucrose, and hydrolyzed sucrose using *Lactobacillus delbrueckii* subsp *delbruekii* at two different concentrations, namely 1% and 10% (w/v). Fermentation was carried out in the presence of calcium carbonate to avoid significant drop in pH, which was

TABLE 2 pH Before and After Fermentation

	Concentration/% (w/v)	pH before fermentation	pH after fermentation
Glucose	1	6.65	5.30
	10	6.80	5.80
Fructose	1	6.94	5.91
	10	6.60	5.20
Sucrose	1	6.74	5.40
	10	6.77	5.20
Hydrolysed	1	6.86	6.50
sucrose	10	6.74	5.30

monitored before and after fermentation (Table 2). The presence of yeast extract is important to provide vitamins and trace elements essential for lactic acid biosynthesis. After the required fermentation period, the solutions were acidified with sulfuric acid, filtered, and concentrated before being subjected to ^{1}H NMR analysis.

Fermentation of Sugar-Cane Mixed Juice and Syrup

The sucrose content and brix of juice and syrup in the freshly collected sugar-cane juice and syrup were characterized prior to fermentation (Table 3). The solutions were diluted with distilled water to provide a 10% (w/v) sucrose solution and fermentation allowed to proceed during a period of 5 days using similar conditions as for the model studies.

The sucrose content[20] in sugar-cane juice and syrup was calculated according to Eq. (1).

$$\frac{[2(p-p')100]\text{pol}\ \ \text{factor}}{C+0.0794(m-13)-0.53t} \quad (1)$$

p = direct pol; p′ = invert pol;
p and p′ of the solution were obtained from a saccharimeter;

TABLE 3 Pol, Brix, and Sucrose Content of Sugar-Cane Juice and Syrup

	Juice	Syrup
Brix (°)	16.0	70.0 (undiluted) 14.0 (diluted)
Sucrose (%)	14.6	60.8 (undiluted) 12.15 (diluted)
Pol factor	0.24473	0.24671
P	29.4	24.8
P′	−9.7	−7.5

m = brix of solution obtained directly from a refractometer;
t = 22°C (temperature of the invert solution);
C is obtained using the brix value, C = 142.80.

^{1}H NMR Analysis of Fermentation Medium

The fermented products from glucose, fructose, sucrose, and hydrolyzed sucrose obtained at 1% and 10% (w/v) were characterized by ^{1}H-NMR. The spectrum recorded for a 1% hydrolyzed sucrose solution after fermentation is shown in Fig. 1a. As can be seen, the solutions contain only L-lactic acid, characterized by a doublet centered at 1.3 ppm and a quartet at 4.2 ppm corresponding to the methyl (CH_3^a) and methine (CH^b) protons of lactic acid, respectively. It is to be noted that commercial L-lactic acid (Fig. 1b) also contains oligomers as depicted by a quartet at 5.1 ppm corresponding to -$CH^{b'}$ of the main chain and quartet at 4.3 ppm representing –$CH^{b''}$ of the ultimate unit of the oligomers. Moreover, in the spectra of 10% solutions, signals corresponding to unfermented saccharides are also observed in the range 2.9–3.7 ppm. The percentage ratio of L-lactic acid to unfermented saccharides was determined by comparing proton intensities in the region 2.9–3.7 ppm to those at 1.3 ppm. The results are shown in Table 4.

The fermentation of hydrolyzed sucrose was found to be more efficient than that of unhydrolyzed sucrose (Table 4). Indeed, after hydrolysis a 1:1 mixture of glucose and fructose is obtained, which lends itself more readily to fermentation. The percentage composition of lactic acid is also shown to increase as a function of time (Fig. 2).

Quantitative Assessment of L-Lactic Acid Produced from Sugar Syrups by ^{1}H NMR

Model Studies

As described in the previous section, the ratio of L-lactic acid to unfermented sugars can be estimated by ^{1}H NMR. However, the simple comparison of proton intensities does not enable a quantitative determination of the amount of L-lactic acid formed since the signals due to the unfermented sugars

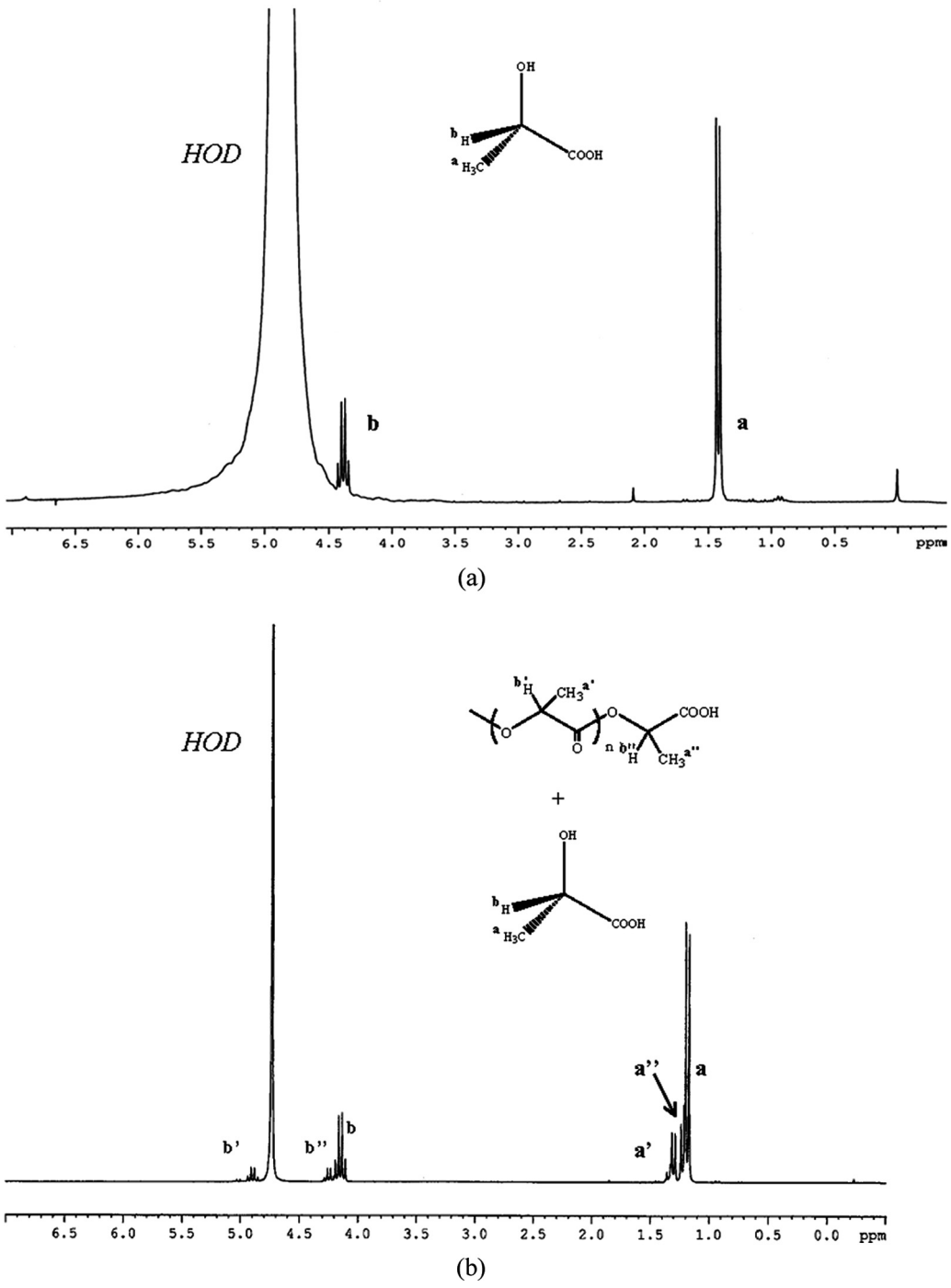

FIGURE 1 ^{1}H NMR (D_2O) spectra of fermented (a) 1% hydrolyzed sucrose; (b) commercial L-lactic acid.

probably correspond to a mixture of sucrose, glucose, and fructose. To overcome this difficulty, the NMR analysis was run in the presence of an internal standard, namely DL-mandelic acid. The latter was added to the solutions after fermentation had been stopped. This acid was chosen for several reasons: (1) it is a solid available at low cost and can be handled with accuracy; (2) it is readily soluble in water; and (3) the aromatic protons of the acid are centered at 7.45 ppm and the methine proton resonate at 5.29 ppm and therefore do not overlap with the protons of either lactic acid or unfermented sugars.

In the first instance, NMR spectra were recorded for varying concentrations of commercial L-lactic acid solutions in the presence of a fixed amount of

TABLE 4 **Molar Percentage Compositions of Lactic Acid and Unfermented Saccharides as Determined by ^{1}H NMR in 10% Fermented Solutions**

Saccharides	Lactic acid (%)	Unfermented saccharides (%)
Glucose	77.8	22.2
Fructose	77.3	22.7
Sucrose	58.9	41.1
Hydrolyzed sucrose	78.4	21.6

mandelic acid. By comparing the intensity of aromatic protons of mandelic acid to that of aliphatic protons (CH_3) of lactic acid, the ratio of lactic acid to mandelic acid was determined. The relaxation delay (RD) used for each NMR pulse was found to be determinant in the correlation between experimental and theoretical ratios. Indeed, at low RD values (2 s), there result significant errors (average 60%) between experimental and theoretical ratios. The best results were obtained at a relaxation delay of 10 s and this value has been used throughout the study. Calibration curves of experimental ratios of lactic acid to mandelic acid as determined by ^{1}H NMR were plotted against the theoretical ratios in the range 0.5 to 2.5 (Fig. 3). As can be seen, a very good correlation is obtained under our conditions presenting a typical equation $\mathbf{y} = 0.9978\mathbf{x}$ ($R^2 = 0.9901$) where **y** corresponds to the experimental and **x** to the theoretical molar ratio of lactic acid to mandelic acid.

Next, it was important to verify whether the presence of sucrose would or would not affect the accuracy of the determination of lactic acid. NMR spectra of solutions containing mixtures of lactic acid, mandelic acid, and a fixed concentration of sucrose were analyzed under conditions preliminarily established and the corresponding calibration curve plotted (Fig. 4). Again the accuracy of the determination was found to be excellent for ratios of lactic acid to mandelic acid between 0 and 2 as noted previously but deviations occur as the concentration of lactic acid compared to mandelic acid increases. To conclude, the presence of sugar does not perturb the accuracy of the determination and it is important to ensure that the amount of mandelic acid used as internal standard is within acceptable ratios.

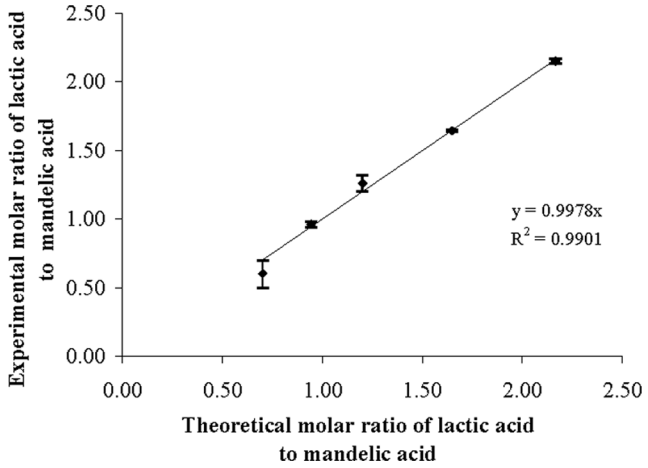

FIGURE 3 **Graph of experimental ratio against theoretical ratio of L-lactic acid to DL-mandelic acid.**

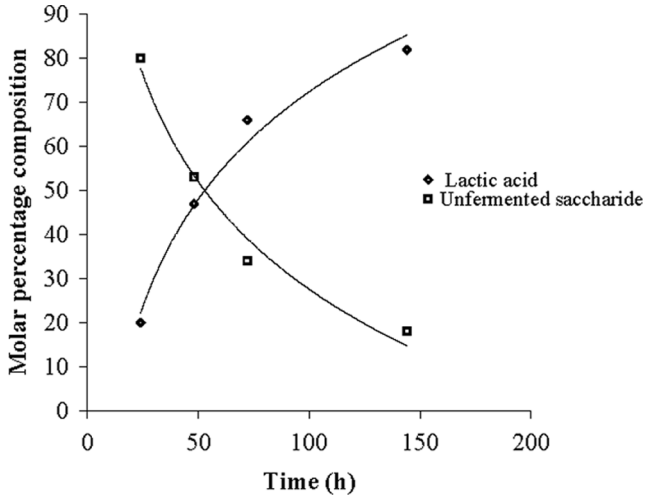

FIGURE 2 **Molar percentage composition of lactic acid and unfermented saccharides after 24 hr, 48 hr, 72 hr, 96 hr, and 144 hr as determined by ^{1}H NMR.**

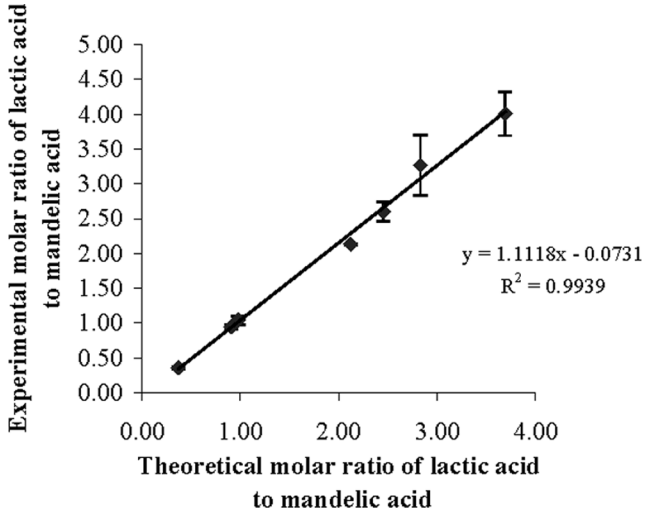

FIGURE 4 **Calibration plot for mixture of lactic acid, mandelic acid, and sucrose.**

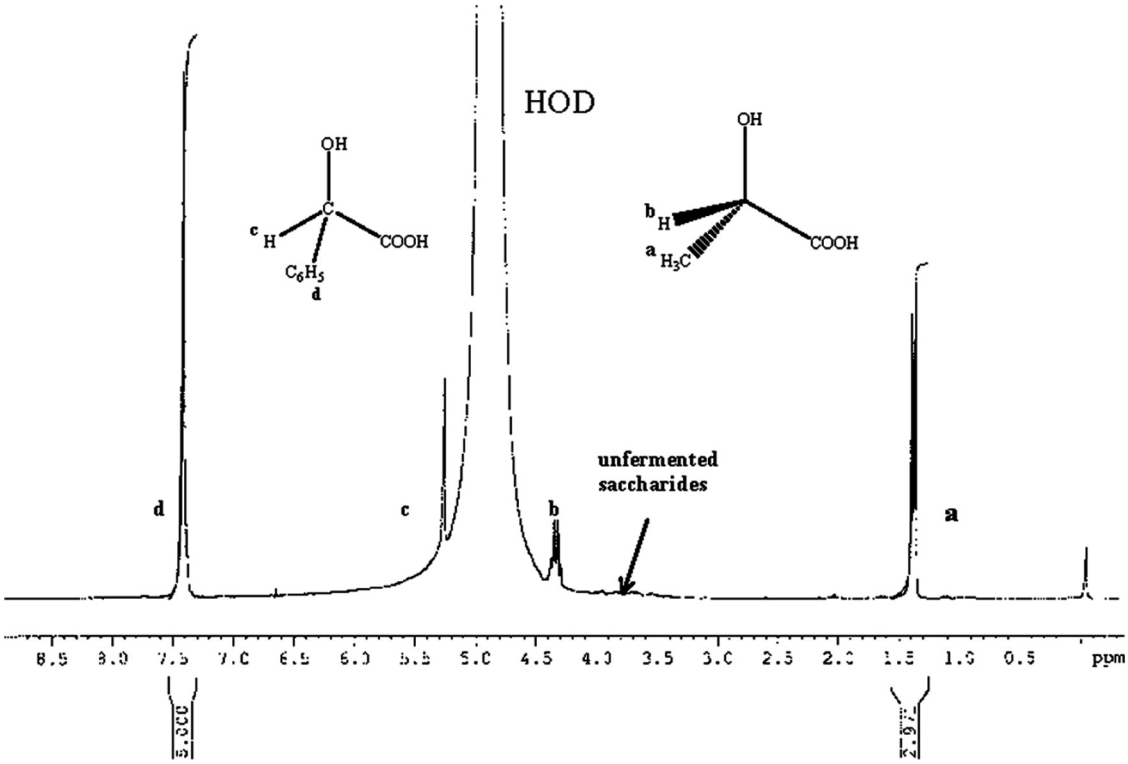

FIGURE 5 ^{1}H-NMR (D_2O) spectrum of fermented juice with D,L-mandelic acid as internal standard.

Application to Fermented Solutions of Sugar-Cane Mixed Juice and Syrup

A typical ^{1}H NMR spectrum of a fermented sugar-cane juice solution to which mandelic acid has been added is depicted in Fig. 5. As can be seen, signals characteristic of L-lactic acid and residual sugars (3.1–4.1 ppm) are present. The amount of lactic acid formed is calculated according to Eq. (2) and corresponds to a value of 25 g/l (productivity = 0.21 $gl^{-1}h^{-1}$).

$$m_{LA} = \left[\left(\frac{{}^{a}I_{CH_3}}{{}^{d}I_{C_6H_5}} \times 1.67\right)\left(\frac{m_{MA}}{152}\right)90\right]\left[\frac{1000}{V_{soln}}\right] \qquad (2)$$

${}^{a}I_{CH_3}$ = intensity of methyl protons of lactic acid
${}^{d}I_{C_6H_5}$ = intensity of phenyl protons of mandelic acid
m_{MA} = mass of mandelic acid
V_{soln} = volume of solution analysed

NMR indeed proves to be quite an interesting technique because it gives quick results with a high level of accuracy under predetermined conditions. In addition, the small volumes of solution used for analysis does not perturb the overall concentration of the fermentation medium and this analysis can be extended to on-line monitoring.

Pure lactic acid was obtained upon further extraction with diisopropyl ether and purification with water and verified by ^{1}H NMR. Negative optical rotatory values confirmed also that the pure L-enantiomer was obtained.

CONCLUSIONS

We have shown that the biofermentation of sugar-cane mixed juice and syrup proceeds with reasonable yields. Direct analysis of fermentation solutions by ^{1}H NMR enables the ratio of L-lactic acid to unfermented sugar to be determined. To assess the amount of L-lactic acid formed quantitatively, NMR was run in the presence of DL-mandelic acid as an internal standard. NMR parameters such as the relaxation delay as well as the ratio of lactic acid to mandelic acid were found to be key factors for accurate determination of lactic acid content. We thus demonstrated that ^{1}H NMR spectroscopy can be used as a fast, easy, reliable, and valuable technique to monitor the on-line fermentation of sugar to lactic acid as compared to existing methods.

The NMR technique does not require sample pretreatment and thus reduces considerably manipulation time. It is also considered environmentally friendly as it enables the use of minimum amount of solvents with practically no waste generation compared, for instance, to chromatographic methods. Extensions of this technique to the quantitative assessment of other value-added chemicals such as butanol resulting from biofermentation processes of sugar-cane solutions are currently underway. In this new paradigm of environmental concern and energy minimization, the biofermentation process coupled with green analytical spectroscopy technique could prove highly beneficial to the promotion of the bio-refinery concept and to sustainable development more globally.

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REFERENCES

1. Kirkovits, A. E.; Edlauer, H. U.S. Patent **1998**, 5,079,164.
2. Payot, T.; Chemaly, Z.; Fick, M. Lactic acid production by *Bacillus Coagulans*—kinetic studies and optimization of culture medium for batch and continuous fermentations. *Enzyme Microb. Technol.* **1999**, *24*(3), 191–199.
3. Ohara, H.; Yahata, M. L-lactic acid production by *Bacillus* sp. in anaerobic and aerobic culture. *J. Fermentation and Bioeng.* **1996**, *81*(3), 272–274.
4. Benthin, S.; Villadsen, J. Production of optically pure D-lactate by *Lactobacillus bulgaricus* and purification by crystallisation and liquid/liquid extraction. *Appl. Microbiol. Biotechnol.* **1995**, *42*, 826–829.
5. Litchfield, J. H. Microbiological Production of lactic acid. *Advances in Appl. Microbiol.* **1996**, *42*, 45–95 and references therein.
6. Shukla, V. B.; Zhou, S.; Yomano, L. P.; Shanmugam, K. T.; Preston, G. F.; Ingram, L. O. Production of D(−)-lactate from sucrose and molasses. *Biotechnol. Lett.* **2004**, *26*(9), 689–693.
7. Timbuntam, W.; Sriroth, K.; Tokiwa, Y. Lactic acid production from sugar-cane juice by a newly isolated *Lactobacillus* sp. *Biotechnol. Lett.* **2006**, *28*(11), 811–814.
8. Kadam, S. R.; Patil, S. S.; Bastawde, K. B.; Khire, J. M.; Gokhale, D. V. Strain improvement of Lactobacillus delbrueckii NCIM 2365 for lactic acid production. *Process Biochemistry* **2006**, *41*(1), 120–126.
9. Cock, L. S.; Rodriguez de Stouvenel, A. *Electronic J. Biotechnol.* [on-line] 15 January **2006**, *9*(1).
10. Kanwar, S. S.; Tewari, H. K.; Chadha, B. S.; Punj, V.; Sharma, V. K. Lactic acid production from molasses by *Sporolactobacillus cellulosolvens*. *Acta Microbiol. Immunol. Hung.* **1995**, *42*(4), 331–338.
11. Kious, J. J. *Lactobacillus and Lactic Acid Production—Report*. August **2000**.
12. Ding, S.; Tan, T. L-lactic acid production by *Lactobacillus casei* fermentation using different fed batch-feeding strategies. *Process Biochem.* **2006**, *41*, 1451–1454.
13. Yun, J. S.; Wee, Y. J.; Ryu, H. W. Production of optically pure L (+)-lactic acid from various carbohydrates by batch fermentation of *Enteroccocus faecalis* RKYI. *Enzyme and Microbial. Technol.* **2003**, *33*, 416–423.
14. *Annual Research and Development Report*, NCL-India, 2003–2004.
15. Rychtera, M.; Melzoch, K.; Habova, V.; Vedlichova, Z.; Patakova, P.; Hamamci, H.; Machek, F. Optimization of Microbial Lactic Acid Production in Integrated Process. *EUROSUSTAIN Proceedings (CD ROM)*, 2002, *8*, 1.
16. Gawehn, K.; Bergmeyer, H. U. *D(−)-Lactate*. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; vol 3.
17. Gutmann, I.; Wahlefeld, A. W. L(+)-Lactate, determination with lactate dehydrogenase and NAD. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; vol 3.
18. Herrero, A. M.; Requena, T.; Reviejo, A. J.; Pingarrón, J. M. Determination of L-lactic acid in yoghurt by a bienzyme amperometric graphite-Teflon composite biosensor. *Eur. Food Res. Technol.* **2004**, *219*(5), 557–560.
19. Narayanan, N.; Roychoudhury, P. K.; Srivastava, A. L(+)-lactic acid fermentation and its product polymerization. *Electronic J. Biotechnol.* [on-line] 15 April 2004, *7*(2), 1–19.
20. George, P. M. *Cane Sugar Handbook: A Manual for Canesugar Manufacturers and Their Chemists*; John Wiley & Sons Inc.: New York, London, Sydney, 1963.