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Organic Acid Production
from Xylose and Glucose
by the yeast Candida utilis

by

© Ashwani Wadhera (B.Sc., Hons.)

A thesis submitted to the Faculty of Graduate
Studies in partial fulfillment of the requirements
of Master of Science

Department of Biology
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The undersigned hereby recommend to the
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....
Chairman, Department of Biology

....
Supervisor

This thesis is dedicated
to my parents,
Krishana and Surinder
and my aunt and uncle,
Usha and Om

ABSTRACT

Hemicellulose can comprise up to 33% of hardwoods and 40% of agricultural residues. Upon hydrolysis of this fraction, D-xylose is the major product. Glucose is also abundant in many agricultural residues. In order to obtain organic acids of commercial importance from these sugars, mutants of Candida utilis were isolated by Cobalt-60 gamma irradiation followed by treatment with nystatin. Three mutants exhibiting little or no growth on citrate, produced 1.7 to 2.5 times more citric acid than the wild type on xylose. The yields ranged between 0.043 mg/ml and 0.065 mg/ml. With use of a rich medium, one mutant produced greater than three times more citric acid than the wild type on xylose. On glucose, two mutants produced 1.9 and 3 times more isocitric acid than the wild type. They did not produce significantly more total citrate plus isocitrate. Absolute yields of citrate plus isocitrate were two to five times higher on glucose than xylose.

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ABBREVIATIONS

A ₄₂₀	absorbance at 420 nm
A ₅₀₀	absorbance at 500 nm
AKG	alpha-ketoglutarate
°C	degree Celsius
CIT	citrate
cm	centimetre
⁶⁰ Co	Cobalt-60
h	hour
M	molar
mg	milligram
min	minute
ml	millilitre
MNNG	N-Methyl-N'-nitro-nitrosoguanidine ⁰
MW	molecular weight
N	normal
nm	nanometre
No.	number
OAA	Oxaloacetate
R _f	relative flow
rpm	revolutions per minute
s	second
SUC	succinate
TCA	trichloroacetic acid
u	micrometre

ul microlitre

UV ultraviolet

w/v weight per unit volume

INTRODUCTION

Renewable biomass, including agricultural wastes contain high proportions of xylose and glucose (Krull and Inglett, 1980). Hemicellulose is one of the major components of renewable biomass and comprises 33% of hardwoods and up to 40% of agricultural residues (Cowling et al., 1977). The pentose is also present in agricultural residues such as sugarcane bagasse, sunflower stalks, wheat straw, corn (leaves, stalks, cobs, fibers,) and in spent sulfite liquor from paper mill wastes (Table 1). Xylose is readily available in a pentose syrup form (Dolenko, 1982). At present there are relatively few competing markets for the complex pentose and hexose mixtures obtained from the hydrolysis of hemicellulose (Flickinger, 1980). Glucose is also abundant in many agricultural wastes. It is found in highest proportions in corn stalk fibers, sugarcane bagasse, wheat straw, comprising 60-70% of the total content.

Xylose from spent sulfite liquor has been utilized to produce single cell protein for human and animal consumption by the Germans during the first world war (Peppler, 1968). Since then, the use of the pentose has been quite limited. This is most likely due to the existence of cheaper substrates and the lack of isolation and characterization of organisms capable of using xylose efficiently. But recently, due to the increasing prices of petroleum

Table 1--Presence of xylose in various agricultural residues.
(Krull and Inglett, 1980).

Agricultural residue	Percent Xylose (%w/v)
Corn	
leaves	35
stalks	32
husks	36
cobs	40
fibers	29
Sugarcane Bagasse	32
Sunflower Stalks	26
Wheat Straw	32

resources, the hemicellulose fraction has received more attention as a substrate for microbial production of fuels (ethanol, butanol, acetone, butane-diol). According to Flickinger (1980), D-Xylose is under utilized due to the lack of a suitable organism to ferment it efficiently. The key to successful conversion of hemicellulose-derived carbohydrates (essentially consisting of xylose) to fuels and chemical lies in using the proper organisms. The conversion of pentoses by anaerobic or facultative anaerobic bacterial systems is unsatisfactory at present due to the co-production of many products (Owen, 1960). Mycelial fungi such as Mucor and Fusarium, that use xylose are impractical due to their slow rates of growth (Ueng and Gong, 1982). The ideal organisms for the conversion of biomass-derived carbohydrates are the yeasts. Yeasts have a fast growth rate. In addition, the use of sugars by yeasts has been well studied, and many industrial strains have been developed (Gong et al., 1981). Candida utilis is favored industrially to modify wood hydrolyzates and spent sulfite liquors, since it attacks more carbon and nitrogen compounds than other common yeasts (Peppler, 1968). It has less stringent nutrient requirements than other yeasts and is able to grow well in minimal medium. Candida utilis is non-pathogenic and has been tested to be safe, even for human consumption as single cell protein (Harrison, 1968).

Citric acid is the most widely used acidulant in the food industry (e.g. in soft drinks, sweets, jellies, jams).

It is used as an effervescent powder and has many uses in the pharmaceutical industry. Sodium citrate is also used in processed cheese manufacture. Esters of citric acid are used as plasticizers (Miall, 1978). The world market for citric acid is 1.6×10^8 kg for sales of \$259 million (Gaden, 1981). In 1981, Canada required citric acid in sales exceeding \$60 million, all of which was imported. Itaconic acid is used in carpet backing and paper coating. Other uses also include acrylonitrile copolymers for synthetic fibre manufacture. It is also used in synthetic resins and related industries. Other organic acids of some commercial significance include, malate, glutamate, alpha-ketoglutarate.

Therefore, it would be advantageous to use Candida utilis to produce organic acids of commercial importance from sugars present in renewable biomass, including agricultural waste hydrolyzates.

Of 434 species of yeast tested, only one-half could use xylose oxidatively and none anaerobically (Barnett, 1976). It is used both as a carbon and energy source (Barnett, 1961). In most yeasts, xylose catabolism is thought to proceed via a two step oxido-reduction, with xylitol as the intermediate (Barnett, 1976). Xylitol is a metabolic by-product of many yeasts with the ability to use xylose (Onishi, 1969). The initial reduction to xylitol, catalyzed by xylose reductase, occurs in the presence of NADPH. Xylitol is then oxidized to form the pentulose, xylulose

Figure 1--Initial pathways of xylose catabolism in yeast.

(Figure 1) in the presence of xylitol dehydrogenase and NAD^+ . Xylulose is then phosphorylated to xylulose-5-phosphate, the common intermediate of the pentose phosphate cycle.

Recent studies support the fact that xylitol may be an intermediate of xylose catabolism. Mutants of Pachysolen tannophilus, defective in xylitol dehydrogenase took up D-xylose and converted it to xylitol. But, they were unable to grow on xylose or xylitol, while they were able to convert xylulose to ethanol (Schneider, 1983). Another yeast, Candida sp. produced high quantities of xylitol from xylose. A mutant derived from this organism generated ethanol instead of accumulating xylitol. This mutant was unaffected in the first enzyme of xylose catabolism, xylose reductase. But, the activities of xylitol dehydrogenase and xylulose kinase increased significantly (Gong and Tsao, 1983). Presumably, NADH is used to reduce acetaldehyde to ethanol. It should be noted however, that these yeasts do not contain a xylose isomerase.

In addition to the oxido-reduction reaction for catabolism of xylose, some yeast strains have a one step conversion of xylose to xylulose (Hofer et al., 1971; Tomoyeda and Horitsu, 1964). This is performed in the presence of xylose isomerase. Such a reaction avoids the production of the metabolic by-product, xylitol.

In Candida utilis, the presence of an inducible xylose isomerase has been demonstrated. It has been described as an adaptive enzyme and is produced when the yeast is grown

on a medium containing xylose (Tomoyeda and Horitsu, 1964). In this strain, there also seems to exist a second pathway, a two step oxido-reduction, with xylitol as an intermediate. It is ~~not~~ known which enzymatic system is predominant in xylose catabolism. But, Candida utilis ATCC 22023 produces less xylitol than other common xylose utilising yeasts (Table 2). This may reflect in part, the participation of xylose isomerase in catabolism of the pentose.

Low concentrations of ethanol (0.6%) are produced from xylose (Schneider et al., 1981). Another yeast, Candida tropicalis produced 0.55% ethanol from xylose (Jeffries, 1981). In contrast, high ethanol concentrations (8%) are obtained in industrial fermentations on glucose (Phaff et al., 1978). This may be due to differences in intermediary metabolism of the sugars utilized, leading to ethanol formation .

Glucose is metabolized via the glycolytic pathway in which the hexose is converted to 2 pyruvates (Figure 2). Pyruvate may be metabolized via one of two modes. It may be oxidized to acetaldehyde, which in turn is reduced to ethanol. This reduction occurs in the presence of alcohol dehydrogenase and requires reducing power in the form of NADH. Alternatively, pyruvate may be decarboxylated to give CO₂ and acetyl CoA, the main precursor of the citric acid cycle. Acetyl CoA then condenses with oxaloacetate to form citrate, thus starting the citric acid cycle (Figure 2). This cycle (coupled to the electron transport pathway) is

Figure 2--Pathways of glucose and xylose metabolism in yeast. Both sugars channel intermediates into the citric acid cycle.

Table 2--By-product formation on xylose by various xylose
utilizing yeasts. Xylitol is the major by-product
(Gong and Tsao, 1983).

Organism (ATCC no.)	Accumulated Xylitol (%)
<u>Candida blankii</u> (18735)	1.82
<u>Candida parapsilosis</u> (28474)	3.32
<u>Candida tropicalis</u> (20215)	2.31
<u>Candida utilis</u> (22023)	1.28

the main energy supplier of the cell. Many of the intermediates of this cycle (citrate, alpha-ketoglutarate, succinate, malate, oxaloacetate) serve as precursors of many cellular constituents.

Xylose metabolism proceeds via the pentose phosphate cycle. Intermediates of this cycle are channelled into glycolysis. Further metabolism continues through the citric acid cycle and electron transport pathway. Regardless of the sugar metabolized, glucose or xylose, the citric acid cycle should be equally operational because of its vital role in energy generation and supply of precursors for the synthesis of cellular constituents. Therefore, it should be possible to obtain citric acid cycle organic acids from Candida utilis grown on xylose or glucose.

Candida utilis is thought to be homothallic and with the ability to sporulate at a low frequency (Perez-Bolanos and Herrera, 1981). In homothallic strains, once haploids are formed via meiosis, they have a strong tendency to diploidize (Esposito and Esposito, 1975). Since the great majority of mutations in yeast have a recessive effect, (Lacroute, 1975) it may have been ideal to use a haploid organism. Nevertheless, amino acid auxotrophs of Candida utilis have been isolated (Delgado, et al., 1976).

As a first step towards obtaining organic acid producers, mutants altered at various steps of the citric acid cycle (Figure 2) were desired. For example, citrate producing mutants may be affected at the second step in the

cycle, in which citrate is converted to isocitrate. Such mutants would be able to grow on isocitrate but would not be able to grow on citrate.

In Sacchromyces cerevisiae, the general procedure for selecting amino acid auxotrophic mutants consists of four steps: (i)mutagenesis, (ii)expression of mutation (iii)mutant enrichment, and (iv)isolation. In order to select a mutant A^- (auxotrophic for A) the following procedure may be followed. After mutagenesis, the cells are grown in minimal medium containing the substance (A) required by the desired auxotroph. The growth period is usually long enough for the cells to recover from the mutagenic treatment and for the auxotrophic mutation to be expressed. After expression of the mutation, the growth of the culture is stopped by starving for nitrogen (usually by the elimination of ammonium sulphate from unsupplemented minimal medium). The cells are then transferred to minimal medium with ammonium sulfate but without the growth requirement. In this medium, the wild type grows vigorously, while A^- mutants are unable to grow. After a 10-15% increase in cell number, (to ensure wild type cells are in log phase), nystatin is added. In dividing cells, nystatin binds to sterols (primarily ergosterol) in the cell membrane, causing distortions of the membrane and subsequent leakage of essential metabolites (Hamilton-Miller, 1973). After an exposure time of one and a half doublings, the majority of growing cells are killed and desired mutants

survive. The surviving population is plated on a medium containing A and then replica plated on to a minimal medium without A. Those able to grow on medium containing A, but unable to grow on medium without A are chosen as the desired mutants (A^-).

In this thesis, various citric acid cycle mutants were isolated using a similar scheme. These were characterized for fermentation ability on xylose and glucose. Effect of media composition and pH on organic acid production was also investigated. In an effort to reduce by-product (xylitol) formation, xylitol utilizing mutants were also derived from Candida utilis.

MATERIALS AND METHODS

(i) Strains, Media, Growth Conditions

All yeast strains are listed in Table 3. Candida utilis Y900 has been used extensively for the production of single cell protein from many carbon sources (Lawford et al., 1979; Sanchez-Marroquin, 1977). Yeast cells were grown in liquid medium with 2% carbon source (xylose, or glucose) and 0.67% yeast nitrogen base without amino acids (YNB). The composition of YNB is given in Table 4. Cultures were incubated at 30 °C and shaken at 200 rpm. For growth on plates, 15 g of Bacto-agar (Difco) was added per litre. Growth was followed by measuring absorbance at 500 nm (A_{500}) with a Bausch and Lomb Spectronic 70 spectrophotometer, using a round cuvette (11 mm inside diameter). One absorbance unit (AU) in A_{500} .ml contains about 4×10^7 cells.

Fermentations using Candida utilis and derived mutants were performed using two types of media. The carbon source consisted of 10% xylose or 10% glucose. The composition of the rich medium is given in Table 5. For fermentations on rich medium, cells were pregrown on a seed medium supplemented with 2% glucose and 0.67% YNB. The composition of the defined medium and the seed medium used for inoculation is given in Table 6. 0.1 ml of the seed culture containing 1×10^7 cells/ml was added to 25 ml of

Table 3--Yeast strains used.

Organism	Strain	Relevant Characteristics	Source	Reference
<u>Candida utilis</u>	Y900	wild type	P.S. Dawson, NRC, Prairie Laboratories	-
	CU 1			
	CU 2			
	CU 3			
	CU 4			
	CU 5			
	CU 6	CIT ⁻ AKG ⁺	This Lab	This paper
	CU 7			
	CU 8			
	CU 9			
	CU 10			
	CU 11			
	CU 12			
	CU 13			
	CU 14			
	CU 15	AKG ⁻ SUC ⁺	This Lab	This paper
	CU 16			
	CU 17			
	CU 18	OAA ⁻ CIT ⁺	This Lab	This paper

Mutants CU 1 through CU 12 are unable to grow on citric acid, but are able to grow on alpha-ketoglutarate. Mutants CU 13 through CU 17 grow slowly (leaky) on alpha-ketoglutarate, but are able to grow on succinate. Mutant CU 18 is unable to grow on oxaloacetate, but is able to grow on citrate.

Table 4--Composition of yeast nitrogen base, without amino acids (Difco).

INGREDIENTS (per 1)	QUANTITY
Nitrogen Source	
Ammonium Sulfate	5 g
Vitamins	
Biotin	2 ug
Calcium Pantothenate	400 ug
Folic Acid	2 ug
Inositol	2000 ug
Niacin	400 ug
p-aminobenzoic acid	200 ug
Pyridoxine hydrochloride	400 ug
Riboflavin	200 ug
Thiamine hydrochloride	400 ug
Compounds Supplying Trace Elements	
Boric Acid	500 ug
Copper Sulfate	40 ug
Potassium iodide	100 ug
Ferric chloride	200 ug
Manganese sulfate	400 ug
Sodium molybdate	200 ug
Zinc sulfate	400 ug
Salts	
Potassium phosphate monobasic	1 g
Magnesium sulphate	0.5 g
Sodium chloride	0.1 g
Calcium chloride	0.1 g
Total volume of final medium from 100 g dehydrated medium	14.9 l

Table 5--Composition of rich medium.

	Quantity (g/l)
Yeast Extract	4
KH_2PO_4	1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1

Table 6--Composition of defined medium and seed medium used for pregrowth of cells. Quantities indicated are per l of medium (Terasawa and Takahashi, 1981).

	SEED MEDIUM	DEFINED MEDIUM
Yeast Extract	0.2 g	-
$\text{Na}_2\text{H}_2\text{PO}_4$	1.5 g	-
NH_4Cl	6.5 g	2.0 g
KH_2PO_4	3.5 g	0.5 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g	0.5 g
Thiamine-HCl	100 ug	100 ug
Biotin	100 ug	100 ug
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	10 mg	10 mg
$\text{MnSO}_4 \cdot \text{nH}_2\text{O}$	0.1 mg	0.1 mg
$\text{ZnSO}_4 \cdot \text{n}7\text{H}_2\text{O}$	0.1 mg	0.1 mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	5 ug	5 ug

fermentation media in 250 ml erlenmyer flasks. Flasks were shaken at 240 rpm and 3% CaCO_3 was added for pH control after 24 h. Fermentations were run for 6 days.

(ii) Mutant Isolation

(a) Mutagenesis

Candida utilis was grown on glucose to exponential phase. 20 ml of this culture was filtered using 0.45 μ cellulose acetate membrane filters. The cells were then resuspended in 0.1 M sodium phosphate buffer (pH 6.5) to a density of approximately 1×10^7 cells/ml. 20 ml of the suspension in 100 ml beakers was mutagenized using a Cobalt 60-gamma irradiation source, supplied by the Atomic Energy of Canada at the National Research Council of Canada laboratories in Ottawa.

(b) Mutant Selection

A gamma ray dose of 40 kilorads, providing a 10% survival rate was chosen for selection of mutants. Three classes of citric acid cycle mutants were isolated: CIT^- AKG^+ , AKG^- SUC^+ , OAA^- CIT^+ .

In order to obtain expression of CIT^- AKG^+ mutants, the mutagenized culture was grown for 10 doubling times on 0.1% alpha-ketoglutarate in 0.67% YNB. In addition to allowing expression of the desired mutants, this step avoids the selection of mutants blocked after alpha-ketoglutarate. The

cells were washed free of alpha-ketoglutarate and were incubated without a carbon source, in yeast nitrogen base. After three hours, citric acid was added to a final concentration of 0.1%. This allows wild type cells and mutants blocked before citrate to divide, while CIT^-AKG^+ mutants are unable to do so. Growth is continued until there is a 15% increase in absorbance. The cells were then incubated in the presence of 15 ug/ml nystatin, for 1.5 h. After washing with 5 volumes of 10 ml distilled water, the cells were grown again for 10 doublings on alpha-ketoglutarate. After carbon starvation and addition of citrate, cells were treated with nystatin as described above. After washing well with distilled water, cells were plated on to 0.1% alpha-ketoglutarate. Colonies appearing on this medium were replica plated on to 0.1% citrate. Those able to grow on alpha-ketoglutarate but not on citrate after three days, were chosen as the desired mutants (CIT^-AKG^+).

Two other classes of citric acid cycle mutants were isolated in a similar fashion. Relevant changes are indicated. In both cases, two cycles of mutant expression, carbon starvation, followed by treatment with nystatin were carried out. Expression of AKG^-SUC^+ mutants was obtained on 0.1% succinate for 14 doubling times. After carbon starvation, alpha-ketoglutarate was added to a final concentration of 0.1%. After a 15% increase in absorbance, nystatin was added. After washing well with distilled

water, cells were plated on succinate and replica plated on to alpha-ketoglutarate. Those able to grow on succinate but exhibiting leaky growth on alpha-ketoglutarate after 3 days were chosen as AKG^-SUC^+ mutants.

OAA^-CIT^+ mutants were expressed by growing the mutagenized culture on 0.1% citrate for 7 doubling times. After carbon starvation, oxaloacetate was added to a final concentration of 0.1%. After a 15% increase in absorbance, nystatin was added. After washing with distilled water, cells were plated on citrate and replica plated on to oxaloacetate. Those able to grow on citrate, but unable to grow on oxaloacetate after 3 days were chosen as the desired mutants (OAA^-CIT^+).

(iii) Detection of Organic Acids

(a) Chromatography

Fermentations were conducted with the addition of CaCO_3 , for pH control. The organic acids were regenerated from their calcium salts, by adjusting the pH to 2 with 18 N H_2SO_4 .

For rapid screening of organic acids produced by various mutants, samples were analyzed by ascending paper chromatography, using No.1 Whatman filter paper. 60 ul of sample was applied and organic acids were separated in a solvent system containing n-butanol, acetic acid, and H_2O (12:3:5) in 12 h. After air drying the chromatograms thoroughly, organic acids were detected using the bromocresol green reagent. This was prepared in the following manner. Bromocresol green was dissolved in ethanol to a final concentration of 0.1%. 1 N NaOH was added to the dye solution until the colour became blue-green. Before use, 1 volume of that solution was mixed with 4 volumes of acetone. The chromatograms were then rapidly drawn through the indicator solution, laid flat on a sheet of paper and the yellow spots (on a green background) indicate the presence of organic acids. Basic components appear on the chromatograms as blue spots and colour development of organic acids was complete after 12 h. For detection of amino acids, chromatograms were run exactly as described above. They were developed by dipping through

0.2% ninhydrin in acetone. The chromatogram was hung up in the cold and left for 12 h for full development.

(b) Chemical Method

Three citric acid cycle acids, citrate, isocitrate and aconitate were measured using the acetic anhydride-pyridine method (Saffran and Denstedt, 1948). It detects these organic acids according to the Furth-Hermann reaction in which yellow color is formed in the presence of pyridine and acetic anhydride. The sample was prepared to contain a final concentration of 5% (w/v) trichloroacetic acid.

Acetic anhydride (1.6 ml) was added to 0.2 ml of the extract prepared above. The mixture was heated at 60 °C for 10 min and cooled to room temperature by immersion in cold water. Pyridine (0.2 ml) was added in a screw cap test tube and this was heated at 60 °C for 40 min. The tube was then cooled in ice and the extinction of the solution was determined at 420 nm (light path 1 cm). Under these conditions, the method measures aconitate, isocitrate and citrate with various specificities (Table 7).

Trans-aconitate gives an extinction coefficient that is 15% higher, and cis-aconitate that is 35% lower than that for citrate. Isocitrate gives an extinction coefficient that is only 25% of citrate. Aconitate was measured separately by carrying out the acetic anhydride-pyridine method at 0 °C: After reaction of the sample with acetic anhydride at 60 °C for 10 min, cold pyridine (0 °C) was added. The reaction was

Table 7--Molar extinction coefficients by the acetic anhydride-pyridine method for determination of three citric acid cycle acids (Saffran and Denstedt, 1948)

Compound	Molar Extinction Coefficient (425 nm)
Citrate	3620
Isocitrate	900
cis-Aconitate	2360
trans-Aconitate	4160

Table 8--Absorbance of citric acid cycle acids at 60 °C and 0 °C.

	Absorbance	
	60 °C	0 °C
Citrate	0.469	0.006
Isocitrate	0.116	0.006
cis-Aconitate	0.435	0.278
trans-Aconitate	0.459	0.478

carried out for 60 min at 0°C. Under these conditions, aconitate gives the color reaction, whereas citrate and isocitrate give little or no color (Table 8).

(c) High Pressure Liquid Chromatography

Citrate and Isocitrate standards at a concentration of 1 mg/ml in doubly distilled water were used. Fermentation samples and standards were filtered through 0.2 μ paper filters. 10 μ l of sample were injected onto a Alltech C-18 column. Organic acids were detected by a single path UV monitor at 214 nm.

(d) Enzymatic Analyses

In order to confirm the organic acids produced by specific enzymatic methods, the fermentation liquors were subject to removal of metals and inorganic salts, which may interfere with such analyses. This was performed using first, a Dowex 50-X8 (H^+) in the hydrogen form, to remove all cations (Mg^{+2} , Ca^{+2} , K^+ , Na^+). After washing with four volumes of 10 ml double distilled water, the eluant was passed through a Dowex-1-10 formate column. This allows organic acids and inorganic anions (PO_4^{-2} , SO_4^{-2} , Cl^-) to adsorb on the column. The organic acids were then selectively eluted using five volumes of 10 ml 8 N formic acid. The sample was then concentrated to 2 ml using a rotoevaporator.

Both citric and isocitric acid were measured

enzymatically, using food analysis kits (Boehringer). Citric acid is converted to oxaloacetate and acetate in a reaction catalysed by the enzyme citrate lyase (CL):

When in the presence of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH), oxaloacetate and its decarboxylation product, pyruvate are reduced to L-malate and L-lactate, respectively by NADH:

The amount of NADH oxidized in the last two reactions is stoichiometric with the amount of citrate.

Using a cuvette (1 cm light path), the oxidation of NADH was measured at 340 nm and 25°C. Absorbances were taken before (A_1) and after (A_2) mixing the sample with citrate lyase using the following procedure. 1 ml of solution 1 (containing glycine buffer pH 7.8, malate dehydrogenase--ca. 136 U, lactate dehydrogenase--ca. 284 U, NADH-6.0 mg) was added to 2 ml of doubly distilled deionized water to make a blank. For the samples containing citric acid, 1 ml of solution 1 was added to 1.8 ml of doubly distilled water, in addition to 0.2 ml of the sample solution. After mixing, the solutions were incubated at room temperature (25°C) for 5 min and the absorbances (A_1) were read at this time. To start the reaction, 0.02 ml of

solution 2 (citrate lyase--12 U) were added and mixed. After completion of the reaction (approx. 5 min), the absorbances (A_2) of the solutions were obtained.

The absorbance differences ($A_1 - A_2$) for both the blank and the sample were then determined. After subtracting the absorbance difference of the blank from the absorbance difference of the sample,

$$\Delta A = \Delta A_s - \Delta A_b$$

the amount of citrate in the sample is given by the general formula,

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \Delta A$$

where, V= final volume (ml)

v= sample volume (ml)

MW= molecular weight of citrate

ϵ = absorbance coefficient of NADH at 340 nm

d= light path (cm)

For citric acid, monohydrate, the concentration is given by:

$$c = \frac{3.02 \times 210.1}{6.3 \times 0.2 \times 1 \times 1000} \Delta A \text{ g citric acid monohydrate/l}$$

If the sample was diluted during preparation, the result was multiplied by the dilution factor.

D-Isocitric acid is oxidatively decarboxylated by nicotinamide-adenine dinucleotide phosphate (NADP) in the presence of the enzyme isocitrate dehydrogenase (ICDH).

The NADPH formed in this reaction is stoichiometric with the amount of D-isocitrate present. Using a cuvette (1 cm light path), the reduction of NADP was measured at 340 nm at 25 °C

1.00 ml of solution 1 (containing 30 ml imidazole buffer--pH 7.1 with 45 mg NADP, 10 mg manganese sulphate) was mixed with 2.00 ml of redistilled water to make up the blank. 1.00 ml of solution 1 was mixed with 1.90 ml redistilled water and 0.10 ml of the sample solution containing isocitrate. After mixing, the absorbances (A_1) were read after 3 min. The reaction was started by addition of 0.05 ml of solution 2 (containing ICDH-5U in 1.8 ml redistilled water). After mixing and allowing the reaction to proceed to completion (approx. 10 min), the absorbances (A_2) of the solutions were again read.

The absorbance differences ($A_2 - A_1$) for both the blank (A_b) and the sample (A_s) were then determined. After subtracting the absorbance difference of the blank from the absorbance difference of the sample,

$$\Delta A = \Delta A_s - \Delta A_b$$

the concentration of isocitric acid may be calculated, using the general formula stated previously for citric acid. Finally then, the concentration of D-Isocitric acid is given by:

$$c = \frac{3.05 \times 192.1}{6.3 \times 0.1 \times 1000} \Delta A$$

If the sample was diluted during the preparation, the result was multiplied by the dilution factor.

RESULTS

A. ISOLATION OF CITRIC ACID CYCLE MUTANTS

(i) Mutagenesis

Candida utilis was mutagenized with ⁶⁰Co-gamma irradiation. The dose response curve is shown in Figure 3. This is generally a very drastic mutagen and causes gross changes in DNA structure. It essentially causes two types of damage to DNA: (i) direct and (ii) indirect. The former results in single or double strand breaks and is rather rare. Indirect damage is mediated by free radicals, which also produce single and double strand breaks (Nasim and James, 1978).

An arbitrary level of 10% survival, provided by a dose of approximately 40 kilorads was chosen for selection of mutants. A lower survival rate may have increased mutant frequency, but this also may have resulted in an increase in secondary mutations. These should be minimised, especially in cases where a correlation between secondary regulatory changes (e.g. organic acid production) with a primary enzymatic defect is required. In such cases, secondary mutations could completely confuse the issue (Lacroute, 1975).

(ii) Nystatin Treatment

In Sacchromyces cerevisiae enrichment with nystatin increases amino acid auxotroph frequencies (Snow, 1966).

Figure 3--Dose response curve of Candida utilis to

⁶⁰Co gamma-irradiation. Cells were grown to exponential

phase in 2% glucose in YNB. After washing the cells with distilled water, they were suspended in phosphate buffer

a cell density of 1×10^7 /ml. 20 ml portions in 100 ml beakers were exposed to various doses of gamma rays.

Use of such an enrichment procedure requires nitrogen starvation before treatment with the antibiotic. But, in this study mutants of the citric acid cycle were desired. The compounds of this cycle, citrate, isocitrate, alpha-ketoglutarate, succinate, fumarate, malate and oxaloacetate consist essentially of carbon skeletons. Therefore, in order to isolate mutants altered at various steps of this cycle, it was necessary to use carbon starvation.

Candida utilis was grown for 10 doublings on alpha-ketoglutarate, starved for carbon and after growth on citrate, various concentrations of nystatin were added. 15 ug/ml nystatin provided a kill of 96% (Figure 4). This concentration was used for isolation of desired mutants.

Three classes of citric acid cycle mutants were selected. CIT^-AKG^+ mutants were chosen as those that grew on alpha-ketoglutarate but not on citrate after three days. AKG^-SUC^+ mutants grew on succinate but were leaky on alpha-ketoglutarate after three days. One OAA^-CIT^+ mutant grew on citrate but not on oxaloacetate after three days.

The relevant isolation statistics of the three classes of citric acid cycle mutants are given in Table 9. All three classes of mutants were isolated after two cycles of mutant expression, carbon starvation and nystatin treatment. It is more than likely that isolates do not represent independent mutational events; clones were most likely isolated. Some CIT^-AKG^+ , AKG^-SUC^+ , OAA^-CIT^+ mutants may be a result of

Figure 4--Dose response curve of Candida utilis to nystatin. Cells were grown for 10 doubling times on 0.1 % alpha-ketoglutarate in 0.67% YNB. After washing with distilled water, they were incubated without a carbon source. After 3 h, citric acid was added. After a 15% increase in absorbance, cells were incubated in the presence of various concentrations of nystatin for 1.5 h.

Table 9--Isolation statistics of three classes of citric acid cycle mutants[†]

Mutant Class	Relevant Phenotype	%Survival after Nystatin Treatment ^{††}	<u>Mutants Detected</u> Total Replica Plated	(%)
I	^{†††} CIT ⁻ AKG ⁺	4.0	93/697	(13.3)
II	AKG ⁻ SUC ⁺	1.3	7/560	(1.2)
III	OAA ⁻ CIT ⁺	0.8	1/450	(0.22)

[†] The mutagenized culture (10% survival by gamma-irradiation) was carried through two cycles of expression, starvation and nystatin treatment. This was performed separately for each of three classes of mutants desired.

^{††} These values indicate % survival after the second treatment with nystatin.

^{†††} The percent of class I mutants (CIT⁻ AKG⁺) detected after gamma-irradiation was found to be 1.8% (14/750).

secondary mutations. In particular, if some class I, II and III mutants are affected in permease activity, they would lack the ability to uptake citrate, alpha-ketoglutarate and oxaloacetate respectively. Nystatin seems to increase proportions of $CIT^- AKG^+$ mutants detected and hence may act as an enrichment agent. This may explain in part, the high number of class I mutants (13.3%) detected when cells were treated with nystatin. It may be speculated that, this class of mutants has a much higher rate of secondary mutations than the two other classes. However, this is difficult to prove.

B. FERMENTATIONS

Citric acid cycle mutants isolated may exhibit various degrees of leakiness. For the overproduction of organic acids, non-leaky mutants were desired. In an effort to select such promising mutants among those isolated, growth rates were compared in liquid medium. CIT^-AKG^+ , AKG^-SUC^+ and OAA^-CIT^+ , mutants were grown on citrate, alpha-ketoglutarate and oxaloacetate respectively. Doubling times of the three classes of citric acid cycle mutants are given in Tables 10, 11, 12. Those strains exhibiting little or no growth over an 8 h incubation period, have been designated by >>> WT for the doubling time. All CIT^-AKG^+ , mutants exhibited some growth on citrate. The majority of mutants tested had doubling times two to four times longer than that of the wild type. Four mutants, CU 1, CU 2, CU 4 and CU 12 grew very slowly on citrate (Table 10). Two AKG^-SUC^+ , mutants (CU 13, CU 14) exhibited slightly longer doubling times than the wild type on alpha-ketoglutarate. Three mutants (CU 15, CU 16, CU 17), grew very slowly on alpha-ketoglutarate (Table 11). One OAA^-CIT^+ mutant, CU 18 grew very slowly on oxaloacetate (Table 12).

Growth of citric acid cycle mutants, generally revealed that class I, II and III mutants have slower growth rates on citrate, alpha-ketoglutarate and oxaloacetate respectively than the wild type. Those mutants exhibiting very slow growth on such media were then tested for fermentation character. Samples were analyzed by paper

Table 10--Doubling times of *C. utilis* and derived CIT⁻ AKG⁺ mutants on citrate.

Strain	Doubling Time (h)
WT	2.3
CU 1	>>> WT
CU 2	>>> WT
CU 3	6.0
CU 4	>>> WT
CU 5	6.0
CU 6	6.0
CU 7	8.5
CU 8	8.0
CU 9	3.2
CU 10	4.0
CU 11	6.0
CU 12	>>> WT

Growth curves were conducted over an incubation period of 8 h. Those mutants exhibiting little or no growth on citrate have been designated to have a doubling time much greater than the wild type (>>> WT).

Table 11--Doubling times of C. utilis and $\text{AKG}^- \text{SUC}^+$ mutants on alpha-ketoglutarate.

Strain	Doubling Time (h)
WT	2.8
CU 13	3.8
CU 14	3.0
CU 15	^a >>> WT
CU 16	>>> WT
CU 17	>>> WT

^a The doubling time of those mutants exhibiting little or no growth over 8 h is designated by >>> WT.

Table 12--Doubling times of C. utilis and one $\text{OAA}^- \text{CIT}^+$ mutant^a on oxaloacetate.

Strain	Doubling Time (h)
WT	1.5
CU 18	>>> WT

^a This mutant exhibited little or no growth over 8 h.

chromatography for organic acids. This is a relatively simple and rapid technique which allows simultaneous product analysis of a large number of fermentation samples. Organic acids were detected as yellow spots on a blue-green background. For fermentations run on glucose and xylose, the total acid yield by the wild type is designated by +. According to the increase in size and intensity of the yellow region(s), for mutant samples, qualitative increases are indicated in Table 13.

Class I mutants, CU 1, CU 4 and CU 12 exhibited the greatest increase in organic acid production on glucose and xylose. CU 8 did not show any signs of organic acids, on either carbon source. Of three class II mutants tested, CU 15 exhibited the largest increase in organic acid production on both carbon sources. The sole class III mutant, CU 18 showed a higher organic acid production on xylose than glucose.

Comparison of R_f values of organic acids produced by these mutants, generally revealed that they may be producing several organic acids (Table 14). Since most mutants generated one broad region on paper chromatograms, it became difficult to identify single organic acids.

Most class I mutants exhibited organic acids with R_f values close to those of citrate, isocitrate and malate on both carbon sources. On glucose, two mutants, CU 2 and CU 12 exhibited organic acids with R_f values similar to aconitate and itaconitate. CU 1, CU 4, CU 12 were discovered

Table 13--Detection of total organic acids produced by mutants on xylose and glucose, using paper chromatography.

Mutant class	Strain	Carbon Source	
		Xylose	Glucose
	WT	+	+
	CU 1	++++	++++
	CU 2	++	++++
	CU 4	++++	++++
I	CU 7	+++	++
	CU 8	-	-
	CU 12	+++	++++
	CU 15	+++	++++
II	CU 16	++	++
	CU 17	++	++
III	CU 18	++++	++

(+) Indicates the size of the yellow region for the wild type fermentations run on xylose and glucose. Increases in size and intensity of these yellow regions generated by mutant samples is indicated by multiples of (+).

Table 14--Probable organic acids detected by paper chromatography.

Mutant Class	Relevant Phenotype	Probable Product(s) Detected
I	CIT ⁻ AKG ⁺	Citrate, Isocitrate, Malate, Aconitate, Itaconitate
II	AKG ⁻ SUC ⁺	Glutamate Alpha-ketoglutarate
III	OAA ⁻ CIT ⁺	Glutamate

† A single yellow region was detected for most fermentation samples. Comparison of R_f values to standards shows that a number of acids may be present.

to have the best fermentation ability on glucose and xylose. Class 2 mutants produced compounds with R_f values similar to those of alpha-ketoglutarate and glutamate on xylose and glucose fermentations. Class III mutant, CU 18 produced a compound with an R_f similar to that of glutamate from xylose and glucose.

Mutants generally exhibited greater organic acid production than the parent, but with successive fermentations, the yields (roughly judged by the size and intensity of the yellow spot on paper chromatograms) seemed to decrease. Although class I mutants CU 1, CU 4 and CU 12 still exhibited greater organic acid production than the wild type, class II and class III mutants did not exhibit signs of overproduction of organic acids. Therefore, it became necessary to reconfirm the original characteristics of the three classes of mutants.

Three class I mutants exhibited leaky growth on citrate. Viable counts of the wild type and $CIT^- ARG^+$ mutants were taken over an incubation period of 5 days. No colonies were detected for the wild type or the mutants before two days on citrate or alpha-ketoglutarate. On alpha-ketoglutarate, the total viable count for the wild type and the mutants was established after 2 days (Table 15). On citrate, the viable count for the wild type was established after 2 days. The size of the colonies was 2 mm. No colonies were detected for the mutants after 2 days. Small colonies (0.5 mm) were observed for CU 1 after 3 days.

Table 15--Viable counts of *C. utilis* Y900 and three mutants CU 1, CU 4, and CU 12 on citrate and alpha-ketoglutarate over an incubation period of 5 days.

Strain	Viable Counts				
	Alpha-Kg ^a	Citrate ^b			
	2-5 days	2 days	3 days	4 days	5 days
WT	1.00×10^8	1.23×10^8	1.23×10^8	1.23×10^8	1.23×10^8
CU 1	7.10×10^7	$< 1 \times 10^3$	1.90×10^4	1.20×10^5	5.00×10^5
CU 4	1.27×10^8	$< 1 \times 10^3$	$< 1 \times 10^3$	2.00×10^4	6.50×10^4
CU 12	8.80×10^7	$< 1 \times 10^3$	$< 1 \times 10^3$	$< 1 \times 10^3$	1.00×10^4

^a Viable counts of mutants and the wild type on alpha-ketoglutarate were established after 2 days.

^b On citrate, the viable count for the wild type was established after 2 days. The size of the colonies was 2 mm. No colonies were detected for the mutants after 2 days. Small colonies (0.5 mm) were observed for CU 1 after 3 days and for CU 4 after 4 days. These increased in size (2 mm) after 5 days. Small colonies (0.5-1.0 mm) were detected for CU 12 after 5 days.

Figure 5--Change in viable counts of CU 1 (O), CU 4 (Δ), and CU 12 (Δ) on citrate over an incubation period of 5 days. Cells were plated on citrate and incubated at 30°C. The dotted line indicates that viable counts are less than 1×10^3 cells/ml.

and for CU 4 after 4 days. These increased in size (2 mm) after 5 days. Small colonies (0.5 mm-1mm) were detected for CU 12 after 5 days. The viable count of these mutants on citrate remained significantly less (factor $10^2 - 10^4$) than that on alpha-ketoglutarate after 5 days. Such results were found to be reproducible. The rate of increase of viable counts of these mutants on citrate is depicted in Figure 5. The growth behaviour of mutants on citrate may indicate degrees of leakiness of aconitase or isocitrate dehydrogenase.

Similarly, growth characteristics of the two other classes of mutants were compared on solid media. Table 16 gives the viable counts on alpha-ketoglutarate and succinate for class II mutants after an incubation period of 2 days. No growth was observed during the first 2 days for either the parent or the mutants. Therefore mutants, like the parent strain exhibited little or no difference in viable counts on alpha-ketoglutarate and succinate. Table 17 gives the viable counts on citrate and oxaloacetate for one OAA⁻ CIT⁺ mutant, after 2 days of incubation. Both the wild type and CU.18 showed similar viable counts on oxaloacetate and citrate. Since class II and class III mutants seem to have lost their original characteristics, they were not investigated further for organic acid production. Mutants of these classes may have an inherent high degree of instability. It is possible that instability is due to

Table 16--Viable counts of *C. utilis* and AKG⁻ SUC⁺ mutants on alpha-ketoglutarate, and succinate after 2 days^a

Strain	Viable Counts	
	Alpha-ketoglutarate	Succinate
WT	9.6×10^6	1.5×10^7
CU 15	1.2×10^7	1.9×10^7
CU 16	2.0×10^7	3.2×10^7
CU 17	2.4×10^7	3.2×10^7

^a No growth was detected for wild type or mutants before two days on alpha-ketoglutarate or succinate.

Table 17--Viable counts of *C. utilis* and one OAA⁻ CIT⁺ mutant on citrate and oxaloacetate^a

Strain	Viable Counts	
	Oxaloacetate	Citrate
WT	1.3×10^7	1.1×10^7
CU 18	1.3×10^7	1.2×10^7

^a No growth was detected for wild type or mutant before 2 days on citrate or oxaloacetate.

mitotic recombination. This event has the potential for the reassortment of recessive alleles, leading to the masking of the mutation. It occurs at considerably higher frequencies than mutation and has been attributed to be the probable cause of instability of diploid cultures (Mortimer and Hawthorne, 1969). But mutants CU 1, CU 4 and CU 12 should also be subject to such recombination pressure. This does not appear to be the case, as judged by growth on citrate and alpha-ketoglutarate plates. Although such mutants are somewhat leaky, they are stable.

C. DETECTION OF FERMENTATION PRODUCTS OF CIT⁻ AKG⁺ MUTANTS

(a) Chemical Assay

For the analysis of organic acids by the chemical assay, various citric acid cycle acids may be detected. Since some of these have similar structures, the chemical configurations are shown in figure 6.

CITRATE

ISOCITRATE

ACONITATE

Figure 6--Chemical structures of several citric acid cycle acids.

Fermentation products of three mutants CU 1, CU4, CU 12 were analyzed by the acetic anhydride-pyridine method. Three citric acid cycle acids, citrate, isocitrate and aconitate were measured. According to this method, total citrate plus isocitrate can be calculated by carrying out the assay at two temperatures, 60 °C and 0 °C. Only aconitate is measured at 0 °C, while all three acids are measured at 60 °C. Therefore total citrate plus isocitrate can be obtained by subtracting the absorbance reading at 0 °C from the absorbance reading at 60 °C. No aconitate was detected on xylose or glucose fermentations (Table 18). A_{420} indicates the intensity of the yellow color at 420 nm. But, for assays conducted at 60 °C, small particulate matter was found to be suspended in the samples. Upon filtering such samples through 0.45 μ membrane filters, this interference was not eliminated. As a result, samples with low intensities of yellow color (xylose fermentations) do not represent a true indication of total organic acids present. In glucose samples, the same interference was also present, but the greater part of the absorbance is attributable to the yellow color. This interference is thought to be due to precipitated proteins, not fixed by trichloroacetic acid. Higher concentrations of trichloroacetic acid could be used, but these interfere with the formation of yellow color (Saffran and Denstedt, 1948). In addition to this point,

Table 18--Analysis of fermentation products on xylose and glucose by the acetic anhydride-pyridine method[†].

	Xylose		Glucose	
	A_{420}		A_{420}	
	60°C	0°C	60°C	0°C
CU 1	0.1397	0	0.1871	0
CU 4	0.0757	0	0.3010	0
CU 12	0.1177	0	0.2441	0
WT	0.0605	0	0.1821	0

[†] Absorbances at 60 °C indicate total citrate, isocitrate and aconitate. Absorbances at 0 °C indicate aconitate.

the assay requires fresh pyridine for optimum performance. Upon exposure to air or light, this chemical renders the assay less effective. Although this method has obvious limitations, it does permit rapid and simultaneous detection of three citric acid cycle acids. But, accurate measurement by other methods is clearly necessary.

(b) High Pressure Liquid Chromatography

In an effort to measure citrate and isocitrate more precisely, two techniques were utilized, high pressure liquid chromatography and enzymatic analysis. High pressure liquid chromatography allows quick and efficient separation of many biological compounds, including organic acids. It has two big advantages over enzyme analysis. Firstly, it requires no prior clean up of sample before analysis, i.e. removal of metals and inorganic salts that may interfere with enzyme activities. In addition it measures a mixture of organic acids simultaneously, in contrast to separate assays for each organic acid by the enzymatic method.

Using high pressure liquid chromatography, citrate and isocitrate standards were discovered to have retention times of 8.5 min and 6.5 min respectively. But in fermentation samples analyzed using the Alltech NH column, these organic acids could not be resolved.

(c) Enzymatic analysis

Next, fermentation samples were analyzed by enzymatic analysis. But, since the defined media contains inorganic salts ($\text{Na}_2\text{H}_2\text{PO}_4$, NH_4Cl , KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and metals ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), enzymatic analysis required removal of such substances because of possible interference. Fermentation samples were treated first with Dowex-50-X8 (H^+) to remove all cations (Fe^{+2} , Zn^{+2} , Mn^{+2} , Mg^{+2} , K^+ , Na^+) and subsequently with Dowex-1-10 to remove inorganic anions (Cl^- , PO_4^{-2} , SO_4^{-2} etc.) and organic acids. After selective elution of organic acids, the sample was concentrated with a rotoevaporator.

Both citrate and isocitrate were measured from xylose fermentations. All three mutants, CU 1, CU 4 and CU 12 produced more citrate than the wild type. CU 4 and CU 12 exhibited increases of 127% and 54% respectively (Table 19).

The mutants did not show significant increases in isocitrate. Only CU 1 and CU 4 exhibited slightly higher isocitrate yields than the wild type; CU 12 showed a decrease in isocitrate yield. Overall, CU 1, CU 4 and CU 12 exhibited increases of 23%, 133% and 38% with respect to total citrate and isocitrate over the wild type.

In an effort to assess if the enzymatic method represents a true indication of the total citrate and isocitrate before removal of salts and metal ions, the chemical assay was utilized to measure total citrate plus

Table 19--Enzymatic determination of citrate and isocitrate after removal of interfering substances from fermentation samples.

	Citrate (mg/ml)	Isocitrate (mg/ml)	Total (mg/ml)	% Increase Citrate + Isocitrate
WT	0.022	0.0032	0.0252	-
CU 1	0.027	0.0040	0.0310	23
CU 4	0.050	0.0048	0.0588	133
CU 12	0.034	0.0008	0.0348	38

Table 20--Absorbances of two fermentations samples, indicating total citrate plus isocitrate, before and after removal of interfering substances.

	Before Treatment †† A ₄₂₀	After Treatment A ₄₂₀
CU 12	0.1483	0.0840
WT	0.0376	0.0297

† fermentations were conducted on rich medium with xylose as carbon source.

†† A₄₂₀ indicates total citrate and isocitrate as detected by the acetic anhydride-pyridine method.

isocitrate before and after such treatment. Although fermentations were conducted on rich medium containing xylose, Table 20 clearly shows that significant losses may result from the method employed for removal of metals and inorganic salts from fermentation samples. Losses were calculated, assuming equal interference from suspended particulate matter in all samples.

The mutant, CU 12, (CIT⁻AKG⁺) mutant exhibited a 44% total loss in citrate plus isocitrate. Since such high losses in organic acids were encountered, it became necessary to modify the measurement of such acids by the enzymatic method.

In order to decrease the inhibition by interfering substances, various dilutions of fermentation samples were utilized to assay for citrate and isocitrate. But, in order to achieve this goal, it became necessary to determine the appropriate dilutions at which no inhibition would be encountered by interfering substances. On the assumption that inhibition by such substances may be equal in wild type and mutant fermentation samples, regardless of sugar metabolized, appropriate dilutions were determined only for wild type fermentations run on glucose. A known amount of citrate was added to dilutions of the wild type sample. The addition, of 0.250 mg/ml citrate to the undiluted wild type sample, gives a lower reading than the amount detected in the standard (Table 21). Therefore, there seems to be an inhibition of detection of citrate in this sample. A 1:2

Table 21--Affect of undiluted wild type fermentation sample on the enzymatic detection of citrate!

	Citrate Detected (mg/ml)
Citrate Standard (0.25 mg/ml)	0.230
Wild Type Sample	0.080
Citrate Standard plus Wild Type Sample	0.152

A known amount of citric acid standard was added to wild type fermentation sample. Citric acid was determined enzymatically in the standard, wild type and the mixture of the two.

Table 22--Affect of diluted wild type fermentation sample (1:2) on the enzymatic detection of citrate.

	Citrate Detected (mg/ml)
Citrate Standard (0.50 mg/ml)	0.517
Wild Type Sample (1:2)	0.050
Citrate Standard plus Wild Type Sample (1:2)	0.555

Wild type fermentation sample was diluted (1:2) with doubly distilled water.

dilution of the wild type, seems to be suitable for detection of citrate (Table 22). The addition of 0.50 mg/ml citrate to such a diluted sample, gives an amount (0.555 mg/ml) that is almost yielded by the addition of the standard and wild type (0.567 mg/ml). On the assumption that inhibition by interfering substances may be equal in wild type and mutant fermentations, a 1:2 dilution was utilized for detection of citrate in mutant fermentation samples.

The enzymatic measurement of isocitrate is conducted under a completely different assay system than that used for citrate. Therefore, it became necessary to determine the appropriate dilution necessary to reduce inhibition by interfering substances. Again, wild type fermentation sample run on glucose was utilized. It was discovered that a 1:1 dilution was suitable for the assay of isocitrate (Table 23). On the assumption that inhibition by interfering substances may be equal in wild type and mutant fermentations, a 1:1 dilution was utilized for detection of isocitrate in mutant fermentation samples.

For fermentation on defined media, with glucose and xylose as carbon sources, citrate and isocitrate were measured using the appropriate dilutions. On xylose, the three mutants produced significantly more citrate than the wild type. CU 1, CU 4 and CU 12 exhibited increases of 88%, 150% and 65% respectively (Table 24). But, little isocitrate was made by these mutants.

Table 23--Affect of diluted wild type fermentation sample (1:1) on the enzymatic detection of isocitrate.

	Isocitrate Detected (mg/ml)
Isocitrate Standard (0.150 mg/ml)	0.154
Wild Type (1:1)	0.040
Isocitrate Standard plus wild type (1:1)	0.198

Table 24--Citrate and Isocitrate yields of three CIT⁻ AKG⁺ mutants as detected by enzymatic analysis.

	Xylose		Glucose		Total (mg/ml)
	Citrate (mg/ml)	Isocitrate (mg/ml)	Citrate (mg/ml)	Isocitrate (mg/ml)	
CU 1	0.049 (.046-.065) ^a	0.007	0.067 (.065-.071) ^b	0.042 (.040-.049)	0.109
CU 4	0.065 (.050-.070)	0.006	0.103 (.097-.114)	0.120 (.104-.127)	0.223
CU 12	0.043 (.034-.054)	0.005	0.161 (.150-.164)	0.075 (.070-.079)	0.236
WT	0.026 (.016-.028)	0.007	0.150 (.140-.155)	0.040 (.038-.041)	0.190

^a All samples were diluted 1:2 and 1:1 for detection of citrate and isocitrate respectively.

^b Total citrate plus isocitrate concentration in glucose fermentations.

^a Values represent range of citrate and isocitrate detected for 4 determinations.

^b Values represent range of citrate and isocitrate detected for 3 determinations.

On glucose, CU 4 and CU 12 produced significantly more isocitrate than the wild type (87% and 199% respectively). But, the mutants made little citrate; in fact CU 1 and CU 4 had significantly lower yields. Only CU 12 exhibited a comparable yield to that of the wild type. The calculation of total citrate plus isocitrate showed that only CU 4 and CU 12 produce more than the wild type (17% and 24% respectively). A comparison of organic acid yield on the two carbon sources, reveals that the mutants produce 2-5 times more on glucose than on xylose. One puzzling fact is that for fermentations on glucose, total acids for CU 1, CU 4, CU 12 and the wild type (as detected by paper chromatography), does not correlate with that detected by enzymatic analysis. But, paper chromatography is a qualitative method and designations given in Table 13 represent at best rough comparisons of total acids. But, more to the point, this method seems to suffer somewhat from the lack of reproducibility. Therefore, comparisons between organic acids detected by this method and the enzymatic assay should be made with some caution. Although mutants were originally judged to have decreased yields by paper chromatography, such comparisons may not be entirely accurate.

D. EFFECT OF MEDIA COMPOSITION AND pH ON CITRIC ACID
PRODUCTION

(a) Media Composition

Citric acid production generally requires two phases. The first phase is that of vigorous growth of cells and nitrogen limitation. This usually occurs within the first 18 h of the fermentation. The second phase is that of acid secretion and deceleration of growth (Mandeeva *et al.*, 1982). Organic acid production continues till exhaustion of the carbon source (Lozinov, 1975). This may last for 4-5 days. It was hypothesized that a richer media, containing yeast extract may allow a quicker growth of cells, with high cell yield and thereby lead to increased citric acid yields.

Rich media containing a few salts and yeast extract was then utilized for fermentations on xylose. Citrate was detected enzymatically. All three mutants produced significantly more citrate than the wild type (Table 25). But only CU 1 and CU 12 citrate yields on rich media increased over those found on defined medium (49% and 91% respectively).

Table 25--Citrate yields by three mutants with fermentations on rich medium and xylose as carbon source. These are compared to those found on defined medium[†].

	Rich Medium	Defined Medium
	Citrate (mg/ml)	Citrate (mg/ml)
CU 1	0.073	0.049
CU 4	0.067	0.065
CU 12	0.082	0.043
WT	0.022	0.026

[†] All samples were diluted 1:2 with doubly distilled water, before detection by the enzymatic method.

(b) pH Control

Glucose fermentations generally result in a drastic drop of pH. After 12 h the pH drops from 5.5 to 2. At such low pH values, citric acid production is clearly inhibited and regulation of pH close to neutral (pH 7) is required for organic acid production. This is performed by the addition of CaCO_3 . Many yeasts produce high yields of citric acid between pH 4.5 and 6.5 (Marchal, et al., 1977). It may be beneficial from the practical point of view of citric acid and cell recovery to allow self acidification of the fermentation media (Akiyama, 1974).

CU 12 was grown in rich media containing xylose for 4 days. The pH profile over this period shows a severe drop within the first 12 h, but increases to 5 after 24 h (Figure 7). It decreases slowly after this, reaching pH 4.5 after 2 days and coming to an equilibrium of pH 4 after 4 days. Therefore, it was postulated that pH control may not be necessary for fermentations run on xylose. In order to test this hypothesis, citrate production was measured using CU 12.

Fermentations were conducted with the omission of CaCO_3 and with the addition of CaCO_3 24 h after inoculation. No detectable citrate was measured in the fermentation without pH control, at various times during the fermentation (Table 26). In contrast, significant quantities of citrate were detected when the fermentation was run under pH control. Therefore, pH control is clearly important in citric acid

Figure 7--pH profile of CU 12 on rich medium over 4 days. Cells were grown on 25 ml rich medium containing 10% xylose in a 250 ml erlenmyer flask. Samples were withdrawn to test for pH at various intervals.

Table 26--Citrate production by CU 12 with and without pH control.

Time (h)	Citrate (mg/ml)	
	No CaCO_3	CaCO_3 added
12	0	-
24	0	-
36	0	-
72	0	-
96	0	0.082

Two fermentations were run on xylose, the first without pH control (no CaCO_3) and the second with pH control (3% CaCO_3 added). In the former fermentation, citrate was determined at 12 h, 24 h, 36 h, 72 h and 96 h. In the latter, citrate was determined after 96 h.

production by CU 12. It seems to be determinantal to citric acid production if the pH is not maintained closer to neutral (pH 7).

DISCUSSION AND CONCLUSIONS

Three classes of citric acid cycle mutants were isolated from Candida utilis. Three class I mutants, (CIT⁻ AKG⁺), CU 1, CU 4 and CU 12 were found to be stable. It is not known exactly why class II (AKG⁻ SUC⁺) and class III (OAA⁻ SUC⁺) mutants proved to be unstable. However, instability in Candida utilis Y900 has been reported by Delgado et al. (1976). Although they isolated 33 amino acid auxotrophs by MNNG mutagenesis, only 4 of these were found to be stable.

Fermentation character of CU 1, CU 4 and CU 12 was investigated on xylose and glucose. Paper chromatography and the chemical method were used as initial criteria for rapid and simultaneous determination of a number of organic acids. But, these methods clearly have their limitations. Enzymatic analysis was used to detect citrate and isocitrate accurately. However, this required dilution of fermentation samples, to reduce interference by inorganic salts and metals. Appropriate dilutions were determined only for wild type fermentation samples. Use of such dilutions for the estimation of citrate and isocitrate in mutant fermentation samples assumes that interfering substances in all fermentation samples can be considered to be essentially the same. This assumption may be valid, but since fermentations were conducted with citrate cycle mutants, it is conceivable that their metabolism differs from the wild type. This may alter, the composition of interfering substances in mutant

fermentation samples. Although dilutions of 1:2 and 1:1 for measurement of citrate and isocitrate reduced the inhibition in the media drastically, it was not eliminated altogether. Therefore, readings probably represent somewhat conservative estimates.

For fermentations on xylose, these mutants exhibited increases in citric acid production over the wild type. CU 1, CU 4 and CU 12 were discovered to have increases of 88%, 150% and 65% respectively (Table 24). These mutants did not produce significantly more isocitrate than the wild type. Such CIT⁻AKG⁺ mutants may be affected in one of two enzymes, aconitase or isocitrate dehydrogenase. Since CU 1, CU 4 and CU 12 do not overproduce isocitrate, they may be altered in aconitase instead of isocitrate dehydrogenase.

For fermentations on glucose, only CU 4 and CU 12 exhibited increases in isocitrate over that of the wild type. Although CU 12 citric acid yield was comparable to that of the wild type, CU 4 citric acid yield was much less than the parent (Table 24). These results seem to suggest that CU 4 and CU 12 are affected in isocitrate dehydrogenase. The defined medium used for such fermentations contains $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Fe^{+2} is an enzyme cofactor of aconitase and in its presence, the citrate to isocitrate equilibrium is shifted towards isocitrate. It is possible that CU 4 and CU 12 are altered in aconitase and excess citrate is converted to isocitrate by aconitase. This is supported by the fact that CU 4 and CU 12 produce much more

citrate than isocitrate on xylose. But isocitrate should also be produced in xylose fermentations, since these also contain Fe^{+2} . The fact that the absolute citrate yield is much less than that on glucose, may not allow significant conversion of citrate to isocitrate. It is not known why CU 1 does not produce more citrate or isocitrate than the wild type on glucose (Table 24).

On rich medium and xylose as carbon source, only CU 1 and CU 12 exhibited increases in citric acid yield over that found on defined medium (49% and 91% respectively). In such cases, rich medium (with yeast extract as an essential component) may allow faster growth of cells. This would result in increased cell yields during the first phase of the fermentation thereby increasing citric acid secretion in the production phase. CU 4 citrate production on rich medium did not increase over that found on defined medium; hence citric acid yield may not be correlatable with cell yields. It is not known why citric acid yield of the wild type decreased somewhat on rich medium, than that found on defined medium.

This thesis has demonstrated that it is possible to isolate mutants of Candida utilis that produce organic acids from xylose and glucose. From xylose, mutants produced mainly citric acid. Although citric acid yields increased several-fold over that of the wild type, yields were generally less than 0.07 mg/ml. From glucose, two mutants produced more isocitrate than the wild type. The total

citrate plus isocitrate yield of mutants was greater than 0.2 mg/ml. But, this did not represent a significant increase over that of the wild type.

It was hypothesized that the citric acid cycle should be equally operational, regardless of the sugar metabolized (xylose or glucose), because of its vital role in energy generation and as a supplier of precursors of many cellular constituents. But, the fact that the absolute yields of citric and isocitric acids are higher on glucose than xylose, seems to indicate that the citric acid cycle may be more prominent when the sugar metabolized is glucose. This may be due to differences in metabolism of these sugars. In particular, by-product (xylitol) formation may be a factor in reducing carbon flow towards the citric acid cycle when fermentations are run on xylose. More basic investigations are required in the study of xylose metabolism, especially with regard to conversion of xylose to xylitol and xylulose. Efficient conversions of xylose to xylulose would reduce xylitol formation and may be useful in channelling carbon towards the production of organic acids.

The mutant CU 12 appears to be the most promising citric acid producer for fermentations conducted on xylose and glucose. In order to obtain optimum yields of citric acid from this mutant, it would be useful to run fermentations on rich medium, supplemented with a mixture of pentose syrup (containing xylose) and glucose. Step-wise mutagenesis has been utilized in Aspergillus niger to

increase citric acid yields (Hannan et al., 1976). Although it is not known if citric acid productivity involves multiple genes in Candida utilis, this could be investigated in improving citric acid yields of CU 12:

APPENDIX

Candida utilis produces, a by-product, xylitol from xylose. Although this organism produces less xylitol than other common yeasts, it may be useful to eliminate this altogether or reduce it to a minimum. But, this organism does not grow on xylitol or does so at a very slow rate. In any xylose fermentation, it may be useful if Candida utilis could utilize xylitol as a carbon source.

As a first step in achieving this goal, xylitol utilizing mutants were desired. Such mutants were chosen in the following manner.

Candida utilis was grown for 3 days in 2% glucose and 0.67% YNB until a cell density of 1.20×10^{10} cells/ml. 100 ml of the culture was filtered on a 0.45 μ membrane filter. Cells were washed free of glucose with 10 volumes of 10 ml, distilled water. The total cells were suspended in 1.0 ml distilled water and 0.1 ml of this suspension was plated on 2% xylitol and 0.67% YNB. Large colonies arising on a background lawn of weak growth after 3 days of incubation at 30°C were chosen as xylitol utilizing mutants. To assess their ability to use xylitol, growth rates were conducted on 2% xylitol (Table 1). All three mutants exhibited a much faster growth on xylitol than the wild type. Such mutants may be useful in any xylose fermentation, in directing carbon towards the formation of some commercially viable product.

Table 1--Doubling times of Candida utilis and three xylitol utilizing mutants on xylitol.

Designation	Doubling Time (h)
WT	> 24
# 1	7.7
# 2	6.2
# 3	4.2

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