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Effect of Nutrient Supplements Addition on Ethanol Production from Cheese Whey Using *Candida pseudotropicalis* Under Batch Condition

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ABSTRACT

Candida pseudotropicalis ATCC 8619 was selected among nine strains of lactose fermenting yeast for the production of ethanol from cheese whey. The effects of three nutrients (ammonium sulfate $(NH_4)_2SO_4$, dipotassium hydrogen phosphate K_2HPO_4, yeast extract, and a combination of them) on the ethanol yield from cheese whey were investigated. The results indicated that no addition of nutrient supplement is necessary to achieve complete lactose utilization during the cheese whey ethanol fermentation. However, addition of a small concentration (0.005% w/v) of these supplements reduced the lag period and the total fermentation time and increased the specific growth rate of the yeast. Higher concentrations (0.01 and 0.015% w/v) of ammonium sulfate and dipotassium hydrogen phosphate inhibited the cell growth and reduced lactose consumption. The highest ethanol (21.17 g/L) was achieved using yeast extract at a concentration of 0.01% w/v, given a conversion efficiency of 98.3%. No indication of alcohol inhibition was observed in this study.

Index Entries: Batch fermentation; ethanol; cheese whey; inhibition; cell growth; nutrient supplement; yeast.

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INTRODUCTION

Ethanol, as a liquid fuel, can be produced by fermentation from virtually any of the abundant carbohydrates found in nature (*1,2*). However, the nature and cost of substrate determine the economic viability of the fermentation process. Although cellulosic materials constitute the most abundant carbohydrate reservoir, cellulose is extremely resistant to breakdown, and economical processes for such materials have not yet reached fruition. Using crops, such as sweet sorghum and sugar beets, or fruit wastes, such as apple pomace, as substrates in ethanol fermentation may not be economically attractive because of the cost of production and the seasonality of crops. However, the dairy industry represents an important part of the food industry and contributes significant liquid wastes that can be used for the production of ethanol while minimizing the environmental problems associated with treatment and disposal. Whey, as a byproduct of the cheese industry, is produced at a rate of 0.22 million metric tons per year in Canada (*3*), of which over half currently is discarded as waste, constituting a significant loss of resources and causing serious pollution problems. Several investigators have studied the feasibility of ethanol production from cheese whey (*4–9*).

Munnecke (*10*) stated that the availability of certain nutrients is essential for growth and maintenance of ethanol-producing microbes. These nutrients have been classified as micronutrients such as (zinc, copper, iron, magnesium, and manganese, which are required in trace quantities) and macronutrients (such as nitrogen, phosphorus, potassium, sodium, and sulfur, which are required in larger quantities (*10–12*). Macronutrients are required primarily for the synthesis of cellular material, whereas micronutrients are required as cofactors in enzymatic reactions. Generally, the nitrogen, phosphorus, and sulfur components, some trace elements, and some vitamins are low in most raw materials (*13*). Thus, it becomes necessary to supplement these raw materials in order to maintain reasonable microbial growth and substrate utilization. Nitrogen is an important ionic growth factor in determining the rate of fermentation, as it controls the synthesis of protein and nucleic acid (*14,15*). Sulfur is a constituent of proteins, such as amino acids, cysteine, and methionine, and also some coenzymes, such as cocarboxylase (*15*). Sulfur forms about 0.4% of yeast dry weight (*16*), whereas phosphorus is the most important ionic factor in determining the rate of fermentation, as it controls the synthesis of lipids and carbohydrates and maintains the integrity of the cell wall (*17*). Ammonium sulfate $(NH_4)SO_4$ is generally a common nutrient supplement for nitrogen and sulfur (*16–18*). Dipotassium hydrogen phosphate (K_2HPO_4) has been used to supplement phosphorus for cell growth during fermentation (*16,18*). The addition of yeast extract improves the nutritional quality of the medium, since it contains several of the B vitamins, other growth promoting substances, organic nitrogen,

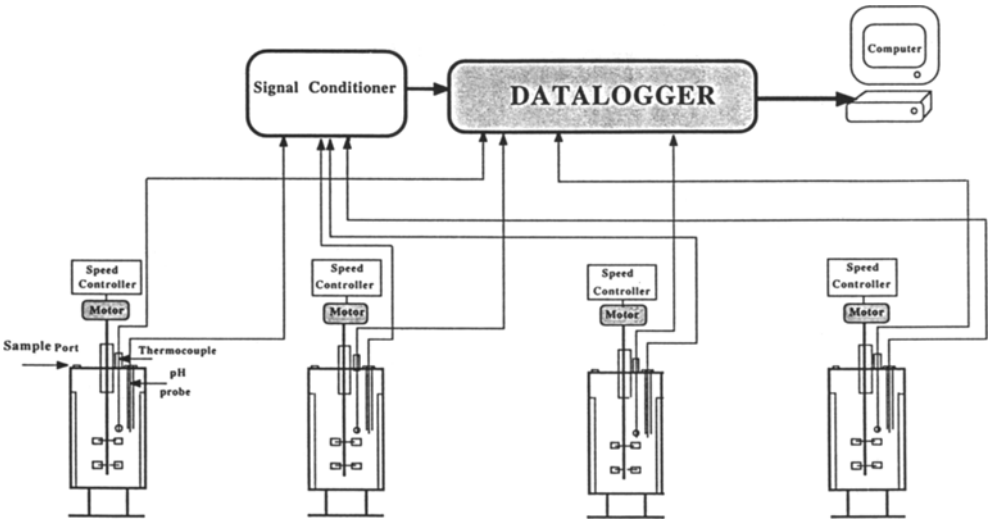

Fig. 1. Batch culture experimental apparatus.

and carbon compounds (*6,19–22*). However, opinions expressed in the literature on the effects and quantities of supplemented nutrients on the cell yield and ethanol yield are not conclusive and are sometimes contradictory (*23*).

OBJECTIVE

The aim of this study was to investigate the effects of some common nutrients and vitamin supplements on the cell growth rate, substrate utilization, and final ethanol concentration during yeast fermentation of cheese whey for alcohol production under batch operating conditions.

EXPERIMENTAL APPARATUS

The experimental setup is shown in Fig. 1. With reference to this figure, the following are detailed descriptions of the experimental apparatus components.

Bioreactor

Four pilot-scale batch bioreactors of 5-L volume each were used in this study. Each bioreactor was constructed from a plexiglas cylinder of 145 mm internal diameter, 6 mm thickness, and 395 mm height (Fig. 2). The bioreactor was covered with a 200-mm diameter circular plate of 6 mm thickness that was secured in place by means of six stainless steel

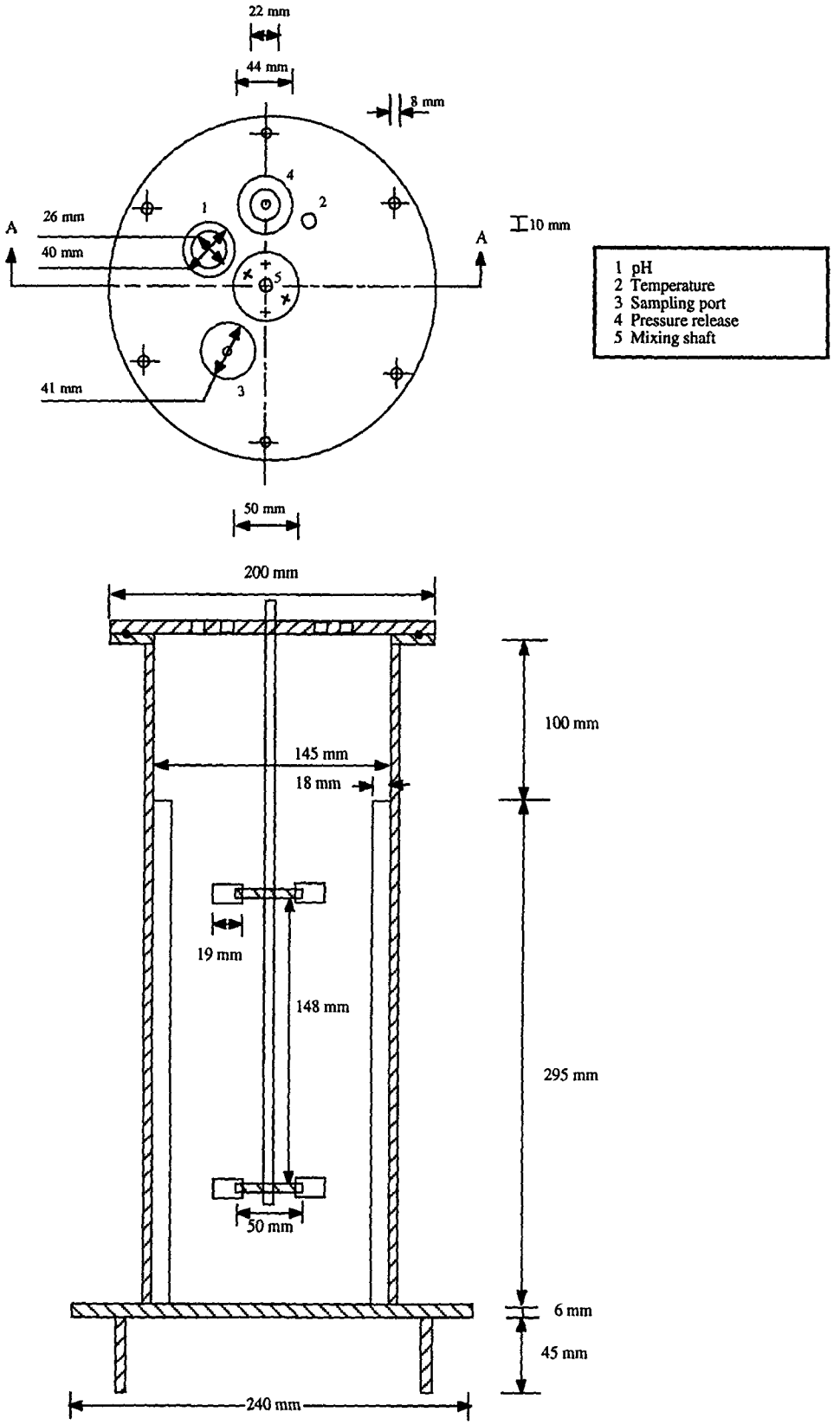

Fig. 2. Batch reactor.

screws and wing nuts fastened at the top outer periphery of the bioreactor. A rubber gasket (O-ring, 2.5 mm thick) was placed in a machined groove on the bioreactor flange under the cover to ensure an airtight seal. Five holes were drilled through each bioreactor cover. The first hole (26 mm in diameter) was used for the pH sensor. The second hole (10 mm in diameter) was used for the temperature sensor. The third hole (2.5 mm in diameter) was used as a sampling port that consisted of a rubber (disk-shaped) septum of 29 mm diameter and 7 mm thickness placed within a machined circular plexiglas plate collar of 41 mm diameter and 12 mm thickness that was secured into the cover by means of four stainless steel screws. The fourth hole (12 mm in diameter) was used as a pressure release vent. The fifth hole (8 mm in diameter) was used for the mixing shaft.

Data Acquisition System

The data acquisition system consisted of data logger, temperature sensors, pH probes, a signal conditioning unit, and a personal computer. A 24-channel data logger (model 525 S.SYSCON) was connected to the signal conditioning unit and to the Tandy 1200 personal computer (model no. 25-3000A) through a serial communication port, as shown in Fig. 2. Five Type-T, special purpose thermocouple sensors (Cole Parmer, Chicago, IL, cat no. L-08530-74) were calibrated and used for temperature measurement. Four pH probes (Fisher Scientific [Pittsburgh, PA] model no 13-620-104) were used for pH measurement. The thermocouples were connected to the data logger directly, whereas the pH probes were connected to the data logger through the signal conditioning unit. A Quick Basic environment was used to develop the software for driving the data acquisition system.

EXPERIMENTAL MATERIALS

Microorganisms

The yeast strain *Candida pseudotropicalis* 8619 obtained from the American Type Culture Collection (ATCC, Rockville, MD) was used in this study. The yeast was rehydrated according to the ATCC procedure using TM broth (Difco, Detroit, MI) with the following composition of medium: 1 L of water, 3 g of Bacto yeast extract, 3 g of Malt Extract, 5 g of Bacto peptone, and 10 g of Bacto-Dextrose.

Nutrient Supplement

Nitrogen, sulfur, potassium, phosphorus, and vitamin B were added to the cheese whey; nitrogen and sulfur as ammonium sulfate, potassium, and phosphorus as dipotassium hydrogen phosphate and vitamin B as yeast extract were used to supplement the cheese whey. Certified A.C.S. $(NH_4)_2SO_4$ and K_2HPO_4 were obtained from Fisher Scientific

Table 1
Some Characteristics of the Cheese Whey

Characteristics	Measured value	Units
Total solids	68298	mg/L
Fixed solids	6748	mg/L
Volatile solids	61550	mg/L
Percent volatile solids	90.1	%
Percent fixed solids	9.9	%
Suspended solids	25160	mg/L
Fixed solids	225	mg/L
Volatile solids	24935	mg/L
Percent fixed solids	0.9	%
Total Kjeldahl nitrogen	1560	mg/L
Ammonium nitrogen	263	mg/L
Organic nitrogen	1297	mg/L
Percent ammonium nitrogen	13.9	%
Total chemical oxygen demand	81050	mg/L
Soluble chemical oxygen demand	68050	mg/L
Insoluble chemical oxygen demand	13000	mg/L
Percent soluble chemical oxygen demand	85.0	%
Lactose	4.8	%
Potassium	1623	mg/L
Sulfur	154	mg/L
Phosphorus	468	mg/L
pH	4.9	

(Fisher Scientific, Pittsburgh, PA, cat. nos. A702-500 and P-288-100). The yeast extract was obtained from Difco (Difco Laboratories, Detroit, MI, cat no 0127-01-7).

EXPERIMENTAL PROCEDURE

Cheese Whey Collection and Storage

Cheese whey was collected from Farmer's Cooperative Dairy Plant located in Truro, Nova Scotia and placed in 60-L plastic containers. The sealed containers were stored at −25°C in a commercial freezing plant (Associated Freezers of Canada, Dartmouth, Nova Scotia) in order to reduce microbial and enzymatic degradation. When the cheese whey was needed, a few containers of cheese whey were removed from the freezer and kept at room temperature in the biotechnology laboratory to thaw. Some characteristics of the cheese whey are presented in Table 1.

Inoculum Preparation

The dehydrated stock culture of *Candida pseudotropicalis* was used to prepare several plates by streaking one loopful of the culture on each plate (plates were prepared with the specified growth media YM agar). The plates were then incubated for 3 d at 26°C in a temperature controlled environmental chamber. The yeast was then transferred from the agar plates into several Erlenmeyer flasks (250 mL) each containing 100 mL of the pasteurized raw cheese whey. The flasks were loosely covered with cotton stoppers and placed in a temperature controlled incubator shaker operating at 200 rpm and 26°C for 48 h. The yeast culture was collected from the flasks and transferred to a large sterilized container and mixed. The flasks were then refrigerated at 2°C until needed. Each reactor was inoculated with 480 mL of inoculum (10% v/v).

Experimental Protocol

The raw cheese whey was pasteurized in 4-L presterilized bottles that were capped with nonabsorbent cotton plugs and covered with aluminum foil. The bottle sterilization process was carried out for 20 min at 121°C and 103.4 kPa in an autoclave (Market Forge Sterilmatic, New York, NY, model no STM-E). The cheese whey filled bottles were placed in a water bath (Haake, Fischer Scientific, model no 4391) at a temperature of 70°C for 45 min, removed from the water bath, and placed in an ice bath for 30 min and then kept at room temperature for 24 h. This pasteurization process was repeated three times to allow for the germination of spores and the destruction of vegetative cells. The pasteurized cheese whey bottles were then stored in a refrigerator at 4°C until needed.

The bioreactor and mixing shaft were chemically sterilized using 2% potassium metabisulfite solution. Then, the bioreactors were washed with hot water several times in order to remove any traces of chemicals and then placed under ultraviolet radiation for 30 min. The bioreactors were placed in large sterilized plastic bags until ready for use.

The amount of nutrient supplement required was weighed (240, 480, and 720 mg for 0.005, 0.010, and 0.015% w/v, respectively) using an electronic balance (Mettler AE200, Fisher Scientific cat no 01-909-414) and placed in the bioreactor. Dipotassium hydrogen phosphate, ammonium sulfate, yeast extract, and all three nutrients were placed into the first, second, third, and fourth bioreactors, respectively. Each bioreactor was filled with the pasteurized cheese whey (4.32 L) and then immediately inoculated with 0.48 L of inoculum to bring the working volume to 4.8 L. The bioreactors were covered and the mixing motor was turned on (the mixing speed was 300 rpm). The data acquisition system was turned on and the computer program was activated. The experimental conditons for each bioreactor were chosen from a menu displayed on the screen. The time interval to display and store the data was set through the datalogger.

The above protocol was repeated with 0.01 and 0.015% w/v nutrient concentrations.

Samples Collection and Analysis

Samples of about 20 mL each were collected over a 148-h period for cell number, ethanol, and lactose analyses. The first sample was drawn from the fermenter immediately at the start "zero hour." Samples were, then, taken every 2 h for a period of 36 h and then every 4 h for the next 48 h. After 84 h from the start, the samples were taken every 6 h until the end of the experimental run.

Lactose concentration was determined using a sugar analyzer (Yellow Spring Instrument YSI Model 27, Fisher Scientific cat no 14-660). Ethanol Concentration was determined using a gas chromatograph (Hewlett Packard, Atlanta, GA, 5890 Series II gas chromatograph). Cell number was determined by measuring the dehydrogenase activity according to the procedure described by Ben-Hassan (*24*).

RESULTS AND DISCUSSION

pH

The initial pH of the cheese whey was 4.9. The pH of the medium decreased slightly with time reaching 3.9 for the supplemented media and 4.6 for the nonsupplemented media after 148 h (Fig. 3). These results are of the same order of magnitude as those reported by Friend et al. (*25*), O'Leary et al. (*26*), and Rogosa et al. (*27*). The decrease in the pH during cheese whey fermentation could be attributed to the dissolved carbon dioxide that formed carbonic acid or to hydrogen ions being formed during the conversion of lactose to ethanol as reported by Chen and Zall (*28*) and King and Zall (*29*). The optimum pH for the growth of the *Candida pseudotropicalis* is 4.5 (*6,21*). However, several investigators found that a slight change in pH had no significant effect on the fermentation rate of the yeast (*25,26,28–30*).

Cell Growth

The effects of nutrient supplements on the cell growth of the yeast *Candida pseudotropicalis* during the batch fermentation of cheese whey are shown in Fig. 4. The four phases of cell growth usually encountered with batch culture operation were observed with all treatments except those of dipotassium hydrogen phosphate and ammonium sulfate at a concentration of 0.015% w/v. These were: the lag phase, which represents the time required for the yeast cells to undergo the necessary changes in chemical composition and to acclimatize to the new envirionment; the exponential

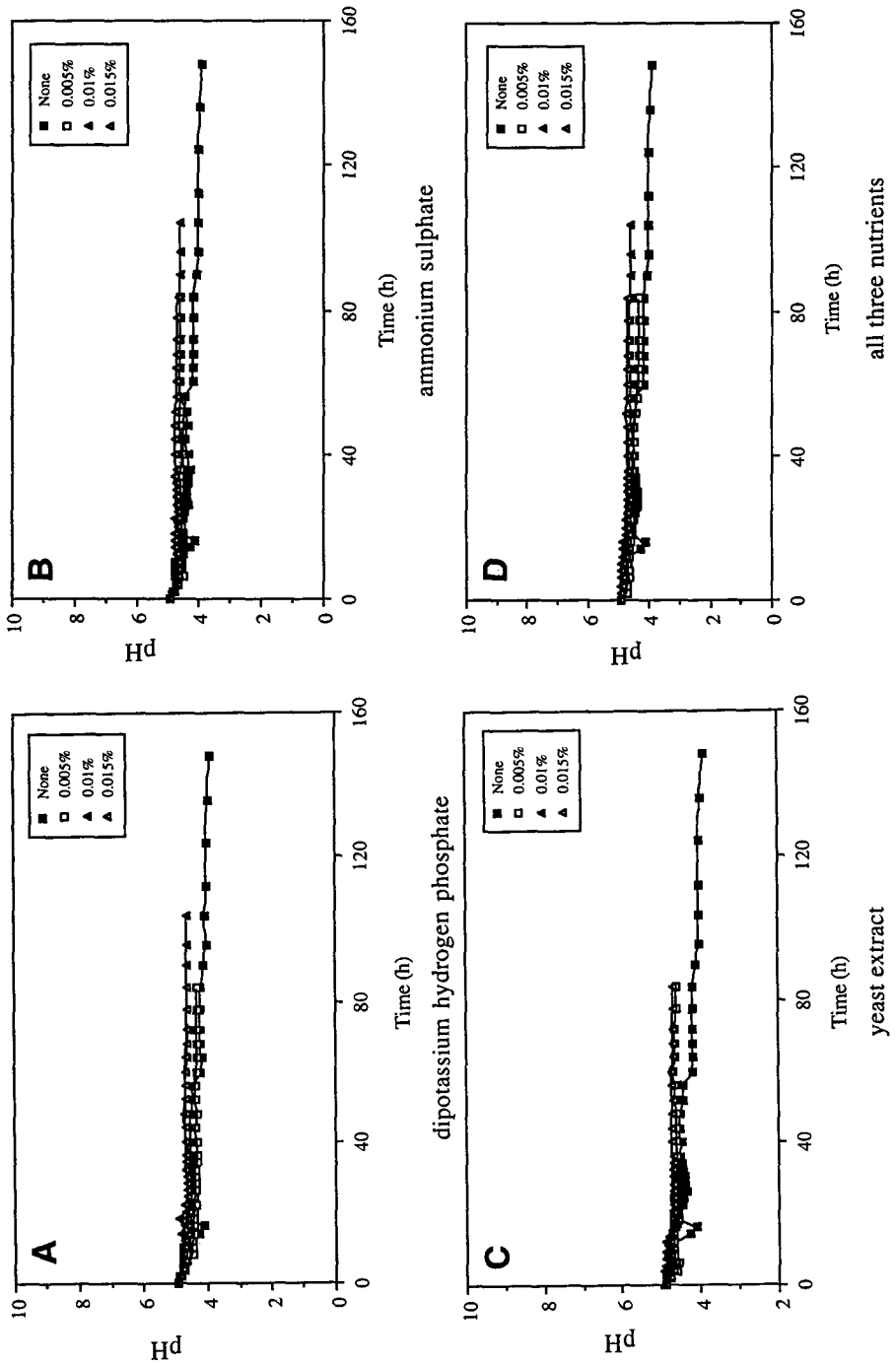

Fig. 3. pH Measurements at different concentration of nutrient supplements.

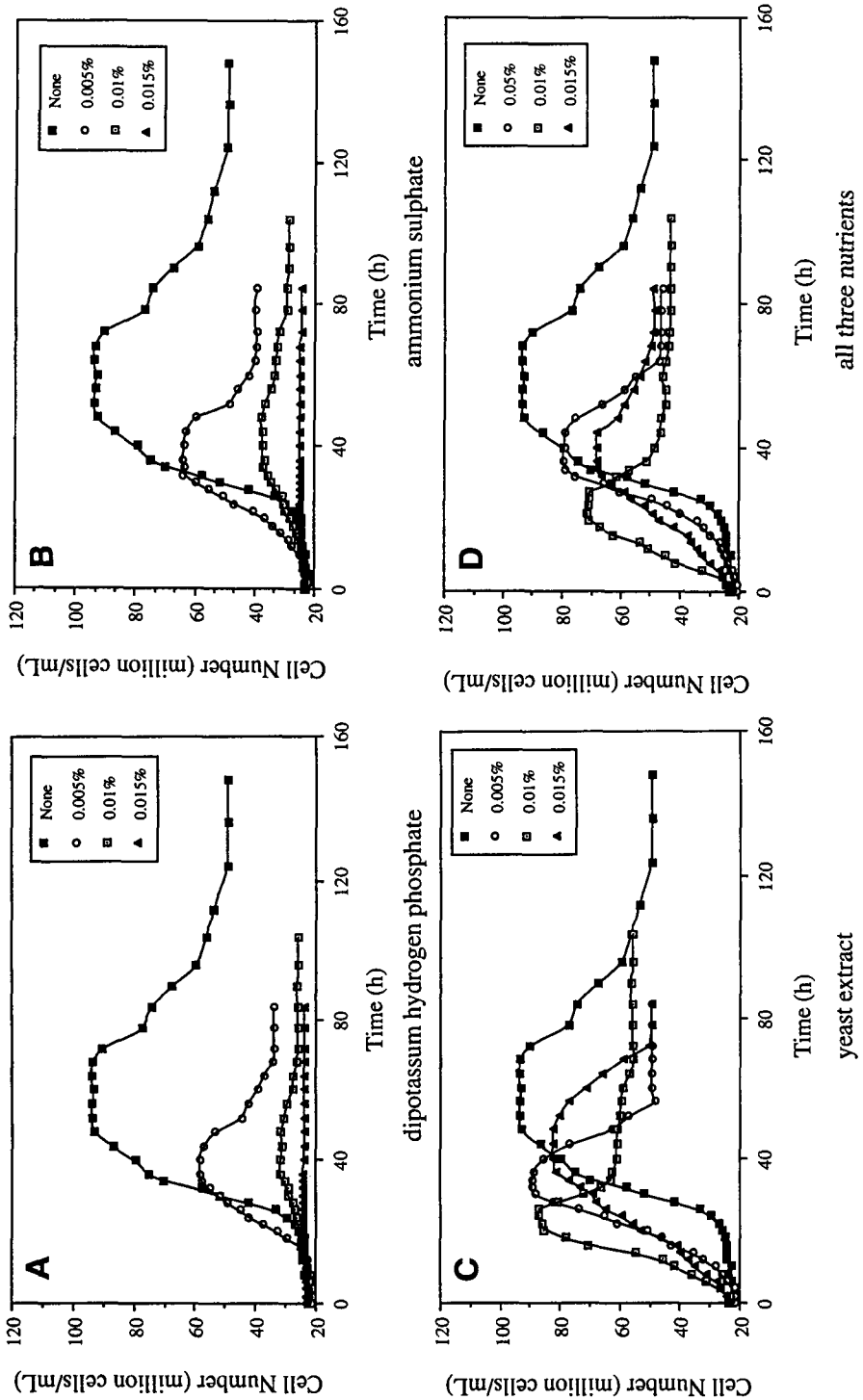

Fig. 4. The effects of nutrient supplements on the growth of the yeast *Candida pseudotropicalis*.

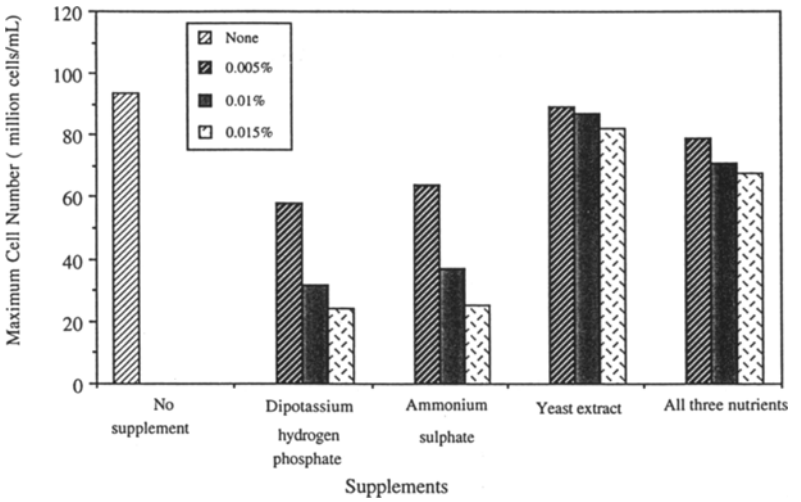

Fig. 5. The effects of nutrient supplements on the maximum cell number of the yeast *Candida pseudotropicalis*.

phase, during which the growth rate had a constant maximum value; the stationary phase, during which the yeast cell number remained constant owing to the balance between growth and death resulting from substrate depletion and high concentrations of toxic metabolites; and the death phase, which represents the decrease in the yeast cell number as a result of continued accumulation of inhibitory byproducts (*31,32*).

Dipotassium hydrogen phosphate and ammonium sulfate supplements appeared to have significant inhibitory effects on the growth and survival of the yeast *Candida pseudotropicalis*. The yeast cell number was reduced significantly when 0.005% w/v of these supplements was used. Increasing the amount of 0.01% w/v resulted in a further decrease in the number of cells, and a complete inhibition of cell growth resulted at the concentration of 0.015% w/v. On the other hand, the addition of yeast extract (0.005% w/v) resulted in a small reduction in the cell number and increasing the amount of yeast extract resulted in only a slighly decrease in the cell number. A similar trend was observed when the three nutrients were added, as it appears that the yeast extract counteracted the inhibitory effects of dipotassium hydrogen phosphate and ammonium sulfate on the cell growth. However, a significantly higher cell number was observed with the addition of the three nutrients as compared to those observed with the addition of dipotassium hydrogen phosphate or ammonium sulfate.

Figure 5 shows the effects of the nutrient supplements on the maximum cell number of *Candida pseudotropicalis*. The highest maximum cell number was achieved with no supplement addition to the cheese whey

followed by yeast extract, all three nutrients, ammonium sulfate, and dipotassium hydrogen phosphate. The lowest maximum cell numbers were obtained with the addition of dipotassium hydrogen phosphate and ammonium sulfate at the concentration of 0.015% w/v.

The presence of potassium (K) is essential for the uptake of $H_2PO_4^-$. The results obtained with the addition of dipotassium hydrogen phosphate are contradictory to those obtained by Izaguirre and Castillo (*21*), who used deproteinized cheese whey (7% lactose concentration) supplemented with 0.01% w/v of dipotassium hydrogen phosphate and reported increased cell concentration (from 1.02 to 1.74 g/L). The cheese whey contained 1623.0 mg/L potassium, of which only 13.5 mg/L are required to achieve a cell concentration of approx 4.4 g/L based on the available lactose in the cheese whey (326 g cell/g K) (*12,16*). Thus, the quantity available in cheese whey is about 120-fold that required for cell growth. The decrease in the cell concentration when dipotassium hydrogen phosphate was used in this study was owing to the increased concentration of potassium in the cheese whey that inhibited the cell growth. Increasing K^+ may have caused inhibition of hexokinase, enolase, membrane ATPase, and nucleic acid uptake as suggested by Maiorella et al. (*11*), Hewitt and Nicholas (*33*), Kotyk and Horak (*34*), and Gawronski (*35*). Jones and Greenfield (*12*) reported that at a potassium concentration above 391 mg/L, a partial inhibition of the yeast cell growth was observed and a total inhibition of yeast cell growth took place at about 78,200 mg/L. Soumalainen and Oura (*36*) reported a decreased fermentation rate at potassium concentration above 156–391 mg/L.

Phosphorus (P) is essential for the growth of yeast and plays an important part in carbohydrate metabolism. It controls the synthesis of lipids and maintains the integrity of cell wall (*36,37*). The cheese whey contained 468 mg/L phosphorus, of which only 82 mg/L are required to achieve a cell concentration of approx 4.4 g/L based on the available lactose in the cheese whey (53.6 g cell/g P) (*12,16*). Thus, the quantity of P available in cheese whey is about 5.7-fold that required for cell growth. Increasing phosphours (by the addition of dipotassium hydrogen phosphate) may have caused inhibition by acidifying cell cytoplasm, as reported by Rothstein (*38*).

Nitrogen is an important ionic growth factor determining the rate of fermentation as it controls the synthesis of protein and nucleic acid (*14,15*). The concentration of ammonium nitrogen in the cheese whey was 263 mg/L, which is fivefold of that required (52 mg/L) for growth (*12,16*). High concentration of ammonium ion may cause inhibition of amino acid uptake, as suggested by Pattabiraman (*39*) and Roon et al. (*40*). Amino acids are known to enhance the product yield in yeast by giving a measure of relief to the carbon demand that would otherwise be provided by the intermediates of sugar metabolism when ammonium ions are used as a nitrogen source (*16*). Sulfur is a constituent of proteins,

such as amino acids, cysteine, and methionine, as well as some coenzymes, such as cocarboxylase (*14*). Cheese whey contained 154 mg/L sulfur (*41*), of which only 1.8 mg/L is required to achieve a cell concentration of approx 4.4 g/L based on the available lactose in the cheese whey (2444 g cell/g S) (*12,16*). Thus, the quantity available in cheese whey was 85.6-fold that required for cell growth. The results obtained with the addition of ammonium sulfate agreed with those obtained by several authors (*11,19,42*). Increased concentration of sulfate (owing to the addition of ammonium sulfate) may have caused inhibition of phosphoglycerate kinase, as suggested by Scopes (*43*). Kotyk and Horak (*34*) and Eddy (*44*) reported a range of 0.16 to 11.2 mg/L for ammonium sulfate concentration, outside which there is either sulfur deficiency or sulfur inhibition.

In this study increasing the amount of yeast extract resulted in slight decreases in the maximum cell number. The use of yeast extract at a concentration of 0.005% (w/v) has been reported to increase the cell concentration from 1.02 to 5.28 g/L during the ethanol fermentation of deproteinized cheese whey (*21*). In another study (*42*), no significant effect on the cell number (which remained at 800 colonies/mL) was observed as a result of yeast extract addition during ethanol fermentation of cheese whey.

The specific growth rate and lag period were determined graphically according to the procedure described by Ghaly et al. (*31*). Table 2 shows the lag period, fermentation time, and specific growth rate at different nutrient supplements. The addition of nutrient supplement substantially reduced the lag period. Shorter lag periods were observed with the yeast extract followed by all three nutrients, dipotassium hydrogen phosphate, and ammonium sulfate. The shortest lag period (1.0 h) was obtained when 0.015% w/v yeast extract was used, whereas the longest lag period (19.5 h) was obtained with no supplement addition to the cheese whey. Ghaly et al. (*45*) reported that the length of the lag phase usually depends on the extent to which the new medium and the environmental factors, such as pH and temperature, are different from those under which the inoculum was prepared. When new substrates are added cells excrete digestive enzymes in order to assimilate the nutrients. Maule (*46*) reported that the length of the lag phase depends largely on the pretreatment and physiological conditions of the yeast. Bailey and Ollis (*47*) stated that the length of lag phase is affected by the nutrient concentration in the medium. Pineault et al. (*48*) observed a shorter lag period during alcohol fermentation from waste sulfite liquor supplemented with the addition of bacto yeast nutrient. They stated that even though the cell numbers did not increase, the cells were physiologically active and metabolizing. The yeast extract used in this study contained amino acids, lipids, and nucleotides, all of which are readily available sources of carbon for cell biomass synthesis (*16*). It also contained utilizable nitrogen source, vitamins, and trace elements.

Table 2
The Lag Period and Specific Growth Rate of Cheese Whey Batch Fermentation at Different Nutrient Supplements

Supplement	Concentration, % w/v	Lag period		Fermentation time		Specific growth rate	
		h	%[a]	h	%[a]	h^{-1}	%[a]
None	0.000	19.5	100.00	56	100.00	0.043	100.00
Dipotassium hydrogen phosphate	0.05	11.0	56.41	34	60.71	0.046	106.98
	0.010	5.5	28.20	30	53.57	0.011	25.58
	0.015	3.5	17.95	24	42.86	0.003	6.98
Ammonium sulfate	0.005	13.5	69.23	36	64.28	0.046	106.98
	0.010	11.5	58.97	32	57.14	0.019	44.91
	0.015	7.0	35.90	26	46.43	0.004	9.30
Yeast extract	0.005	4.5	23.08	30	53.57	0.056	130.23
	0.010	2.0	10.26	22	39.28	0.075	174.42
	0.015	1.0	5.13	34	60.71	0.037	86.05
All three nutrients	0.005	7.5	38.46	32	57.14	0.050	116.28
	0.010	3.0	15.38	24	42.86	0.057	132.56
	0.015	2.0	10.26	36	64.28	0.035	81.40

[a]Percentage of the value with no supplement addition (0.043/h).

Addition of a small amount (0.05% w/v) of dipotassium hydrogen phosphate or ammonium sulfate slightly increased (7%) the specific growth rate of the yeast *Candida pseudotropicalis*. Further increases in the concentration of these nutrients substantially decreased the specific growth rate. The lowest specific growth rates (0.003/h for dipotassium hydrogen phosphate and 0.004/h for ammonium sulfate) were obtained when using 0.01% w/v dipotassium hydrogen phosphate and ammonium sulfate. On the other hand, the addition of yeast extract at concentrations of 0.005 and 0.01% w/v resulted in increases in the specific growth rate of 30% (from 0.043 to 0.056/h) and 75% (from 0.043 to 0.075/h), respectively. Further increases in the yeast extract concentration decreased the specific growth rate by 14%.

The rapid cell growth achieved using yeast extract is owing to the presence of all the necessary yeast growth factors: amino acids, purines, pyrimidines, and vitamins, which help to propagate the cell rapidly (*49–51*). The improved viability of the yeast and the increase in the specific growth rate, owing to the addition of the yeast extract to the cheese whey, decreased the total fermentation time by 51% (form 90 h to 44 h). This decrease in the fermentation time would be reflected in the overall cost of the fermentation process and thus have a significant impact on the economics of ethanol fermentation from cheese whey.

Lactose Utilization

Figure 6 shows the profile of lactose concentration during the fermentation of cheese whey with different nutrient supplements. The lactose concentration first decreased slowly at the start of the fermentation process (during the lag period), then decreased rapidly (during the exponential growth), and finally decreased slowly (during the stationary phase). When no nutrient supplement was added to the cheese whey, lactose was almost completely utilized after a fermentation time of 68 h. When ammonium sulfate was used at concentrations of 0.005 and 0.01 w/v, the lactose was completely utilized after 40 and 48 h, respectively. On the other hand, about 99 and 97% of lactose was utilized after 44 and 52 h when dipotassium hydrogen phosphate was used at concentrations of 0.005 and 0.01% w/v, respectively. However, only 57% of the original lactose concentration in cheese whey was utilized (during the first 36 h) when the higher concentrations (0.015% w/v) of dipotassium hydrogen phosphate and ammonium sulfate was used; no further reduction in lactose was observed.

When yeast extract was added to the cheese whey, the lactose was completely utilized after 44, 44, and 48 h for the concentrations of 0.005, 0.01, and 0.015% w/v, respectively. Similar patterns were observed when all three nutrient supplements were added; complete lactose utilization was achieved after about 44, 52, and 52 h at the concentrations of 0.005, 0.01, and 0.105% w/v, respectively.

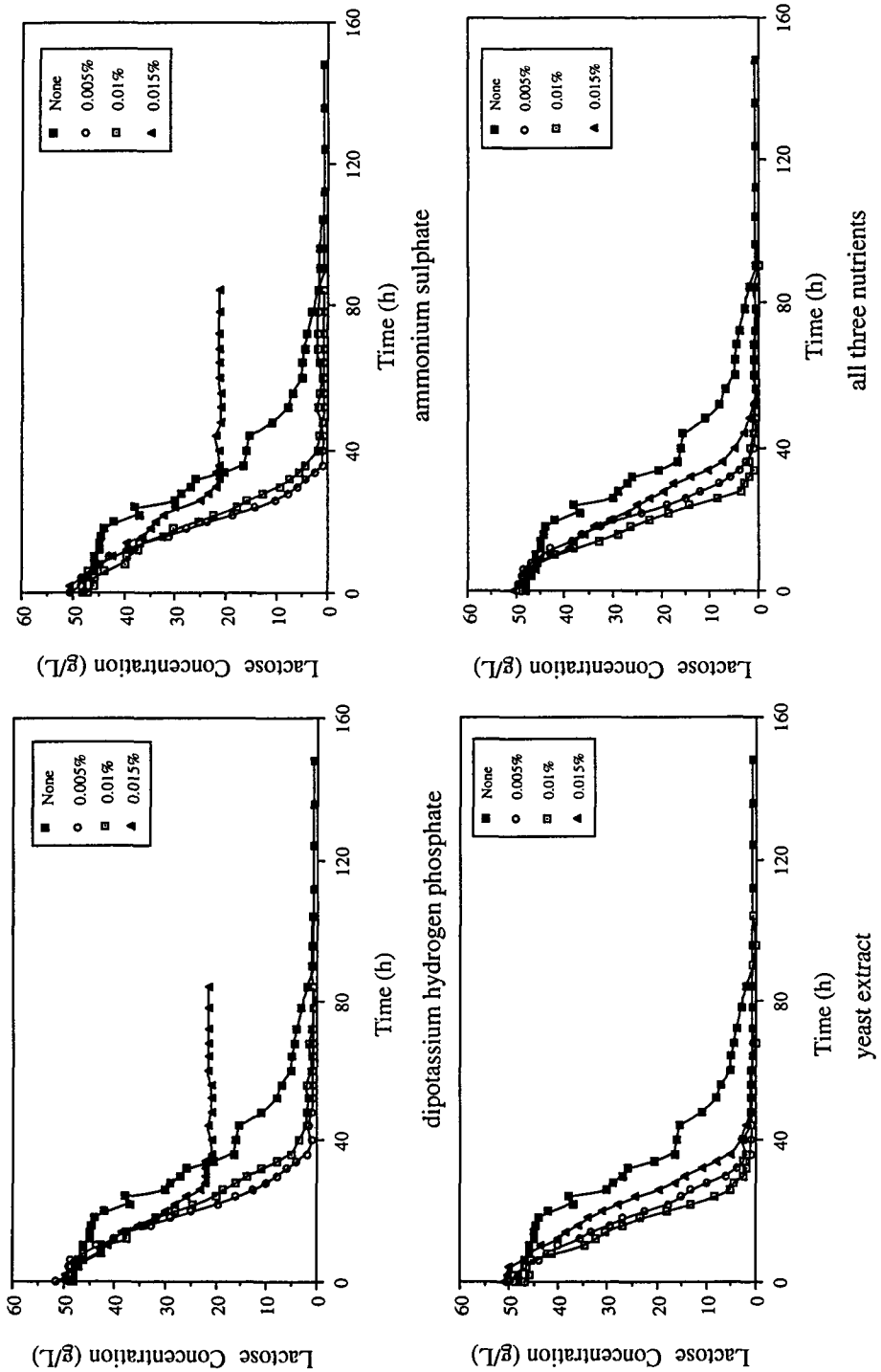

Fig. 6. The effects of nutrient supplements on the lactose consumption by the yeast *Candida pseudotropicalis*.

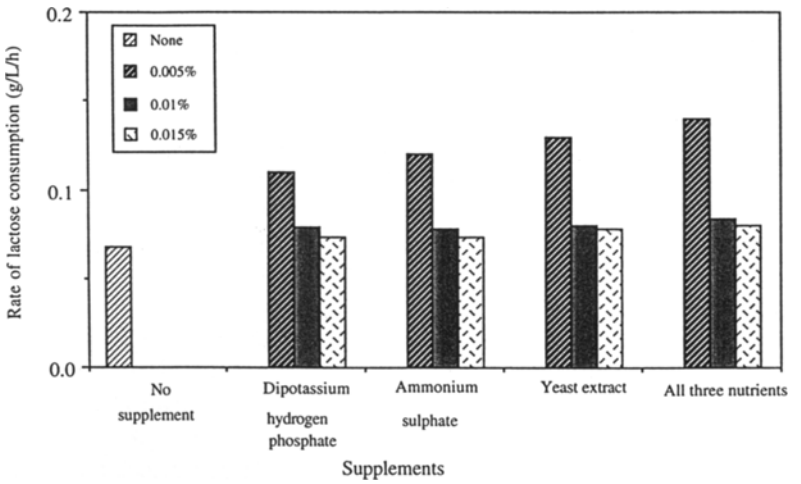

Fig. 7. The effects of nutrient supplements on the average rate of lactose consumption by the yeast *Candida pseudotropicalis* during the exponential growth phase.

Using lactose concentration of 25%, Mahmoud and Kosikowski (*19*) reported that the highest percentage of lactose utilization during alcohol fermentation was found to be 60.9%. Izaguirre and Castillo (*52*) reported that 88% of available concentrated lactose (7%) was utilized during the ethanol fermentation process by *Candida pseudotropicalis*. The results obtained in this study are in good agreement with those obtained by Izaguirre and Castillo (*21*).

The present study indicated that addition of nutrient supplements may not be required to achieve complete lactose utilization during cheese whey fermentation for ethanol production. Similar results were obtained by De Bales and Castillo (*53*), who reported no significant differences in lactose utilization by *Candida pseudotropicalis* when different concentrations of ammonium sulfate and dipotassium hydrogen phosphate were added to cheese whey. The substantially low lactose utilization of 43 and 42% when using high concentrations (0.015% w/v) of ammonium sulfate and dipotassium hydrogen phosphate was attributed to cell inhibition resulting from increased nutrient concentrations. However, the addition of a small amount of nutrient supplements 0.005% w/v increased lactose consumption and thus reduces the fermentation time. Figure 7 shows the effect of nutrients supplement on the average rate of lactose consumption by *Candida pseudotropicalis* during the exponential growth phase. As the concentration of the nutrient supplement increased, the rate of lactose consumption decreased sharply for all supplements. The lowest rate of lactose consumption (0.068 g/L/h) was obtained with no supplement addition to the cheese whey, whereas the highest rate of lactose consumption (0.14 g/L/h) was obtained when 0.005% w/v of all the three nutrients were added to the cheese whey.

Ethanol Production

The effect of nutrient supplements on the ethanol concentration profile of the cheese whey fermentation is presented in Fig. 8. In all treatments, the ethanol concentration increased with the fermentation time. Maximum ethanol concentration was reached at about the same time that the cell concentration reached its maximum. This suggested that the ethanol fermentation process has growth associated kinetics as described by Bailley and Ollis (*47*) and Sinclair (*54*).

The maximum ethanol obtained from cheese whey with no supplement was 20.69 g/L. With the addition of nutrient supplements to the cheese whey at concentrations of 0.005, 0.01, and 0.015% w/v, the maximum ethanol production at the end of the exponential phase was 21.10, 21.10, and 12.85 g/L, for ammonium sulfate; 21.29, 21.04, and 12.20 g/L for dipotassium hydrogen phosphate; 20.99, 21.17, and 21.86 g/L for the yeast extract; and 20.78, 20.42, and 21.22 g/L for all three nutrients, respectively. Ethanol inhibition was not observed during the fermentation process, because the maximum ethanol concentration obtained in this study was less than the ethanol inhibition level of 30 g/L (*55*). In a recent review, Jones (*56*) stated that for many of the highly tolerant strains of yeast no inhibition of the growth rate was observed below 20 g/L ethanol concentration. Holzberg et al. (*57*) suggested a threshold concentration of ethanol of 26 g/L, below which there is no inhibition,

Figure 9 shows the effect of the nutrient supplements on the average ethanol production rate during the exponential growth phase. The rate of ethanol production in all treatments were higher than the control run (no supplement addition to the cheese whey). The lowest rate of ethanol production (0.039 g ethanol/L/h) was obtained with no nutrient supplements addition to the cheese whey, whereas the highest rate of ethanol production (0.08 g ethanol/L/h) was achieved when 0.01% w/v of the three nutrients was added to the cheese whey.

Lactose is used by yeast cell for energy and growth according to the following equation:

Energy release:

$$88\,C_{12}H_{22}O_{11} + 88\,H_2O \xrightarrow{\text{Yeast}} 352C_2H_5OH + 352\,CO_2 + \text{Energy} \quad (1)$$

Growth:

$$12C_{12}H_{22}O_{11} + 20\,NH_4^+ \xrightarrow{\text{Yeast}} 10C_{13}H_{20}O_7N_2 + 14CO_2 + 34H_2O + 76H^+ \quad (2)$$

Theoretically, 100 g of lactose would be expected to yield 47.3 g of ethanol and 9.2 g of cells based on the following net equation:

$$100C_{12}H_{22}O_{11} + 88\,H_2O + 20\,NH_4^+ \xrightarrow{\text{Yeast}} 352C_2H_5OH + 10C_{13}H_{20}O_7N_2 + 366CO_2 + 34H_2O + 76H^+ \text{ Energy} \quad (3)$$

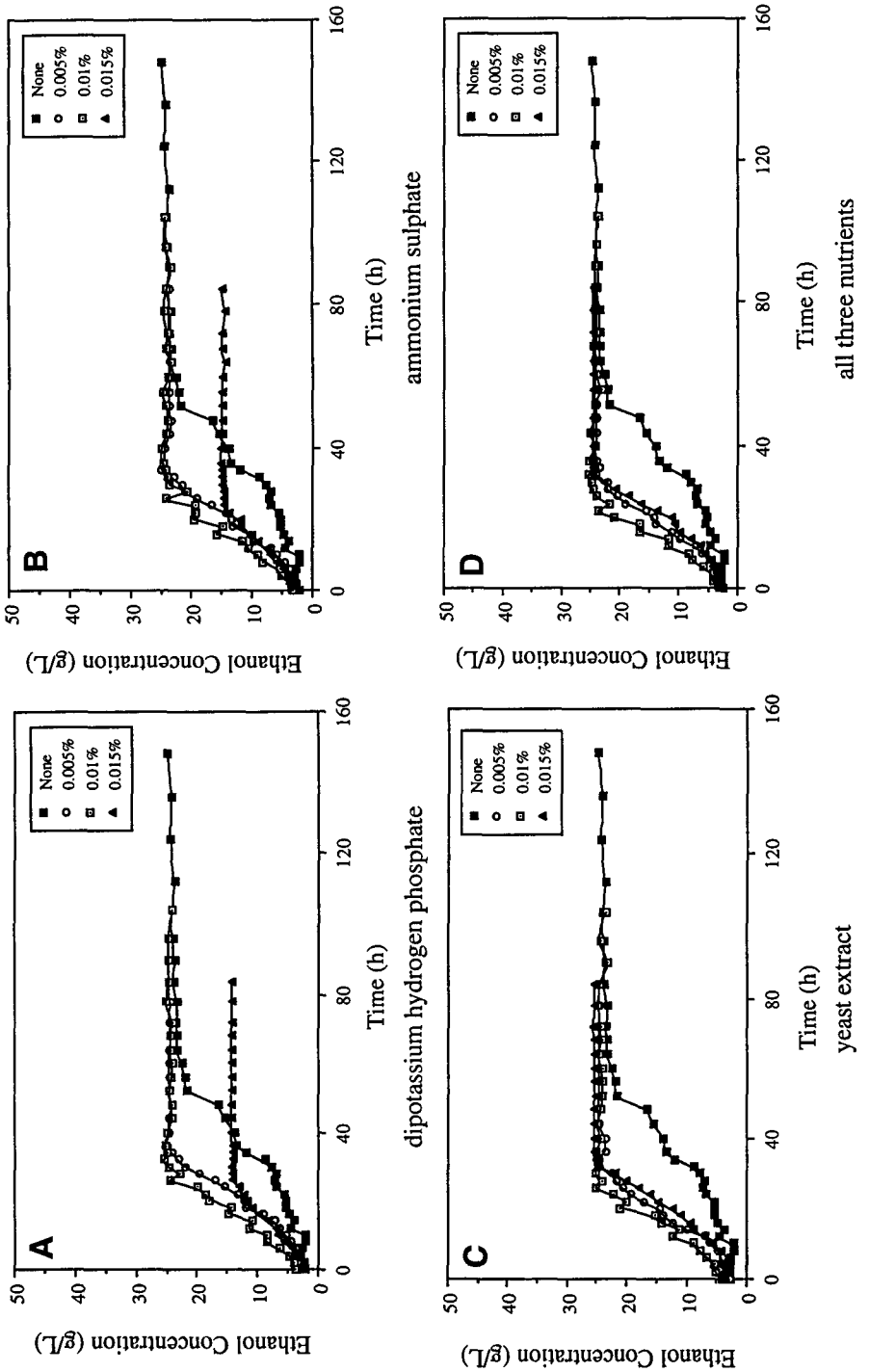

Fig. 8. The effects of nutrient supplements on ethanol concentration during cheese whey batch fermentation.

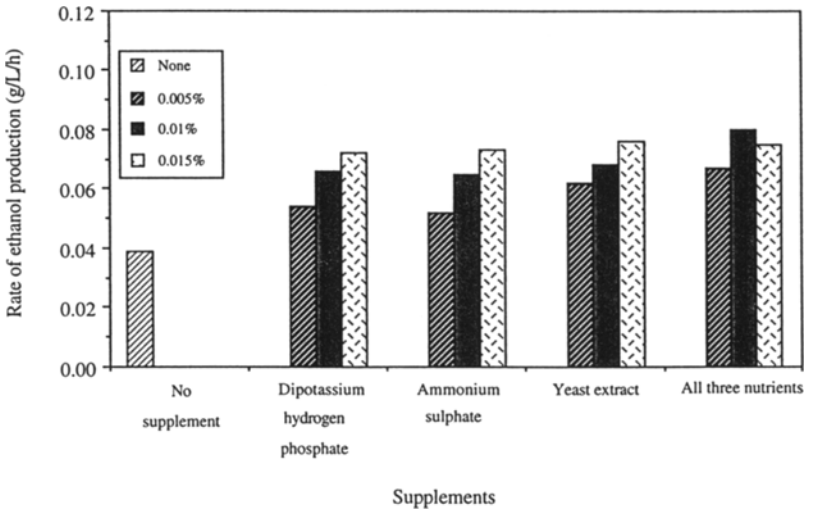

Fig. 9. The effects of nutrient supplements on the average ethanol production rate by the yeast *Candida pseudotropicalis* during the exponential growth phase.

Table 3 shows the effects of the nutrient supplements on the specific ethanol yield (g ethanol/g lactose), cell yield (g cell/g lactose) and conversion efficiency. The lowest specific ethanol yield (0.417 g ethanol/g lactose) was obtained when dipotassium hydrogen phosphate was used at a concentration of 0.005% w/v, whereas the highest specific ethanol yield (0.465 g ethanol/g lactose) was achieved when yeast extract was used at a concentration of 0.01% w/v.

The conversion efficiencies (experimental yield/theoretical yield) obtained in this study was 92.18% for cheese whey with no supplement; 89.22, 97.04, and 93.70% with ammonium sulfate; 88.16, 94.40, and 90.50% with dipotassium hydrogen phosphate; 91.54, 98.30, and 91.54% with yeast extract; and 88.79, 89.85, and 89.64% with all three nutrients for the concentrations of 0.005, 0.01, and 0.015% w/v, respectively. The conversion efficiencies obtained in this study are higher than those of 80% reported by several authors (*25,26,58*). Singh et al. (*59*) reported an ethanol conversion efficiency of 90% during ethanol fermentation of cheese whey concentrated to 16% by reverse osmosis. Marwaha and Kennedy (*7*) reported 87% ethanol conversion efficiency during alcohol production from whey permeate by immobilized and free cells of *Kluyveromyces marxianus*. Chen and Zall (*28*) reported ethanol conversion efficiency ranging from 78 to 89% using yeast extract (0.3–0.7%) during cheese whey batch fermentation. Gil et al. (*60*) noted that using yeast extract resulted in increased ethanol yield during continuous ethanol fermentation. Rogosa et al. (*27*) reported that the yield of ethanol obtained during cheese whey fermentation by *Torula cremoris* (Candida pseudotropicalis) averaged 90.73% in the laboratory and 84% in the plant.

Table 3
Cell Yield and Ethanol Yield of Cheese Whey Batch Fermentation at Different Concentrations of Nutrient Supplements

Supplement	Concentration, % w/v	Cell yield, g cell/g lactose	Ethanol yield, g ethanol/g lactose	Conversion efficiency
NOne	0.000	0.048	0.436	92.18
Dipotassium hydrogen phosphate	0.005	0.022	0.417	88.16
	0.010	0.005	0.447	94.40
	0.015	0.001	0.428	90.50
Ammonium sulfate	0.005	0.205	0.422	89.22
	0.010	0.009	0.459	97.04
	0.015	0.002	0.443	93.70
Yeast extract	0.005	0.042	0.433	91.54
	0.010	0.045	0.465	98.30
	0.015	0.035	0.433	91.54
All three nutrients	0.005	0.036	0.420	88.79
	0.010	0.032	0.425	89.85
	0.015	0.029	0.424	89.64

CONCLUSION

The highest final yeast cell concentration (2.79 g/L) was obtained with raw cheese whey with no nutrient supplement addition. This indicated that the cheese whey had sufficient nutrients for the growth of the yeast *Candida pseudotropicalis* under an anaerobic condition. The strongest cell growth inhibition was observed with the highest concentration (0.015% w/v) of dipotassium hydrogen phosphate and ammonium sulfate, whereas the highest maximum specific growth rate (0.075/h) was observed with the addition of yeast extract at a concentration of 0.01% w/v. Addition of 0.01% w/v of ammonium sulfate, dipotassium hydrogen phosphate, yeast extract, and all three nutrient supplements decreased the lag period by 41.0, 71.8, 89.74, and 84.61%, respectively. Using yeast extract at a concentration of 0.01% w/v decreased the fermentation time from 90 h (no nutrient supplement addition) to 44 h. Complete lactose utilization was observed with all levels of the yeast extract, all levels of the three nutrients and the 0.005 and 0.01% w/v levels of ammonium sulfate and dipotassium hydrogen phosphate. Only 57 and 58% of the initial lactose concentration were utilized when these nutrient supplements were added to the cheese whey at a concentration of 0.015% w/v owing to the cell inhibition caused by the high nutrient supplement concentrations. The highest ethanol production (21.17 g/L) was achieved when yeast extract was added to the cheese whey at the concentration of 0.01% w/v, giving a conversion efficiency of 98.3%. No indication of alcohol inhibition was observed in the study.

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