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Analysis of substrates and metabolites in fermentation broth by ion chromatography

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Abstract

Reliable methods to quantitate microbial fermentation nutrients and metabolites are essential for improved process monitoring and control. Analysis of culture media components by ion chromatography was completed for several recombinant and pathogenic microorganisms. Specific applications include monitoring of carbohydrates, alcohols, and inorganic cations. Isocratic methods were used with pulsed amperometric detection for carbohydrates and alcohols, and conductivity detection for inorganic cations. Sample preparation was a simple dilution of filtered broth with water. All isocratic analysis times were 20 min. An additional gradient method was utilized for certain carbohydrate mixtures. These analyses accurately monitor over fifty nutrients and metabolites present in fermentation media.

1. Introduction

Reliable methods to quantitate common microbial nutrients and metabolites are essential for improved fermentation process monitoring and control. Measurement of carbohydrates, alcohols, and inorganic cations are very important when investigating and understanding microbial physiology and for many aspects of fermentation process development. Improvements in process performance may be achieved by supplementation with organic [1] or inorganic [2] nutrients, by developing defined media to replace complex media formulations [3], and by defining fermentation endpoints. Process consistency can also be documented. Methods reported for carbohydrate monitoring include enzymatic [4], colorimetric [5], HPLC–refractive index detection [6,7], and HPLC–UV [8] assays. Alcohols have been measured using enzymatic [9], near-infrared spectroscopy [10], and gas chromatography [4] assays. Atomic absorption [4] and colorimetric [11] assays have been used for inorganic cation monitoring.

This paper describes four ion chromatography methods which quantitate nutrients and metabolites present in common fermentation media including carbohydrates (galactose, glucose, ribose, fructose), sugar alcohols (glycerol, inositol, mannitol, sorbitol), ethanol, and inorganic cations (calcium, magnesium, ammonium, potassium, sodium). Sample preparation for all analyses is a simple dilution of filtered broth with water before injection. All isocratic assays have analysis times of 20 min or less. These methods have been successfully used to analyze chemical-

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ly defined and complex culture media for various recombinant and pathogenic microorganisms fermentations.

2. Experimental

2.1. Chemicals

Sodium hydroxide (50% w/w) and hydrochloric acid (11.6 *M*) were obtained from Fisher Scientific (Malvern, PA, USA). Glacial acetic acid (HPLC grade, CH_3COOH), and perchloric acid (70%, Ultrex ultrapure reagent, $HClO_4$) were obtained from Baker (Phillipsburg, NJ, USA). Tetrabutylammonium hydroxide (55% aqueous solution, TBAOH), was obtained from Sachem (Austin, TX, USA). D,L-2,3-Diaminopropionic acid (DAP) was obtained from Sigma (St. Louis, MO, USA). All stock carbohydrate and alcohol standards were prepared from analytical reagent grade material obtained from either Pfanstiehl (Waukegan, IL, USA) or Fluka (Ronkonkoma, NY, USA). All inorganic cation standards were prepared from chloride salts (purity >97%) obtained from Sigma. All solutions were prepared with double distilled, chemically purified water (Millipore, Bedford, MA, USA).

2.2. Carbohydrate chromatographic system and eluents

Carbohydrate analysis was performed using a Dionex Ion Chromatography (Sunnyvale, CA, USA) system which consisted of a gradient pump, pulsed amperometric detector with gold electrode, autosampler, and a data handling system. The separation was accomplished using a CarboPac PA1 analytical column (Dionex, 250 × 4 mm, P/N 35391) and a CarboPac PA1 guard column (Dionex, 50 × 4 mm, P/N 43096) with a 50-μl filled loop injection.

The isocratic carbohydrate analysis used 150 m*M* NaOH mobile phase at a flow-rate of 1 ml/min. Settings for pulsed amperometric detection (PAD) were potentials E1 = 0.05, E2 = 0.65, E3 = −0.95, times T1 = 2, T2 = 2, T3 = 5 and range = 2. Total run time was 20 min.

The gradient carbohydrate analysis used water (A) and 50 m*M* NaOH–3 m*M* CH_3COOH (B) for mobile phases with an eluent flow-rate of 1 ml/min. The mobile phase composition was held constant for 13.8 min at a composition of 94%A–6%B, was varied linearly to 0%A–100%B over the next 11.2 min, and then returned to the original composition by the end of the run (40 min). The settings for the PAD detector were E1 = 0.05, E2 = 0.60, E3 = −0.65, T1 = 5, T2 = 2, T3 = 1 and range = 2.

2.3. Alcohol chromatographic system and eluents

Isocratic alcohol analysis was performed using a Dionex DX-100 chromatography system along with a PAD apparatus (platinum electrode), autosampler, and a data handling system. The separation was performed using an IonPac ICE-AS1 analytical column (Dionex, 250 × 4 mm, P/N 35330) with a 100 m*M* $HClO_4$ mobile phase, a flow-rate of 0.8 ml/min, and a 750-μl reaction coil. The PAD settings were: E1 = 0.2, E2 = 1.25, E3 = −0.1, T1 = 4, T2 = 1, T3 = 4, and range = 1. A 10-μl sample was injected using a filled loop injection. The total run time was 20 min.

2.4. Inorganic cation chromatographic system and eluents

Isocratic inorganic cation analysis was performed using a Dionex DX-100 chromatography system, conductivity detector, autosampler, and data handling system. The separation was achieved using an IonPac CS10 analytical column (Dionex, 250 × 4 mm, P/N 43118) and an IonPac CG10 guard column (Dionex, 50 × 4 mm, P/N 43119) with a 20 m*M* HCl–4 m*M* DAP mobile phase at a flow-rate of 1 ml/min. The regenerant system used a Dionex AutoRegen cation cartridge with 0.1 *M* TBAOH. A range setting of 30 was used with the conductivity detector. The injection method was a 10-μl filled loop. Total run time for the analysis was 20 min.

2.5. Data system

A Dionex Advanced Computer Interface (ACI), Model III was used to transfer data to an AST Premium 486/33TE computer. Data reduction and processing was accomplished using Dionex AI-450 software, version 3.3.

2.6. Preparation of standards and samples

Stock carbohydrate and alcohol standards were prepared at a concentration of 10 mg/ml in water. Stock inorganic cation standards were prepared at a concentration of 1 mg/ml for NaCl and KCl and 2 mg/ml for NH_4Cl, $MgCl_2$, and $CaCl_2$ in water. All standards were stored in 1.8 ml aliquots at −70°C in 2-ml Wheaton vials (Wheaton, Millville, NJ, USA) with screw caps. Standards were not sensitive to these storage conditions (data not shown). Dilutions of stock standards were made daily to prepare 10, 25, 50, 100, 250 and 500 μg/ml carbohydrate standards. Alcohol stock standards were diluted to prepare 10, 25, 50, 100, and 250 μg/ml. Inorganic cation stock standard were diluted daily to prepare either 0.5, 1.25, 2.5, 5, 12.5 and 25 μg/ml standards for NaCl and KCl or 1, 2.5, 5, 10, 25, and 50 μg/ml standards for NH_4Cl, $MgCl_2$, and $CaCl_2$. Diluted stock standards were used to generate standard curves.

Fermentation samples were prepared by making dilutions of filtered fermentation broth in water. A dilution of at least 1:25 was used for all samples.

3. Results and discussion

The isocratic carbohydrate analysis allowed quantitation of important microbial substrates and metabolites including pentoses (ribose, arabinose), hexoses (galactose, glucose, and fructose), disaccharides (lactose, sucrose, maltose), and sugar alcohols (mannitol and sorbitol). Separation of these compounds can be achieved in 20 min with the general elution order being alcohols, pentoses, hexoses and then disaccharides. The order of elution of monosaccharides largely corresponds to their bulk pK_a values [12]. Ethanol and glycerol can be detected with the carbohydrate method, but since their retention times are very similar, accurate quantitation may not be possible in cases where both components are present as metabolites. When monosaccharides are the primary carbon source, the run time may be shortened when chemically defined media are used which do not contain the later eluting disaccharides. When di- or trisaccharides are used as the primary carbon sources, higher molarity mobile phase may be used to shorten run times.

Example chromatograms depicting standards analyzed using isocratic and gradient methods have been described previously [13]. Typical standard curves for a pentose, hexose, disaccharide and sugar alcohol can be found in Fig. 1. Standard curve ranges are typically 10–500 μg/ml for carbohydrates and 10–250 μg/ml for alcohols. The large dynamic range of the standard curves minimizes the number of repeats due to inappropriate dilutions. Because of the large dynamic range, a cubic fit is used to better describe the standard curve.

Table 1 shows intraday and interday validation data for galactose, a common fermentation nutrient. Similar data (not shown) were obtained for five other carbohydrates. All validation data

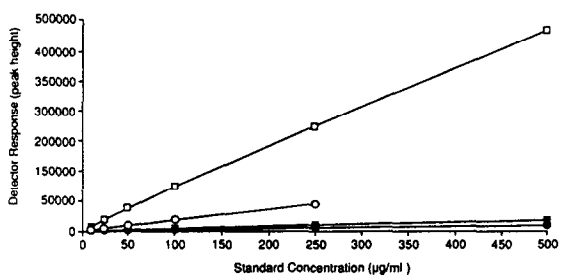

Fig. 1. Typical calibration curves for ribose, galactose, lactose, and glycerol generated using the isocratic carbohydrate analysis. Standard curve range 10 to 500 μg/ml. Ribose (■): amount = $1.40 \cdot 10^{-15}y^3 + 2.42 \cdot 10^{-10}y^2 + 1.03 \cdot 10^{-3}y + 4.87$, $R^2 = 0.999988$; galactose (□): amount = $-2.77 \cdot 10^{-19}y^3 + 3.76 \cdot 10^{-12}y^2 + 6.22 \cdot 10^{-5}y + 0.32$, $R^2 = 0.999999$; lactose (●): amount = $6.31 \cdot 10^{-15}y^3 - 6.11 \cdot 10^{-10}y^2 + 2.18 \cdot 10^{-3}y + 0.004$, $R^2 = 0.999989$; glycerol (○): amount = $4.20 \cdot 10^{-17}y^3 - 9.93 \cdot 10^{-12}y^2 + 2.53 \cdot 10^{-4}y + 0.25$, $R^2 = 0.999999$.

Table 1
Intraday and interday validation data for the isocratic carbohydrate analysis

Standard concentration (μg/ml)	Validation type	n	Galactose R.S.D. (%)
10	Intraday	6	1.8
25	Intraday	6	0.8
50	Intraday	6	1.0
100	Intraday	6	0.4
250	Intraday	6	1.1
500	Intraday	6	0.2
10	Interday	67	5.8
25	Interday	67	2.1
50	Interday	67	1.8
100	Interday	67	0.9
250	Interday	67	0.2
500	Interday	67	0.05

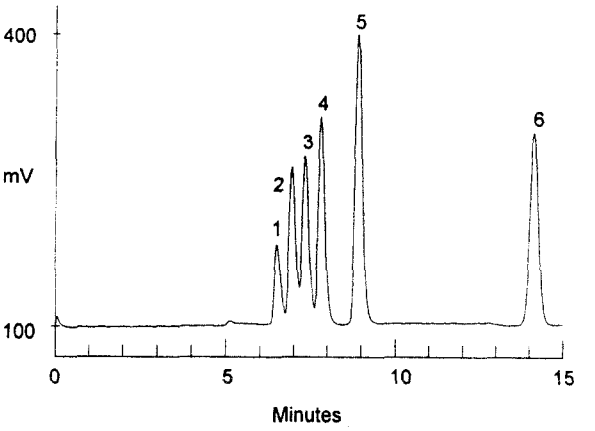

Fig. 2. Chromatogram of a mixture of alcohol standards (100 μg/ml) separated using an isocratic method. Injection volume 10 μl. Peaks: 1 = inositol, 2 = arabinitol, 3 = erythritol, 4 = galactitol, 5 = glycerol, and 6 = ethanol.

were generated using standards spiked into water. Data for spike recovery into fermentation medium is reported elsewhere [13]. The interday validation occurred over a two and one half year period and was generated using six different chromatography columns. Several thousand injections of standards and fermentation samples were run during this time without compromising assay accuracy, indicating that this is a rugged and reliable assay.

An ion-exclusion method was implemented to accurately quantitate glycerol and ethanol. This method was also isocratic with an analysis time of 20 min. The general order of elution correlated with molecular mass; retention of low-molecular-mass alcohols (*e.g.* ethanol) is based mainly on inclusion whereas higher-molecular-mass alcohols are retained by adsorptive interactions with the resin [14]. A chromatogram depicting elution of five alcohol standards is shown in Fig. 2. Fig. 3 shows typical standard curves for glycerol and ethanol over a range of 10 to 250 μg/ml. As with the carbohydrate analysis, this large dynamic range minimizes sample repeats.

Intraday and interday validation data for ethanol and glycerol analyses are shown in Table 2. The interday validation occurred over a ten-month period and was generated using one chromatography column. Over 2200 injections of standards and fermentation samples were run during this time with retention time changes of <1% for glycerol and <2% for ethanol.

Five common inorganic cations important in certain microbial fermentations were analyzed using a cation-exchange method. The isocratic cation analysis allowed quantitation of monovalent (sodium, ammonium, and potassium) and divalent (magnesium and calcium) inorganic cations. Separation of these compounds was performed in 20 min with monovalent cations eluting first. Retention time increased as the size of

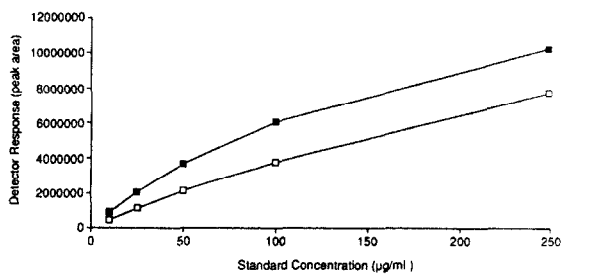

Fig. 3. Typical calibration curves for ethanol and glycerol generated using the isocratic alcohol analysis. Standard curve range 10 to 250 μg/ml. Glycerol (●): amount = $9.54 \cdot 10^{-20}y^3 + 2.72 \cdot 10^{-13}y^2 + 1.14 \cdot 10^{-5}y - 0.49$, $R^2 = 1.000000$; ethanol (□): amount = $-4.89 \cdot 10^{-20}y^3 + 2.10 \cdot 10^{-12}y^2 + 1.88 \cdot 10^{-5}y + 0.93$, $R^2 = 0.999995$.

Table 2
Intraday and interday validation data for the isocratic alcohol analysis

Standard concentration (μg/ml)	Validation type	n	R.S.D. (%)	
			Ethanol	Glycerol
10	Intraday	6	3.1	5.3
25	Intraday	6	4.0	5.2
50	Intraday	6	2.4	2.6
100	Intraday	6	6.1	1.9
250	Intraday	6	0.7	0.6
10	Interday	40	2.6	3.3
25	Interday	40	2.4	2.5
50	Interday	40	0.8	1.1
100	Interday	40	1.1	0.2
250	Interday	40	0.1	0.1

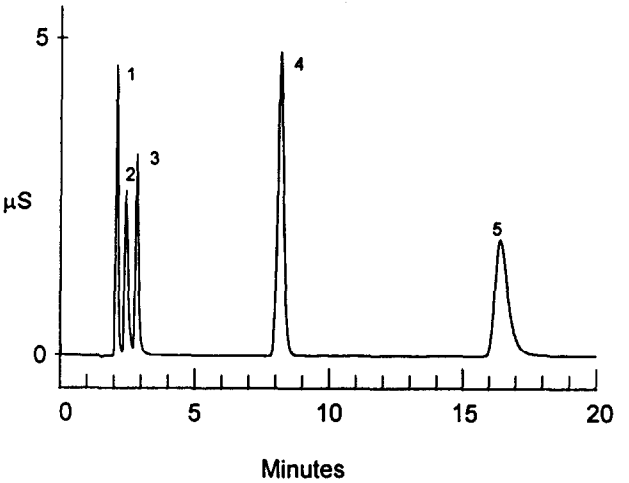

Fig. 4. Chromatogram of a mixture of inorganic cation standards (5 μg/ml or 10 μg/ml concentration for sodium and potassium or ammonium, magnesium and calcium, respectively) separated using an isocratic method. Injection volume 10 μl. Peaks: 1 = sodium, 2 = ammonium, 3 = potassium, 4 = magnesium, and 5 = calcium.

the ion in the hydrated state increased [15]. An example chromatogram showing the elution of these five inorganic cation standards is found in Fig. 4 and typical standard curves are found in Fig. 5. Again, a large dynamic range for analysis was used (0.5 to 25 μg/ml for sodium and potassium or 1 to 50 μg/ml for ammonium, magnesium and calcium).

Intraday and interday validation data for cation analysis (Table 3) shows R.S.D.(%) ≤ 5% for all components. The interday validation occurred over seventeen months and was generated using two different chromatography col-

Table 3
Intraday and interday validation data for the isocratic cation analysis

Standard concentration (μg/ml)	Validation type	n	R.S.D. (%)				
			Ammonium	Magnesium	Calcium	Sodium	Potassium
0.5	Intraday	6				0.02	5.0
1	Intraday	6	3.0	0.6	2.3	4.2	1.8
2.5	Intraday	6	2.5	0.7	1.3	2.0	2.1
5	Intraday	6	4.4	1.3	1.2	0.8	1.8
10	Intraday	6	2.0	0.4	0.5		
12.5	Intraday	6				2.8	1.2
25	Intraday	6	5.0	1.1	0.8	3.3	0.9
50	Intraday	6	3.2	1.3	1.3		
0.5	Interday	23				6	5.1
1	Interday	23	7.4	4.7	3.9	2.9	2.2
2.5	interday	23	3.2	2.3	4	2.6	2.2
5	Interday	23	2.9	1.3	1.6	0.7	0.6
10	Interday	23	1.6	0.4	0.5		
12.5	Interday	23				0.4	0.06
25	Interday	23	0.5	0.2	0.13	0.4	0.05
50	Interday	23	0.6	0.07	0.14		

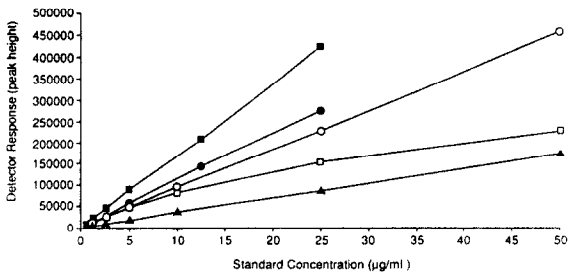

Fig. 5. Typical calibration curves for sodium chloride, ammonium chloride, potassium chloride, magnesium chloride, and calcium chloride using the isocratic cation analysis. Standard curve range 0.5 to 25 μg/ml for sodium and potassium and 1 to 50 μg/ml for ammonium, magnesium and calcium. Sodium (■): amount = $-6.03 \cdot 10^{-17}y^3 + 3.38 \cdot 10^{-11}y^2 + 5.54 \cdot 10^{-5}y - 0.01$, $R^2 = 0.999995$; ammonium (□): amount = $8.53 \cdot 10^{-16}y^3 + 3.58 \cdot 10^{-10}y^2 + 8.90 \cdot 10^{-5}y - 0.11$, $R^2 = 0.999998$; potassium (●): amount = $1.02 \cdot 10^{-16}y^3 - 1.75 \cdot 10^{-11}y^2 + 8.68 \cdot 10^{-5}y + 0.14$, $R^2 = 0.999973$; magnesium (○): amount = $-5.63 \cdot 10^{-17}y^3 + 3.78 \cdot 10^{-11}y^2 + 1.04 \cdot 10^{-4}y - 0.12$, $R^2 = 0.999992$; calcium (▲): amount = $-1.06 \cdot 10^{-15}y^3 + 2.37 \cdot 10^{-10}y^2 + 2.74 \cdot 10^{-4}y - 0.18$, $R^2 = 0.999972$.

umns. Sample analysis was routinely performed using dilutions of 1:1000 or 1:2000 to lower analyte concentrations to within the standard curve range. Sample matrix effects were negligible because of the large dilutions used for analyses. Over 2000 injections of standards and fermentation samples were analyzed during this time.

The isocratic carbohydrate analysis was used to generate a time course profile for a *Haemophilus influenzae* fermentation in a complex medium containing glucose (Fig. 6). Preferential utilization of some minor carbohydrates supplied by complex medium components, which are not easily measured by other methods, can be monitored using this analysis [13]. This information may be helpful when trying to elucidate biochemical pathways, improving process performance by nutritional supplementation, or formulating a chemically defined medium.

In instances where both glucose and galactose are used as carbon sources by the microorganism, a gradient carbohydrate method was required to quantitate the individual hexoses because of their similar retention times in the

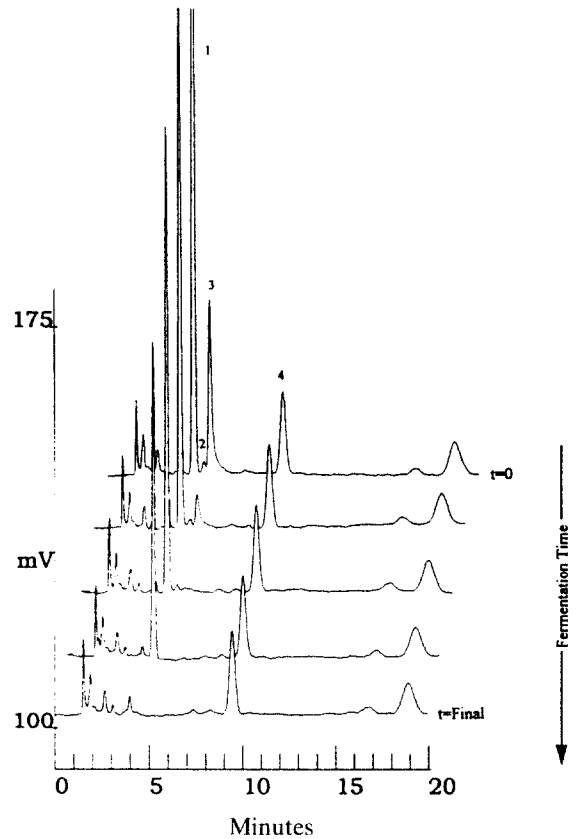

Fig. 6. A time course of representative chromatograms show isocratic carbohydrate analyses of fermentation broth samples for *Haemophilus influenzae* grown in complex media [16]. Peaks: 1 = glucose, 2 = fructose, 3 = ribose, 4 = sucrose.

isocratic analysis [13]. Fig. 7 shows a time course from a *Saccharomyces cerevisiae* fermentation where both glucose and galactose were present at the beginning of the fermentation. These chromatograms show the utilization of galactose after the depletion of glucose which is consistent with the known repression of galactose metabolism by glucose in yeast.

Another important component of investigating and understanding certain physiological events is the generation and subsequent utilization of alcohols, especially glycerol and ethanol. A time course showing glycerol and ethanol levels in a *S. cerevisiae* fermentation is found in Fig. 8. The glycerol peak can be seen to increase throughout

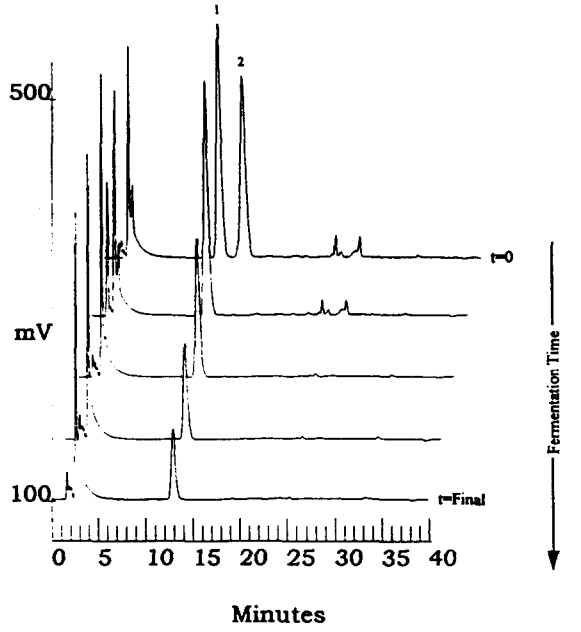

Fig. 7. A time course of representative chromatograms show gradient carbohydrate analyses of fermentation broth samples for *Saccharomyces cerevisiae* grown in complex media containing yeast extract and soy peptone. Peaks: 1 = galactose, 2 = glucose.

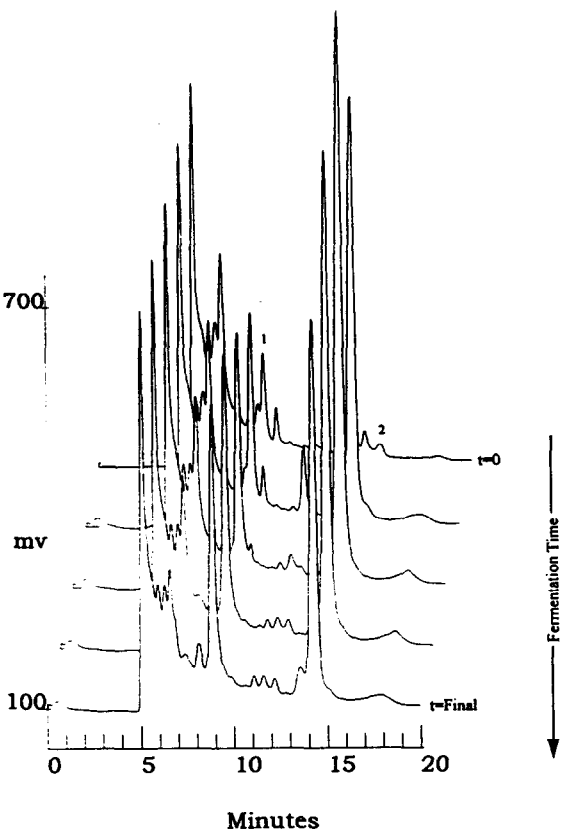

Fig. 8. A time course of representative chromatograms show isocratic alcohol analyses of fermentation broth samples for *Saccharomyces cerevisiae* grown in complex media containing yeast extract and soy peptone. Peaks: 1 = glycerol, 2 = ethanol.

the fermentation while the ethanol peak is absent in the $t = 0$ sample, increases in concentration, and then decreases. This information is useful for documenting process consistency since aerobic utilization of ethanol is dependent upon adequate aeration and other nutritional influences. Other alcohols can also be identified using this method and data could be obtained for these alcohols if their levels changed significantly over the time course of a fermentation.

While often overlooked, inorganic cations are essential for microbial growth and fulfill specific metabolic and structural roles. For example, potassium is used for active transport by yeast and ammonia can be generated or consumed in many microbial processes. Fig. 9 shows a series of chromatograms from a time course of fermentation where five inorganic cation levels were measured. The time course shows that some cation concentrations change dramatically; potassium becomes undetectable and then reappears at the end of the fermentation, while others, such as magnesium, retain a constant concentration throughout the fermentation. This information can be used to design improved medium formulations.

The four ion chromatography methods described here are very versatile for monitoring many common microbial fermentation substrates and metabolites in complex or chemically defined media for various recombinant and pathogenic microorganisms. The methods described are reliable, rugged, and demonstrate very good intraday, interday, and inter-column reproducibility. Because of the ease of sample preparation and the short analyses times, these

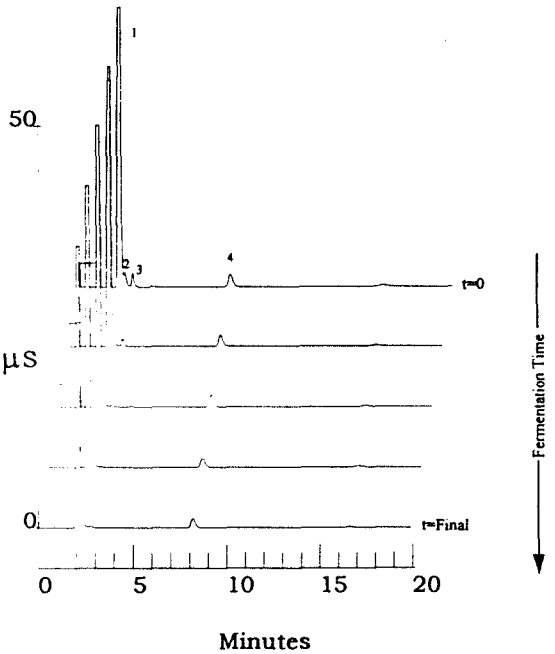

Fig. 9. A time course of representative chromatograms show isocratic cation analyses of fermentation broth samples for *Saccharomyces cerevisiae* grown in chemically defined media [17]. Peaks: 1 = sodium, 2 = ammonium, 3 = potassium, 4 = magnesium.

methods are potentially useful for on-line monitoring or process control.

4. Acknowledgements

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5. References

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