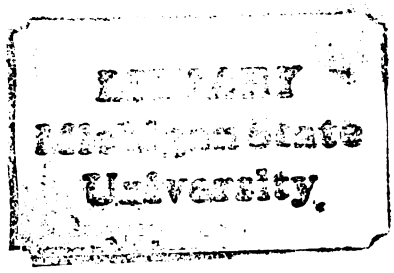

This is to certify that the

thesis entitled

PRODUCTION OF D-ERYTHRO-ASCORBIC ACID BY YEAST

presented by

Jung-Hsiang Tai

has been accepted towards fulfillment
of the requirements for

M.S. degree in **Food Science**

James Porter

Major professor

Date May 12, 1983

PRODUCTION OF D-ERYTHROASCORBIC ACID BY YEAST

By

Jung-Hsiang Tai

A THESIS

Submitted to
Michigan State University
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MASTER OF SCIENCE

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ABSTRACT

PRODUCTION OF D-ERYTHROASCORBIC ACID BY YEAST

By

Jung-Hsiang Tai

Production of ascorbic acid analogues by yeast was investigated. A strain of Candida krusei producing a high yield of an ascorbic acid analogue was screened from 57 yeasts. The analogue produced by C. krusei was identified as D-erythroascorbic acid.

Investigation of nutrient requirements for production of D-erythroascorbic acid by C. krusei indicated a medium containing 5% glucose, 1% NH_4NO_3, 0.2% phosphate buffer and 0.05% $MgSO_4 \cdot 7H_2O$ was optimal. Vitamins and other minerals had no effect on the production of the analogue. The fermentation was optimal when carried out at 23 °C, pH 6.0 with high aeration rate and 15% inoculum. Under these conditions, the fermentation was complete after 7 days.

Ethylmethanesulfonate was used as mutagen to select high productivity mutants. Two mutants which showed improved productivity (140% and 160%) were isolated. An L-ascorbic acid sensitive mutant was also isolated.

ACKNOWLEDGMENTS

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INTRODUCTION

The common feature of ascorbic acid analogues in their chemical structures is the five-atom lactone ring containing the dienolic systems. The dienolic systems in these acids cause their acidity, reducing properties, and instability in alkaline solution. Synthesis of ascorbic acid analogues has consisted in devising methods for the formation of 2-keto and 3-keto hydroxy acids followed by their enolization and lactonization. Bioconversion of D-sorbitol to L-sorbose by Acetobacter suboxydans in the L-ascorbic acid synthesis and the direct fermentation in D-araboascorbic acid production from D-glucose by Penicillium sp. are two examples of biochemical synthesis of these compounds.

The biosynthesis of ascorbic acid analogues by yeasts has also been reported. Bleeg (1966) reported on L-ascorbic acid from L-galactono-γ-lactone and D-glucose by Saccharomyces cerevisiae and its mitochondria. Yasuda (1967) reported on D-erythroascorbic acid from D-xylose by Candida sp. Heick et al. (1969, 1972) also reported on L-ascorbic acid formation from D-glucose by many strains of yeasts.

Microorganisms can grow in a wide range of physical and chemical enviroments; their growth and other physiological activities are a response to their physiochemical

enviroment. Development of a microbiological process begins from a test tube or flask scale fermentation where basic medium composition and physical variables are screened. Genetic techniques are then applied to obtain high yield of the product. The most common means of improving yield after physiological variables have been optimized is by the process of mutation.

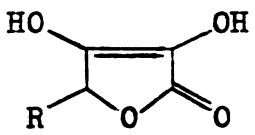
The objectives of this study were to establish appropriate nutritional and physiological conditions for high yield D-erythroascorbic acid production by Candida krusei. In this report the role of carbon and nitrogen sources, the effects of inorganic salts, vitamins and amino acids, aeration, temperature, and pH on D-erythroascorbic acid production have been investigated. Ethylmethanesulfonate mutation and selection of analogue-resistant mutants as means of increasing yield were also investigated.

LITERATURE REVIEW

Ascorbic acid analogues (Figure 1) are the group of compounds that have a five-atom lactone ring containing the dienolic systems. Their chemical properties and absorption data closely resemble to those of L-ascorbic acid, the well-known antiscorbutic vitamin C (Smith, 1946). The unique feature of this group of compounds lies in the dienolic system and it is the system which is responsible for the remarkable reducing properties displayed by these substances. The discovery of this group of compounds has been closely related to scurvy.

Historical Aspects of Ascorbic Acid Analogues

Scurvy is one of the oldest diseases known to mankind. It was the major cause of death among the early sea voyagers who could not get the fresh supply of fruit and vegetables. The value of citrus fruit for preventing scurvy has been recognized for centuries. The first clinical experiment on scurvy was carried out by Lind in 1747 that showed conclusively the therapeutic value of lemon juice in curing the disease. Nearly half a century elapsed before his proposals were adopted in 1795. A dramatic fall in the incidence of the disease then ensued, and by 1800 the conquest of scurvy

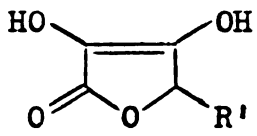


R =

(1)	(2)	(3)	(4)	(5)
CH_2OH	HO-C-H CH_2OH	HO-C-H CH_3	H-C-OH CH_2OH	HO-C-H COOH
D-Erythro-ascorbic acid	L-Ascorbic Acid	6-Deoxy-L-Ascorbic Acid	D-Arabo-ascorbic Acid	6-Carboxy-L-ascorbic Acid

(6)	(7)	(8)	(9)
H-C-OH HO-C-H CH_3	HO-C-H HO-C-H CH_3	HO-C-H HO-C-H CH_2OH	HO-C-H H-C-OH H-C-OH CH_2OH
L-Fuco-ascorbic Acid	L-Rhamno-ascorbic Acid	L-Gluco-ascorbic Acid	D-Glucohepto-ascorbic Acid

Fig. 1.--Structures of ascorbic acid and its analogues



R' =

(10)	(11)	(12)	(13)
CH_2OH	H-C-OH CH_2OH	HO-C-H CH_2OH	H-C-OH H-C-OH CH_2OH
L-Erythro-ascorbic Acid	D-Xylo-ascorbic Acid	L-Arabo-ascorbic Acid	D-Gluco-ascorbic Acid

(14)	(15)	(16)
HO-C-H H-C-OH CH_2OH	H-C-OH HO-C-H CH_2OH	HO-C-H HO-C-H CH_2OH
D-Galacto-ascorbic Acid	L-Gulo-ascorbic Acid	L-Allo-ascorbic Acid

Fig. 1.--(Continued)

was practically complete.

Modern work on scurvy began when Holst and Frolich produced the disease in guinea-pigs by giving them a restricted diet in 1907. In 1912 Hopkins showed that animals could not thrive on purified isolated food constituents and he introduced the concept of "accessory food factors"; the same year Casimir Funk postulated the existence of "vitamins" and subsequently suggested the absence of different vitamins was responsible for the onset of deficiency diseases. In 1917 Zilva and Wells described an antiscorbutic agent in lemon juice for preventing scurvy. The antiscorbutic agent was named as water-soluble C at first; in 1920 Drumond renamed this substance as vitamin C. In 1922 Sherman and his co-workers devised a bioassay method for vitamin C. This method was based on the determination of the minimum amount of samples needed to prevent signs of scurvy in guinea-pigs. In 1924 Zilva and his colleagues extracted a 300-fold concentration of the active compound from lemon juice. In 1928 Szent-Gyorgyi isolated what he called "hexuronic acid" in crystallin form, from cabbage and adrenal cortex. During the course of this work, he established $C_6H_8O_6$ as the molecular formula, the presence of an acidic functional group, and the facile and reversible nature of its oxidation. In 1932 Waugh and King isolated the vitamin from lemon and showed it to be identical with the "hexuronic acid" of Szent-Gyorgyi; they also showed that the antiscorbutic activity of hexuronic acid was similar to that of vitamin C obtained

from orange juice. In 1933 Haworth determined the structure of this vitamin, Reichstein and other researchers synthesized this vitamin, Szent-Gyorgyi and Haworth changed the name of hexuronic acid to L-ascorbic acid. In 1965, the trivial name L-ascorbic acid was recognized by the IUPAC-IUB Commission on Biochemical Nomenclature as an acceptable name for vitamin C (King, 1961; Sebrell and Harris, 1967; Sharman, 1974; Cameron and Pauling, 1979; Hodges, 1980; Crawford and Crawford, 1980).

Ascorbic Acid Analogues and the Antiscorbutic Activity

Investigation by Reichstein et al. (1933a, 1933b, 1934) made it possible to study the antiscorbutic action of synthetic L-ascorbic acid and some of its analogues by feeding the vitamin C-exhausted guinea pigs the diets supplemented with compounds to be tested for their antiscorbutic activities. Although L-ascorbic acid is the most active antiscorbutic substance, some other analogues (Table 1) also show this feature with less activity, some of the analogues do not possess the antiscorbutic activity. A comparison of the structures and the antiscorbutic activity of these compounds reveals that for antiscorbutic activity the asymmetric carbon must lie to the left of lactone oxygen, when their formulas are written according to the convention in Figure 1 (Harris, 1967), no exception to this rule is yet known.

Table 1.--Physical properties and antiscorbutic activity of ascorbic acid and its analogues

Compound	M.P. (°C)	$[\alpha]_D^{o a}$	Activity[b]
D-Erythroascorbic Acid (1)	-	-	-
L-Ascorbic Acid (2)	192	+23	1
6-Deoxy-L-ascorbic Acid (3)	168	+37	0.3
D-Araboascorbic Acid (4)	174	-17	0.05
6-Carboxy-L-ascorbic Acid (5)	206-210	-	-
L-Fucoascorbic Acid (6)	-	-	0.02
L-Rhamnoascorbic Acid (7)	199	+28	0.2
L-Glucoascorbic Acid (8)	140 (hydrate)	+24	0.025
D-Glucoheptoascorbic Acid (9)	-	-	0.01
L-Erythroascorbic Acid (10)	161	+9	0
D-Xyloascorbic Acid (11)	192	-23	0
L-Araboascorbic Acid (12)	170	+17	0
D-Glucoascorbic Acid (13)	140 (hydrate)	-22	0
D-Galactoascorbic Acid (14)	134	-6	0
L-Guloascorbic Acid (15)	184	-22	0
L-Alloascorbic Acid (16)	177	+29	0

[a]Rotation in water.

[b]Antiscorbutic activity relative to L-ascorbic acid.

Occurrence of Ascorbic Acid Analogues in Organisms

L-Ascorbic acid, D-araboascorbic (isoascorbic) acid and D-erythroascorbic acid have been found in organisms. L-Ascorbic acid is present in most higher plants (King, 1973), and some algae (Talpasayi, 1967; Aaronson, et al., 1977), and is most highly concentrated in the more actively growing regions of plant tissues, as in root tips, seed sprouts, flowering and fruiting parts and green leaves.

Only a few species of animals need to consume L-ascorbic acid in the diet, whereas most other species are able to synthesize their own. The biosynthetic ability is lost in the primates, guinea pig, flying mammals, late birds, fish, insects, invertebrates (Kutsky, 1981). Herein lies one of the great genetic mysteries, which is still incompletely solved (Chatterjee, 1973).

Bourne and Allen (1935) suggested that vitamin C-like reducing substance is distributed in lower organisms. Geiger-Hubber and Galli (1945) found the presence of an ascorbic acid analogue in Aspergillus sp.. Kalyanasundaram and Saraswathi-Devi (1955) found it in Fusarium. Bleeg (1966), Humpers (1967) and Heick et al. (1969, 1972) reported the occurrence of L-ascorbic acid among yeasts. Shigeoka (1977a) reported the occurrence of L-ascorbic acid in Euglena, a protozoa. However, extensive studies in relation to lower organisms are needed to develop the information more fully.

D-Araboascorbic acid has been rarely found in biological materials or products. Isherwood et al. (1953, 1954) found

it in cress seedling in D-altrono-γ-lactone solution and in the rat injected with manno-γ-lactone. Takahashi et al. (1960) found its presence in various Penicillium sp.. Yagi et al. (1967) screened about 5000 strains of fungi and bacteria, only Penicillium, but no other genera, was obtained as D-araboascorbic acid producers. D-Erythroascorbic acid was found in Candida sp. by Yasuda (1967).

Biosynthesis of Ascorbic Acid Analogues

L-Ascorbic acid is biosynthesized by a wide variety of plants and animals. It has been shown (Loewus, 1971) that in the biosynthesis of L-ascorbic acid, D-glucose is converted into L-ascorbic acid by two separate pathways. One in which C-1 of D-glucose becomes C-6 of L-ascorbic acid, and one in which C-1 of D-glucose becomes C-1 of L-ascorbic acid. Those animals which cannot synthesize this compound contain no L-gulonolactone oxidase activity (Chatterjee et al., 1975), the terminal enzyme of L-ascorbic acid biosynthesis. A thorough exploration of L-ascorbic acid synthesis by the simpler form of life, such as bacteria, yeasts and molds, has not been made.

Animals synthesize L-ascorbic acid apparently from D-glucose via the D-glucuronic acid pathway of metabolism (Isherwood, Chen and Mapson, 1954). In this pathway (Reaction I) glucose is oxidized to D-glucuronic acid, which is reduced to L-gulonic acid. L-gulonic acid is then lactonized to L-gulonolactone, which is oxidized to L-ascorbic

acid through the intermediate 2-keto-L-gulonolactone. The step from 2-keto-gulonolactone follows by rearrangement without requirement of a specific enzyme (King, 1973).

Reaction I: D-glucose → D-glucuronic acid → L-gulonic acid → L-gulonolactone → (2-keto-L-gulonolactone) → L-ascorbic acid

Synthesis of L-ascorbic acid in plants is accomplished by a greater variety of reactions than those identified in animal tissue. Mapson and his co-workers (1956) demonstrated that L-gulonolactone, L-galactonolactone, D-glucuronolactone, and methyl-D-galacturonate were converted to L-ascorbic acid in cress seedlings. From their studies, they proposed two pathways for L-ascorbic acid synthesis in plants. One of these is the same as that of animals. The other pathway (Reaction II) began from galactose is briefly summarized as follows:

Reaction II: D-galactose → methyl-D-galacturonate → L-galactonolactone → L-ascorbic acid

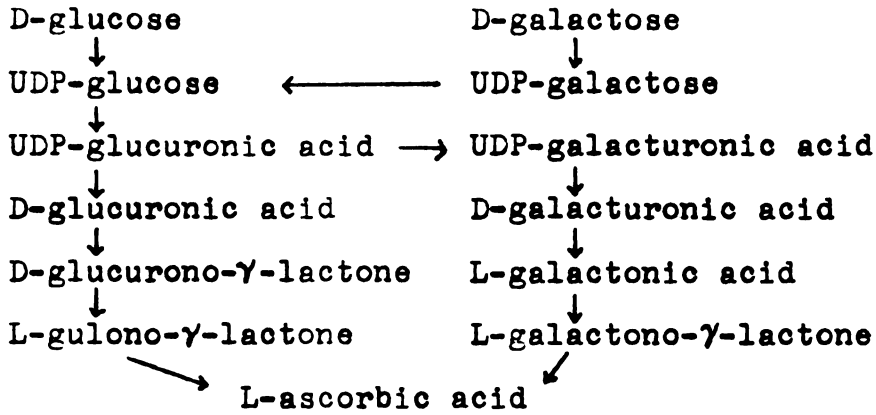
The experimental results of Loewus (1961) in strawberry did not unequivocally support the above mechanisms as major pathways, he proposed the third pathway (Reaction III):

Reaction III: D-glucose → D-glucose 6-phosphate → D-gluconate 6-phosphate → (3-keto-D-gluconate 6-phosphate → L-ascorbic acid

L-ascorbic acid synthesis in lower organisms is not yet clear, however, Bleeg (1966), Nishikimi et al. (1978) found an enzyme in yeast which is similar to a key enzyme

for L-ascorbic acid biosynthesis in animals. Shigeoka and his co-workers (1977b) also proposed a pathway (Reaction IV) in Euglena.

Reaction IV:



The biosynthetic pathway of D-araboascorbic acid in Penicillium notatum has been elucidated by Takeshi and Mitsumoto (1961, 1965). This compound was synthesized from D-glucose, D-gluconate, D-glucono-γ-lactone, D-glucono-δ-lactone, sucrose, maltose, and starch, but not from other substances such as D-glucuronate, 2-ketogluconate and glycerol. The proposed pathway is shown in Reaction V:

Reaction V: D-glucose → D-glucono-δ-lactone → D-glucono-γ-lactone → D-araboascorbic acid

An indophenol reducing substance formed by Serratia marcescens was reported (Bereusi and Illenyi, 1938) as L-ascorbic acid. Takahashi et al. (1976) demonstrated this substance to be D-erythroascorbic acid while S. marcescens was cultured in 2% xylose. Other analogues from D-galactose and D-glucose were identified as D-xyloascorbic acid and D-araboascorbic acid respectively. The biosynthetic pathway of D-erythroascorbic acid (Reaction VI) in Candida utilis

was proposed by Murakawa et al. (1977) as follows:

Reaction VI: D-arabinose → D-arabono-δ-lactone

D-arabono-γ-lactone → D-erythroascorbic acid

Synthesis and Production of Ascorbic Acid Analogues

Four main methods are available for the synthesis of ascorbic acid analogues containing the characteristic five-membered unsaturated dienolic ring (Smith, 1946; Theander, 1980; Hay, Lewis and Smith, 1967a). The synthetic methods based on the formation of 2-keto or 3-keto hydroxy acids followed by their enolization and lactonization are as follows:

1. Cyanohydrin synthesis from glycos-2-uloses.
2. Enolization and lactonization of glyculosonic acids or esters.
3. Condensation of hydroxy aldehydes with ethyl glyoxylate or mesoxalate.
4. Condensation of esters of hydroxy acids.

Among the analogues, L-ascorbic acid and D-arabo-ascorbic acid productions have been sold commercially. The most important starting-material for the synthesis of L-ascorbic acid is D-glucose.

Commercial production of L-ascorbic acid uses the Reichstein-Grussner synthesis (Hay, Lewis and Smith, 1967b; Crawford and Crawford, 1980). In this procedure (Reaction VII) D-glucose is chemically hydrogenated to produce D-sorbitol. Non-isolated sorbitol thus produced is then

biochemically dehydrogenated by Acetobacter suboxydans to L-sorbose. The isolated sorbose is then condensed with acetone to form diacetonesorbose. This is chemically oxidized to diacetone-2-keto-L-gulonic acid, which, after hydrolysis, enolization, and lactonization yields L-ascorbic acid. This method would be further simplified if the problem of direct oxidation of sorbose to 2-keto-gulonic acid were solved chemically or biochemically.

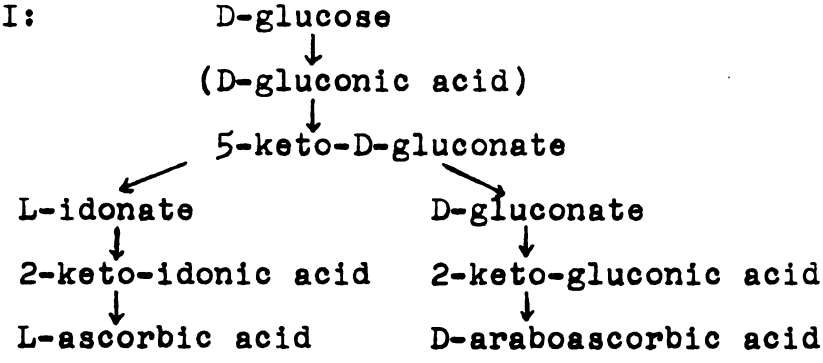
Reaction VII: D-glucose → D-sorbitol → L-sorbose → diacetonesorbose → diacetone-2-keto-L-gulonic acid → L-ascorbic acid

The strains with powerful ability of L-sorbose formation from D-sorbitol are Gluconobacter roseum and Acetobacter suboxydans. In this fermentation, 18% of D-sorbitol is added into medium, after 30 hours fermentation, 95% of D-sorbitol can be converted to L-sorbose.

Newer processes of L-ascorbic acid production (Kulhanek, 1970) based on two stage fermentations (Reaction VIII). Glucose is converted by biochemical dehydrogenation in the presence of calcium carbonate to calcium 5-keto-D-gluconate by Acetobacter suboxydans. Calcium 5-keto-D-gluconate was catalytically hydrogenated to a mixture of calcium D-gluconate with calcium L-idonate in a 1:1 ratio. A second fermentation with Pseudomonas fluorescens or other bacteria oxidize the calcium L-idonate to 2-ketoidonic acid, which, can be transformed into L-ascorbic acid by enolization and lactonization. The D-gluconate processed analogously yields

D-araboascorbic acid. Therefore the reaction mixture has to be processed further in order to separate out either hexonate, or, at least, to isolate the L-idonate component from the mixture.

Reaction VIII:



The newer procedure cannot compete with the Reichstein-Grussner synthesis. Preparation of L-ascorbic acid by this procedure would become more interesting if successful realization of stereospecific hydrogenation of 5-keto-D-gluconic acid to L-idonic acid could be done under commercially acceptable conditions.

L-Ascorbic acid has been identified as a metabolite of a variety of microorganisms. The direct fermentation has not been developed yet, at the present time, L-ascorbic acid is not produced in high yield by any microorganisms (Crawford and Crawford, 1980).

A chemical process is used to produce D-araboascorbic acid by converting from methyl-D-arabino-hexulosonate (Miles Laboratories, 1964). In this process, methyl-D-arabino-hexulosonate is mixed with H_2O or alcohol, and sodium carbonate. The mixture is refluxed in an inert atmosphere, then acidified with H_2SO_4 after cooled down, to give a

87-95% sodium D-araboascorbate.

The direct fermentation of glucose to give D-araboascorbic acid has been developed (Yagi, et al., 1967). The Penicillium spp. are used in this fermentation to give 40% yield. Over 80% yield could be obtained when washed mycelium is used in dilute glucose solution (about 1% glucose). This fermentation is carried out at 28 °C for 5 to 7 days. The control of pH is most important and maintained at pH 3.8 to 4.5 in this fermentation.

D-Erythroascorbic acid was synthesized by Prince and Reichstein (1937) from D-arabinose (Reaction IX). In this synthesis a bioconversion from D-arabitol to D-xyloketose by sorbose bacteria was involved.

Reaction IX: D-arabinose → D-arabitol → D-xyloketose → acetone-D-xyloketose → 2,3-monoacetone-D-xylosonic acid → 2,3-acetone-D-xylosonic acid → D-erythroascorbic acid

Uses of Ascorbic Acid Analogues

Massive information on the uses of ascorbic acid analogues (mainly L-ascorbic acid and D-araboascorbic acid) can be found in the Chemical Abstracts. They are extensively used as food additives during the processing of foods and beverages (Bauernfend and Pinkert, 1974; Birch and Parker, 1974) to enhance nutritional values, or to replace the vitamin C loss during harvesting, processing, storage or home preparation, or as an oxygen carrier or water-soluble

antioxidant, whose role in the oxidation/reduction reactions inherently results in improved keeping quality, color, flavor and texture, and improved processing of the food product.

Ascorbic acid analogues are also widely used for non-food purposes, such as, the electroplating of alloys; the detection of metal ions, nitrate and nitrite, phosphorous; the catalyst in polymerization of vinyl chloride, acrylonitrile, cyanoacrylate adhesives; photographic emulsion for iron spot prevention; pharmaceutical uses; and many others.

The U.S. market for L-ascorbic acid was 90 million pounds per year in 1981(Hochhauser, 1983). The direct fermentation methods to produce ascorbic acid analogues will be desirable if the yield can be increased dramatically through genetic manipulation such as mutation-selection or gene-splicing techniques. In this research, the high yield ascorbic acid analogue producing yeasts will be selected to study the nutrient requirements and optimal physical conditions for fermentation, then the mutation-selection will be applied to improve the production.

MATERIALS AND METHODS

Media

Media mainly used in this study were complete yeast extract-peptone-dextrose (YEPD) medium, Yeast Nitrogen Base (YNB) with carbon compounds, Yeast Carbon Base (YCB) with nitrogen compounds, minimal medium, and storage medium.

Media for petri plates were prepared in 2-liter flasks, each flask containing 1 liter of medium (enough for about 50 plates). After autoclaving for 15 minutes under 15 pounds pressure at 250 ^{o}F, these media were poured into petri plates and the surface of the agar plates was allowed to dry for 2 days before use. These plates could be stored in plastic bags at 10 ^{o}C for over 3 months. Media for liquid cultures were prepared in beakers and dispensed into 13.5x 150 mm test tubes or 500 mL flasks by syringes. The tubes were stoppered with steel caps, the flasks with cotton plugs, autoclaved them for 15 minutes. These media were ready to use after cooling to room temperature.

1. Complete (YEPD) Medium

This medium contained 1% yeast extract, 2% peptone, 2% glucose and 2% agar. It was used in the propagation of yeast cells from stock cultures, surface plate count, replica plating, and maintaining the mutants and revertants

before examing the D-erythroascorbic acid productivity. The agar was omitted for liquid medium, this was used for routine growth to harvest large amount of cell mass. The harvested cells were then used for the inoculation or mutation process.

2. YNB

This synthetic medium contained 6.7 g Bacto-Yeast Nitrogen Base (Difco, 1953) and different carbon compounds in one liter solution. This medium contained all vitamins and minerals required for yeasts growth, 2% glucose was added as carbon source while used as screening medium, 2% of various carbon compound was added while used in the study of carbon assimilation ability.

3. YCB

This medium contained 13.1 g Bacto-Yeast Carbon Base (Difco, 1953) and different nitrogen compounds in one liter solution. The inclusion of vitamins in this base was necessary for the utilization of nitrogen compounds by certain yeasts which cannot assimilate these compounds in the absence of vitamins. This medium was used in the study of effect of nitrogen source on D-erythroascorbic acid production by adding 0.5% various nitrogen compounds to the medium.

4. Minimal Medium

This medium contained only carbon source, nitrogen source, phosphate salts, and magnesium sulfate. The amounts of these compounds were changed to study the media

composition for D-erythroascorbic acid production. Supplemental nutrients were added to study the growth requirements of mutants and the resistant effect of analogues. The pH was adjusted by adding 1 N NaOH or 1 N HCl; 2% agar was added for use in petri plates.

A concentrated phosphate buffer (10% w/v) suggested by Fink (1970) was used as stock solution. To prepare this buffer, 87.5 g KH_2PO_4 and 12.5 g K_2HPO_4 were dissolved in water to make a total volume 1 liter. To prepare minimal medium, this buffer was diluted to the desired concentration with other components.

5. Storage Medium (2xYEPD)

This medium contained double concentration of all components in YEPD except for agar (2%). Cultures maintained on this medium and stored at 4 °C could be kept for one year (Fink, 1970).

Yeast Cultures

The yeast cultures used in the screening procedure were transferred from the stock cultures of Food Microbiology Lab., Department of Food Science and Human Nutrition, Michigan State University. *Candida krusei* was selected for the study of D-erythroascorbic acid production from 57 yeast strains because of its high yield in the production of ascorbic acid analogues. Wild type yeast cells were used to study the media composition and cultivation conditions. Mutants were obtained by treating the wild type cells with

ethylmethanesulfonate (EMS). Revertants were obtained by mutating one of the mutants that showed very low erythroascorbic acid producing ability. An L-ascorbic acid sensitive mutant was isolated from the revertants. The resistant mutant from this sensitive mutant was obtained by EMS mutation.

All of the wild type yeast cultures were maintained on the YEPD agar slants. All of the mutants and revertants were maintained on YEPD agar plates before the D-erythroascorbic acid productivity was examined. The valuable mutants and revertants were purified and maintained on YEPD agar slants. All of the cultures were stored at 4 °C and were transferred to YEPD agar plates 3 days prior to use.

Cultivation of Yeasts

1. Broth Cultures

Liquid media were prepared either in test tubes or 500 mL Erlenmeyer flasks two days prior to inoculation. A loopful of yeast cells were inoculated into 10 mL YEPD liquid medium in test tubes the day before experiments. The inoculated broth was then incubated at 30 °C on a Iwashiya refrigerator shaker overnight. The cells were harvested by an Damon IEC HN-SII centrifuge at the speed of about 1500 rpm and washed twice with sterile distilled water by recentrifugation and resuspension. The cells were then resuspended in the working medium to the same volume after washing. A 5% transfer of the cell suspension was made into

the working medium and then placed on the shaker under controlled temperatures to reach the stationery phase.

2. Plate Cultures

Agar plates were made 2 days prior to use. After the agar surface was dried enough to absorb the liquid of inoculum quickly, 0.1 mL drops of adequate dilutions were dropped onto the agar surface with a calibrated sterile 1 mL pipet, spreading smoothly and uniformly with a flame-sterilized L-shape glass rod till the inoculum liquid was absorbed. The ends of the glass rod did not hit the flange of agar plate. The plates were inverted and incubated at 32 ^{o}C incubator till desired colony size formed. Dilution to obtain 30 to 300 colonies on one agar plate for surface count, and 200 to 300 colonies for replica plating were used.

Growth Parameter

Turbidity was used for measuring growth. Yeast cells grown in YEPD liquid medium overnight were harvested by centrifugation, washed with sterile distilled water twice and resuspended in 0.1 N phosphate buffer. Duplicate surface plate counts of 10^{-4}, 10^{-5}, 10^{-6} dilutions were made on YEPD agar plates and incubated at 32 ^{o}C for 2 days. At the same time, prepared a series of dilutions by 1:1, 1:2, 1:3, 1:4, et al. from the original cell suspension so that a range of turbidities at OD_{600} from 0-0.8 with B&L 20 Spectrophotometer obtained. A standard curve of OD_{600} vs.

cell concentration (CFU/mL) was plotted.

Routinely, the growth of yeast cultures in liquid medium were measured by determining the OD_{600} of the 26-fold diluted culture broth (0.2 mL broth + 5 mL distilled water, using 5.2 mL water as blank), and CFU/mL then determined from the curve.

Estimation of D-Erythroascorbic Acid

1. Qualitative Estimation

A paper chromatography method developed by Miki et al. (1962) was used to separate ascorbic acid analogues, the RF values were compared with Miki's (1962) and Murakawa's (1977). In this method, Whatman paper No. 4, 20x40 cm, was dipped into 3% (w/v) metaphosphoric acid solution to which 3% glycerol (v/v) was added for 10 minutes, dried in the air for 1 hour. Culture broth and standard ascorbic acid analogues were streaked along a line on the prepared paper. The paper was kept standing for 30 minutes in a moist atmosphere with a relative humidity of 40-70%. This paper was then placed in a glass chamber containing the mobile solvent, water-saturated methyl ethyl ketone (upper part) which had been stored for more than 24 hours after preparation. Development was carried out at 25 °C for 5 hours by an ascending method. After completion of the development, the paper was taken out and air dried for about 10 minutes at room temperature. Then 0.013% 2,6-dichlorophenol indophenol (sodium salt) dissolved in water

was sprayed. A white spot against pink background was indicative of an analogue and was marked before the background faded away.

2. Quantitative Estimation

The D-erythroascorbic acid in culture broth was measured by a modification of L-ascorbic acid analysis by Sullivan and Clarke (Omaye, Turnbull and Sauberlich, 1979), the dipyridyl method. Reagents used in this methods were 2,2'-dipyridyl, aqueous 0.5%; orthophosphoric acid, 85%; ferric chloride, aqeous 1% made fresh each 3 days; trichloroacetic acid (TCA), aqueous 10%, made fresh each use.

To 0.5 mL of culture broth, 1.0 mL of 10% TCA was added and the samples centrifuged for 10 minutes. The following reagents were added in sequence to 540 µL of the supernatant: 160 µL of 85% orthophosphoric acid, 2.74 mL of 0.5% 2,2'-dipyridyl, and 560 µL of 1% ferric chloride. The samples were allowed to stand at room temperature for 30 minutes for the ferrous-dipyridyl chromophore to develop. Samples were then read at OD_{525} in B&L 20 Spectrophotometer. Standards of L-ascorbic acid ranging from 10 µg/mL to 120 µg/mL were used for a standard curve; the total amount of ascorbic acid analogues were measured as the L-ascorbic acid equivalent with the same reducing power. The productivity of these analogues were expressed as µg/mL equivalent.

Mutation and Selection

1. Isolation and Purification of Mutants

Mutants isolated from the master plates were purified by streak plate technique. The YEPD agar plates and sterile flat toothpicks were used. The mutant picked out from the master plate with a sterile toothpick was spread evenly over a small area (pool) toward the edge of the plate. Another sterile toothpick was used to make two strokes from the pool of the material. One of the strokes was made only to the center of the plate. Using the third sterile toothpick, a series of strokes were made at right angles to the first two strokes. A series of strokes were made at right angles to previous series with sterile toothpick, the strokes being made towards the pool. Care should be taken that the toothpick did not touch the pool at this stage. On incubation of plates at 32 ^{o}C, discrete single colonies were picked from the streaked plates, and spread onto YEPD agar plates. After erythroascorbic acid production was examined, purified cultures were inoculated onto storage medium and stored at 4 ^{o}C after good growth was reached on agar slants.

2. Replica Plating (Lederberg and Lederberg, 1952)

A cylinder of wood with a diameter $3\frac{5}{16}$ inches was used as replica plating block to accommodate the petri plate. A metal ring $3\frac{3}{8}$ inches in diameter was used to secure the velvet cloth. The replica cloths were made from velveteen cut into $5\frac{1}{2}$ inches squares. These cloths were piled up in a metal container, autoclaved for 50 minutes. In replica

plating, a sterile square was placed, nap up, on the cylindrical wood and held firmly in place with the metal ring pushed over the fabric and around the rim of the support. The YEPD agar plate carrying the initial colonies was inverted onto the fabric with slight digital pressure to transfer the growth. The imprinted plate then provided the pattern for transferring replica-inocula to subsequent minimal agar plates impressed in the same way. The minimal agar plates were then incubated at 32 °C for 2 days. After incubation mutants that showed growth on complete medium but not on imprinted minimal medium were isolated and purified.

3. EMS Mutation (Fink, 1970)

Ethylmethanesulfonate was used as mutagen in the mutation process to give high yields of mutants in this study. The EMS was marked and stored in a freezer. The mutation process required 12 days per cycle and was as follows:

Day 1. Wild type yeast cells from a YEPD agar plate were inoculated into 10 mL of liquid YEPD in an 13.5x150 mm test tube and grown at 30 °C overnight in a shaker.

Day 2. The cells were harvested by centrifugation, washed three times with sterile distilled water and resuspended in 10 mL sodium phosphate buffer at pH 8.0 (pH of buffer was very important in the EMS reaction), and 0.6 mL EMS was added. After shaking the tube with a vortex mixer, the mixture was incubated for 50 minutes at 32 °C without agitation. Immediately after incubation, the cells

were washed three times with sterile distilled water. Each time the cells were transferred to a new sterile culture tube. After the third wash, the cells were resuspended in 10 mL sterile water. Then 1.0 mL of the cell suspension was diluted into 10 mL YEPD liquid medium and shaken for 2 days at 30 °C.

Day 4. After the culture was grown up, the cells were diluted 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}-fold. Then 0.1 mL of each dilution was spread over the entire surface of YEPD agar plate. These plates were incubated at 32 °C. Between 200 and 300 colonies appeared on each petri plate as desired. Diluted cell suspensions were stored at 4 °C.

Day 6. The dilution that yielded agar plates between 200 to 300 colonies was selected to plate out on up to 100 plates and incubated at 32 °C for 2 days.

Day 8. These plates were used as master plates for replica plating. Master plates were stored at 4 °C; imprinted plates were incubated at 32 °C for 2 days.

Day 10. Mutants from master plate were picked with flat sterile toothpicks and purified by restreaking.

Day 12. The YEPD agar plates were inoculated with a single colony from each of these mutant strains. The plates, each containing 30 mutants, were incubated at 32 °C for 2 days and stored at 4 °C.

The mutants thus obtained were tested for the D-erythro-ascorbic acid productivity in minimal broth medium supplemented with 5% YEPD liquid medium for growth. The mutants

were tested for the growth requirements.

4. Analogues Resistant Mutants

The resistance of C. krusei to several chemical analogues of D-erythroascorbic acid was tested. Each analogue was added into minimal agar plates at different concentrations. Yeast cells grown in YEPD liquid medium were harvested and washed 3 times. The yeast cells were resuspended in sterile water. 0.1 mL of 10^{-2}, 10^{-3}, 10^{-4} dilutions were spread both onto minimal agar plates and the plates supplemented with chemical analogues. After incubation at 32 ^{o}C for 2 days, colonies were observed for inhibition by chemical analogues. The analogues that showed inhibition were used to select the resistant mutants by EMS mutation. Mutants thus obtained were examined for the D-erythroascorbic acid productivity.

RESULTS AND DISCUSSION

Screening of D-Erythroascorbic Acid Producing Yeast

The screening of D-erythroascorbic acid producers among yeasts was carried out on a test tube scale. YNB with 2 % glucose as the sole carbon source was used as the screening medium. The pH of the medium was adjusted to 5.0. A loopful of inoculum from each yeast culture was transferred into 2 mL sterile medium contained in a 13.5x150 mm test tube. The cultures were then incubated in a temperature-controlled shaker, continuously shaken (130 strokes per minute) at 30 °C for 5 days. The fermentation broth of each culture was then assayed for their ascorbic acid analogues content. The total amount of ascorbic acid analogues was expressed as μg/mL productivity for each yeast strain. Table 2 shows the results of screening study.

There were 57 yeast strains examined in the screening procedure. These strains belong to two families, Endomycetaceae (ascosporogenous yeasts) and Cryptococcaceae (anascosporogenous yeasts), 18 strains of yeasts in both families gave a positive result for ascorbic acid analogue production. Because of the limitation of analytical sensitivity, the medium used, and cultivation conditions, it is not possible to say that only these 18 strains among the

Table 2.--The occurrence of ascorbic acid analogues in various species of yeasts

Yeast	Ascorbic acid analogues content (μg/mL)
A. Ascosporogenous yeasts	
Debaromyces harsenii (YMA FPL 13)	Neg[a]
Hansenula anomala	Neg
H. anomala (U. of Calif.)	Neg
H. californis (NRRL Y 1425)	50
H. saturnus	15
H. wingei (ATCC 14355)	15
H. wingei (ATCC 14356)	10
Pichia fermentuns	10
Saccharomyces boulardii (IZ 1904)	Neg
S. carlsbergensis (ATCC 9080)	15
S. carlsbergensis (IZ 210)	Neg
S. carlsbergensis (IZ 626)	Neg
S. carlsbergensis (IZ 1327)	Neg
S. carlsbergensis (IZ 1430)	Neg
S. carlsbergensis (IZ 1828)	Neg
S. carlsbergensis (IZ 1831)	Neg
S. carlsbergensis (IZ 1834)	Neg
S. carlsbergensis (IZ 1973)	Neg
S. cerevisiae (ATCC 964)	Neg
S. cerevisiae (ATCC 4126)	Neg
S. cerevisiae (IZ 299) var. turbidans	Neg
S. cerevisiae (IZ 310)	Neg
S. cerevisiae (IZ 629)	Neg
S. cerevisiae (IZ 672)	Neg
S. cerevisiae (IZ 755)	Neg
S. cerevisiae (IZ 765)	Neg
S. cerevisiae (IZ 864) var. ellip.	Neg

Table 2.--(Continued)

Yeast	Ascorbic acid analogues content (μg/mL)
Saccharomyces cerevisiae (IZ 986)	Neg
S. cerevisiae (IZ 987)	Neg
S. cerevisiae (IZ 1716)	Neg
S. cerevisiae (NRRL Y 129)	Neg
S. cerevisiae (NRRL Y 564)	Neg
S. cerevisiae (NRRL Y 567)	Neg
S. cerevisiae (NRRL Y 635)	10
S. cerevisiae (NRRL Y 897)	Neg
S. cerevisiae (NRRL Y 898)	Neg
S. cerevisiae (NRRL Y 978)	10
S. cerevisiae (NRRL Y 2034)	10
S. cerevisiae (ascorspore)	10
S. cerevisiae (yeast cake)	Neg
S. cerevisiae steinberg (wine)	Neg
S. diastaticus (NRRL Y 2044)	Neg
S. kluyveri (U. of Calif.)	10
S. kluyveri (strain C 26)	15
S. oleaginosas (U. of Calif.)	15
S. rouxii (ATCC 2619)	10
S. uranium (NRRL Y 347)	Neg
S. uvarum (NRRL Y 1347)	Neg
S. uvarum (NRRL Y 6004)	Neg
Schizosaccharomyces japonicus var. versatilis	Neg
Schizosaccharomyces octosponus	15
Schizosaccharomyces pombe	Neg
B. Anascosporogenous yeasts	
Brettanomyces claussnil (NRRL Y 1414)	Neg

Table 2.--(Continued)

Yeast	Ascorbic acid analogues content (μg/mL)
Candida krusei	30
Candida utilis (NRRL Y 900)	20
Rhodotorula rubla	15
Torulopsis sphaerica	Neg

[a]No significant amount measurable.

yeasts used could produce ascorbic acid analogues.

The two highest yielding strains, Candida krusei and Hansenula california (NRRL Y 1425) were selected for further screening. Requirements for carbon and nitrogen compounds by these two strains were examined. In the study of carbon compounds, 2% carbon source was added in YNB, 16 carbon compounds were used (Table 3), while other conditions were the same as first screening. In the study of nitrogen compounds, 0.5% nitrogen source was added in YCB, 8 nitrogen compounds were used (Table 4), while other conditions were the same as first screening.

C. krusei could assimilate citric acid, fructose, glucose, glycerol, mannose and sucrose for growth and biosynthesis of ascorbic acid analogues. Sucrose was the poorest carbon source of this group. Although citric acid and mannose gave lower growth yield than fructose, glucose, and glycerol, the ascorbate productivity were about the same. C. krusei could not grow on arabinose, galactose, inositol, lactose, maltose, manitol, sorbitol, sorbose, D-(+)- and L-(-)-xylose. This yeast strain could grow on different nitrogen sources but not on KNO_2. The productivity of ascorbic acid analogues in this yeast depends not on growth level but on nitrogen source. The best nitrogen source for growth was ammonium citrate, but the productivity was pretty low comparing with that of ammonium nitrate which was the best nitrogen source for ascorbic acid analogue production. Ammonium chloride and ammonium acetate also

Table 3.--The availability of carbon compounds in ascorbic acid analogues production by *C. krusei* and *H. california*

Carbon source	*C. krusei*		*H. california*	
	Growth (OD_{600})	Productivity (μg/mL)	Growth (OD_{600})	Productivity (μg/mL)
D-(-)-Arabinose	N.G.[a]	Neg[b]	N.G.	Neg
Citric acid	0.37	30	N.G.	Neg
Fructose	0.51	30	0.46	50
Galactose	N.G.	Neg	0.13	Neg
Glucose	0.48	30	0.49	50
Glycerol	0.53	35	0.51	55
i-Inositol	N.G.	Neg	N.G.	Neg
Lactose	N.G.	Neg	N.G.	Neg
Maltose	N.G.	Neg	0.31	20
Mannitol	N.G.	Neg	0.40	50
Mannose	0.35	30	0.33	45
D-Sorbitol	N.G.	Neg	0.21	20
L-Sorbose	N.G.	Neg	0.22	35
Sucrose	0.27	10	0.25	20
D-(+)-Xylose	N.G.	Neg	0.35	25
L-(-)-Xylose	N.G.	Neg	N.G.	Neg

[a]No growth.

[b]No significant amount measurable.

Table 4.--The availability of nitrogen compounds in ascorbic acid analogues production by C. krusei and H. california

Nitrogen source	C. krusei Growth (OD_{600})	C. krusei Productivity (μg/mL)	H. california Growth (OD_{600})	H. california Productivity (μg/mL)
NH_4Cl	0.45	Neg[a]	0.43	27
NH_4NO_3	0.48	40	0.46	Neg
$(NH_4)_2HPO_4$	0.46	18	0.39	30
NH_4OAc	0.52	Neg	0.51	Neg
$(NH_4)_2$·Citrate	0.53	15	0.50	Neg
KNO_3	0.15	Neg	0.37	Neg
KNO_2	N.G.[b]	Neg	0.35	Neg
$(NH_4)_2CO$	0.25	Neg	0.40	10

[a]No significant amount measurable.

[b]No growth.

gave good growth, but no measurable amount of ascorbic acid analogues was found.

H. californica could utilize fructose, glucose, glycerol, maltose, mannitol, mannose, sorbitol, sorbose, sucrose and D-(+)-xylose for both growth and ascorbic acid analogues production. It also showed slow growth on galactose, but no measurable amount of ascorbic acid analogues was produced in this carbon source. This yeast could not grow on arabinose, citric acid, inositol, lactose or L-(-)-xylose. It could assimilate all nitrogen compounds in Table 4 for growth, but only ammonium chloride, ammonium phosphate and urea could be used for ascorbic acid analogues production, the best source being ammonium phosphate.

The accumulation of ascorbic acid analogues in C. krusei seemed to be a constant metabolic pathway because it produced these compounds during growth on citric acid, a carbon source from which hexose must arise by gluconcogenesis, however, H. californica could not grow under the same condition.

The study of nitrogen availability for both yeast strains showed that the nitrogen source is a critical factor in the ability of these cells to produce ascorbic acid analogues. It might possibly be the reason that many yeast strains could not produce measurable amounts of ascorbic acid analogues in YNB plus glucose. This assumption needs more investigation to prove, and is beyond the scope of this research.

A vitamin-free minimal medium containing only glucose, optimized nitrogen sources, phosphate salts and magnesium salt was devised for C. krusei and H. california. In this medium, C. krusei could produce up to 120 μg/mL ascorbic acid analogues, 4 times as much as the screening medium, while the other strain could produce only 1/3 the amount in the minimal medium.

The fermentation broth of C. krusei was streaked on Whatman No. 4 paper along with L-ascorbic acid and D-arabo-ascorbic acid. This paper was then developed in water-saturated methyl ethyl ketone. There was only one white spot for the fermentation broth on the paper (Figure 2) after spraying with 0.013% 2,6-dichlorophenol indophenol. Comparing the RF value of this spot with that of Murakawa's et al. (1977) showed this analogue to be D-erythroascorbic acid, a five carbon ascorbic acid analogue. This compound was the only ascorbic acid analogue in glucose fermentation by C. krusei.

Since there is no quantitative analysis for D-erythro-ascorbic acid available, Takahashi and his co-workers (1976) used the quantitative method for L-ascorbic acid analysis based on its reducing property to define the total amount of ascorbic acid analogues in fermentation broth. In this study, the 2,2'-dipyridyl method is used for the quantitative determination of D-erythroascorbic acid. The amount of this compound is therefore expressed as L-ascorbic acid equivalent (μg/mL).

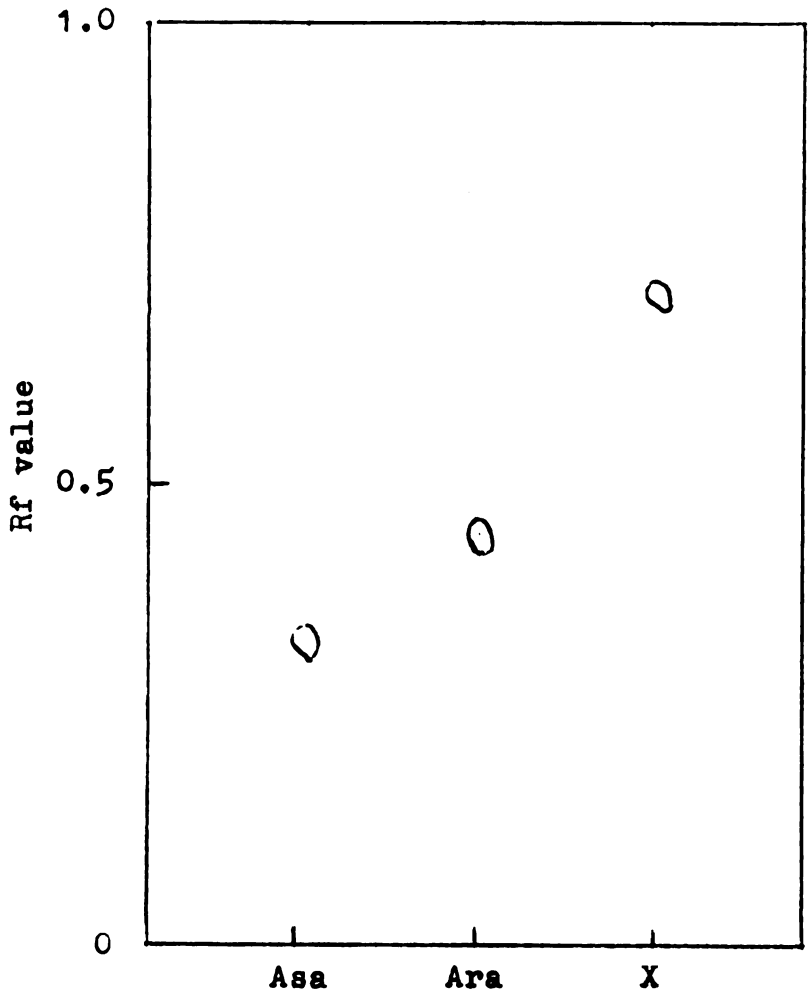


Fig. 2.--Paper chromatogram of C. krusei fermentation broth. Developing solvent was methylethylketone saturated with water, and spraying agent was 0.013% 2,6-dichlorophenol indophenol.
Asa, L-ascorbic acid; Ara, D-araboascorbic acid; X, C. krusei fermentation broth.

Media Composition

C. krusei was the only strain selected for D-erythroascorbic acid production. A minimal medium was used to study the effects of carbon concentration, nitrogen concentration, minerals, vitamins, phosphate salts, amino acids on the D-erythroascorbic acid production. The media thus developed were used to study the cultivation conditions, time course, and genetic improvements of D-erythroascorbic acid production by the yeast.

1. Effect of Phosphate Concentration

Phosphate plays important roles in physiological functions, such as energy transport in the form of ATP; phospholipids in cell membrane; skeleton in DNA and RNA; buffering and osmotic pressure of intracellular fluids.

The concentration of phosphate buffer was varied from 0.05 to 0.5% (w/v) with 3% glucose, 1% NH_4NO_3, 0.05% $MgSO_4 \cdot 7H_2O$ in the minimal medium. It was found (Figure 3) that 0.2% (w/v) phosphate salts gave the optimal productivity.

2. Effect of Carbon and Nitrogen Concentration

Glucose and ammonium nitrate were used as carbon source and nitrogen source respectively. The carbon concentration in minimal medium was changed from 1% to 15% to study its effect on D-erythroascorbic acid production; 1% ammonium nitrate, 0.2% phosphate salts, 0.05% $MgSO_4 \cdot 7H_2O$ were also added. The results (Figure 4) show that a between 5% and 9% glucose gave the highest productivity.

The nitrogen concentration in minimal medium was

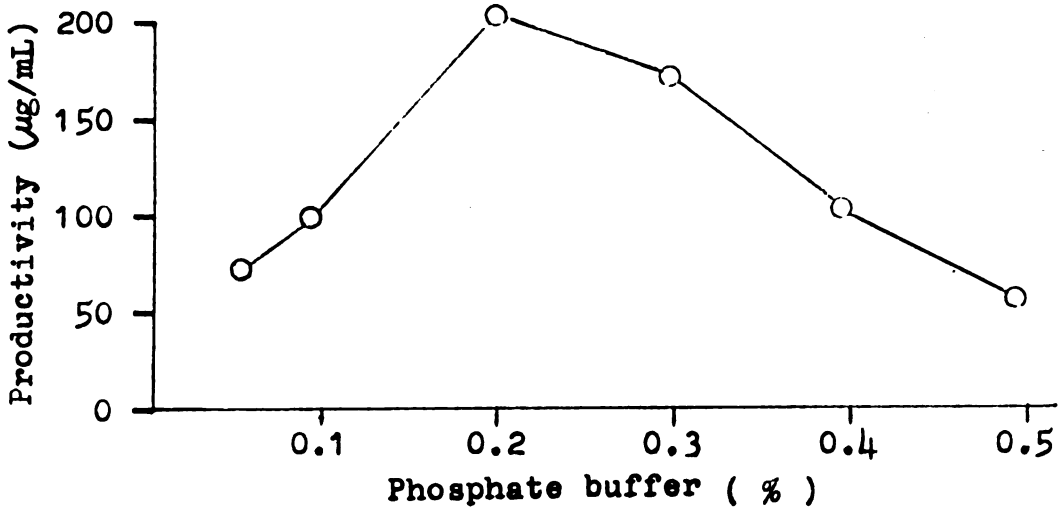


Fig. 3.--The effect of phosphate concentration on the erythroascorbic acid production by C. krusei.

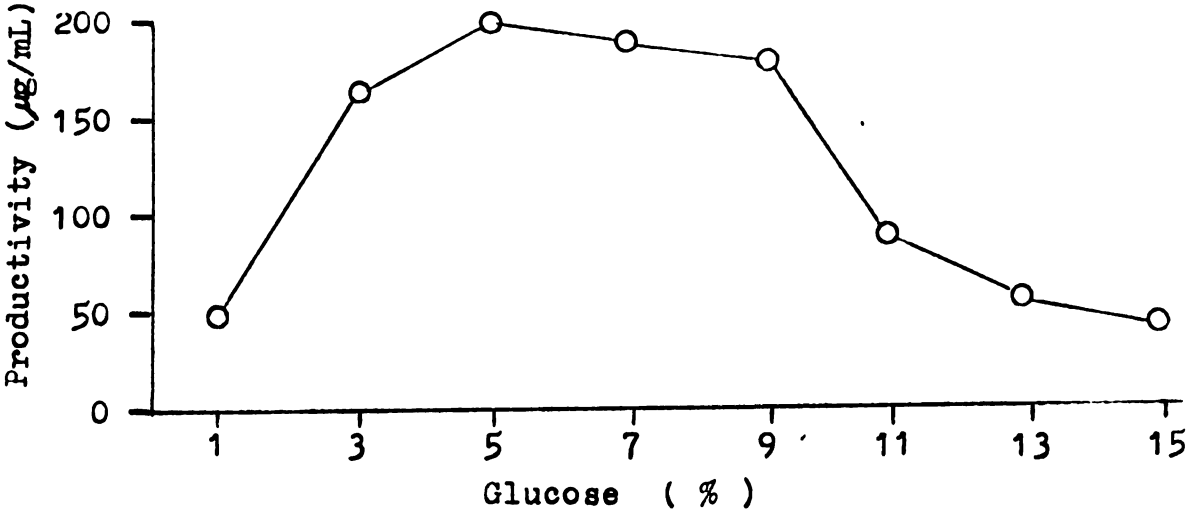


Fig 4.--The effect of carbon concentration on erythroascorbic acid production by C. krusei.

changed from 0.25% to 3% in the minimal medium with 3% glucose, 0.2% phosphate salts and 0.05% $MgSO_4 \cdot 7H_2O$ to study its effect on D-erythroascorbic acid production. The result (Figure 5) showed 1% ammonium nitrate was the optimal concentration.

In order to have more information on carbon concentration, an experiment was done by adding 20 mL minimal medium into 500 mL Erlenmeyer flasks with the glucose concentration 5%, 7% and 9% respectively. The result of this fermentation at 23 ^{o}C is shown in Figure 6. It indicates that the growth in higher sugar concentration reached optimal earlier, the erythroascorbic acid level is the same.

3. Effect of Trace Elements

In this study, 5 inorganic salts and 9 vitamins were individually added to minimal medium with 5% glucose, 1% NH_4NO_3, o.2% phosphate salts and 0.05% $MgSO_4 \cdot 7H_2O$. The temperature of fermentation was controlled at 30 ^{o}C.

In preparing concentrated stock solutions of inorganic salts for dilution into synthetic medium, each inorganic salt was stored at low pH to prevent precipitation. All these stock solutions were sterilized by autoclaving and distributed to minimal medium before use.

The vitamin solutions were also prepared and sterilized before adding into synthetic medium. Folic acid, biotin, Ca-pantothenate, riboflavin, pyridoxin-HCl and i-inositol are heat resistant and thus sterilized by autoclaving; p-aminobenzoic acid, niacin, and thiamin-HCl were sterilized

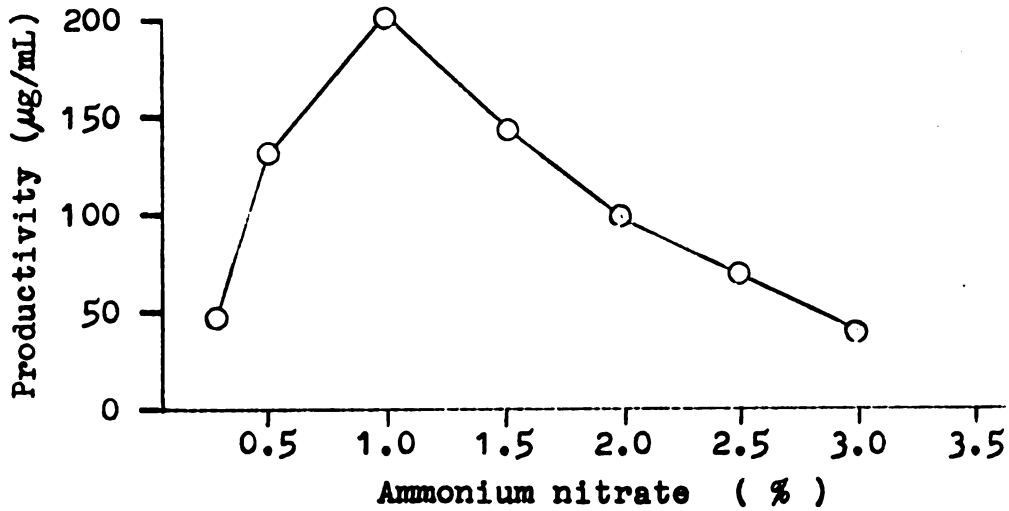


Fig. 5.--The effect of nitrogen concentration on erythroascorbic acid production by C. krusei.

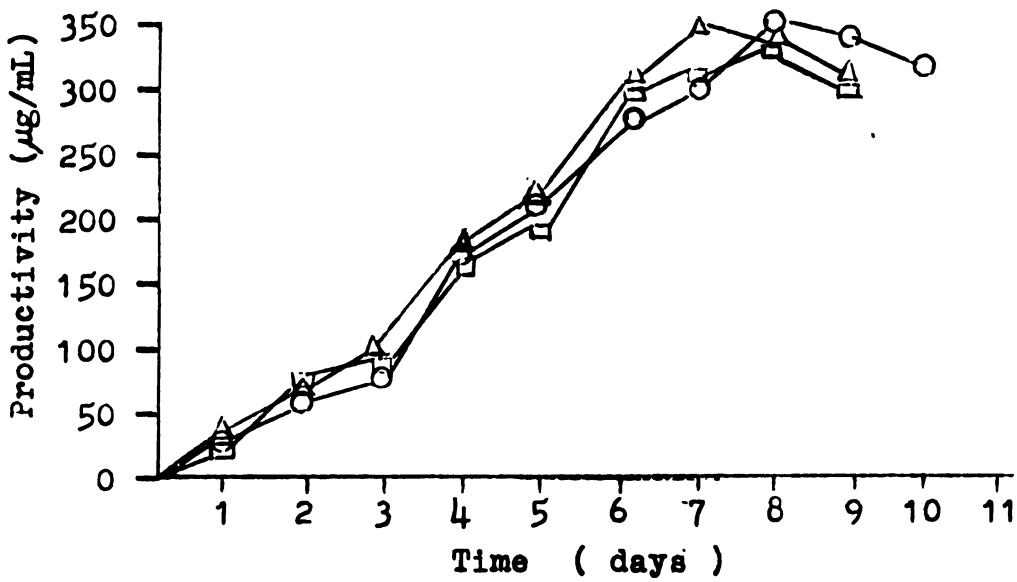


Fig. 6.--Glucose concentration and erythroascorbic acid production, 5% (O), 7% (Δ), 9% (□).

by filtration through milipore filters. These solutions were then added to the separately autoclaved medium.

The effect of trace elements on D-erythroascorbic acid production is shown in Table 5. None of these nutrients showed increase in yield at the given concentration, while ferric salt, cobalt salt, folic acid, niacin, p-aminobenzoic acid, pyridoxin-HCl and thiamin-HCl gave negative results. C. krusei can grow well without any of these elements in the medium.

A further investigation with different concentrations of some trace elements was done. The fermentation was carried out at 23 °C, other conditions were not changed. Table 6 and Table 7 show the results of this investigation. No prominent increase in productivity could be found although some of these nutrients gave better growth response.

When the magnesium salts was eliminated in the minimal medium, there was no difference in growth and productivity when compared with original medium.

4. Effect of Amino Acid

The natural occurring α-amino acids were used in this experiment a 1% stock solution of each amino acid was sterilized by filtration and added separately to autoclaved minimal medium to the final concentration of 0.1% amino acid, 15% of yeast cells were transferred into these media. This fermentation was carried out in a shaker at 23 °C for 5 days. The results in Table 8 indicate the following:

Table 5.--Effect of trace elements on erythroascorbic acid production by *C. krusei* at 30 °C

Trace elements	Concentration (μg/L)	Productivity (μg/mL)
None	0	210
$CuSO_4 \cdot 5H_2O$	40	200
$FeCl_3$	200	160
$MnSO_4$	400	200
$ZnSO_4 \cdot 7H_2O$	400	210
$CoCl_2 \cdot 6H_2O$	400	100
Biotin	2	180
Ca-Pantothenate	400	200
Folic acid	2	150
Inositol	2000	200
Niacin	400	120
P-Aminobenzoic acid	200	150
Pyridoxin-HCl	400	120
Riboflavin	200	200
Thiamin-HCl	400	150

Table 6.--Effect of minerals concentration on erythroascorbic acid production by C. krusei at 23 °C

Minerals	Concentration (μg/L)	Growth (OD_{600})	Productivity (μg/mL)
None	0	0.64	300
$CuSO_4 \cdot 5H_2O$	25	0.74	310
	50	0.74	320
	100	0.66	310
	200	0.63	300
$ZnSO_4 \cdot 7H_2O$	500	0.64	320
	1000	0.70	330
	1500	0.70	330
	2000	0.68	280
$MnSO_4$	500	0.62	310
	1000	0.70	310
	1500	0.68	310
	2000	0.68	320

Table 7.--Effect of vitamins concentration on erythroascorbic acid production by C. krusei at 23 °C

Vitamins	Concentration (μg/L)	Growth (OD_{600})	Productivity (μg/mL)
None	0	0.64	300
Riboflavin	250	0.65	300
	500	0.65	300
	750	0.65	290
	1000	0.66	290
i-Inositol	2500	0.70	300
	5000	0.68	320
	7500	0.66	320
	10000	0.64	300
Biotin	15	0.62	260
	30	0.63	280
	60	0.62	280
	120	0.63	280

Table 8.--Effect of amino acids on erythroascorbic acid production by C. krusei

Amino acid (0.1%)	Growth (OD_{600})	Productivity (μg/mL)
None	0.68	300
Neutral amino acids		
Alanine	0.75	360
Valine	0.75	280
Leucine	0.64	170
Isoleucine	0.70	200
Proline	0.75	300
Phenylalanine	0.68	140
Tryptophane	0.64	170
Methionine	0.75	160
Glycine	0.85	360
Serine	0.80	340
Threonine	0.80	310
Cysteine	0.66	80
Tyrosine	0.66	220
Acidic amino acids		
Aspartic acid	0.80	190
Glutamic acid (MSG)	0.90	260
Basic amino acids		
Lysine	0.75	400
Arginine	0.75	330
Histidine	0.76	350

(1) The 3 basic amino acids were effective nutrients not only for growth but for productivity, (2) the 2 acidic amino acids decreased the productivity although they gave high growth response, (3) the sulfur-containing amino acids group, methionine and cysteine gave low productivity, cysteine gave only about as much 20% productivity as that of lysine, (4) the amino acids containing phenyl group, phenylalanine, tryptophane and tyrosine, gave decreased productivity, (5) the amino acids with branched carbon chain, valine, leucine and isoleucine, showed less productivity than those without branched carbon chain, alanine, glycien, serine and threonine. The highest productivity was obtained by lysine, the lowest by cysteine. Such information might be useful in the study of biosynthetic pathway.

Physical Conditions

1. Effect of Aeration and Agitation

The aeration and agitation in this fermentation was accomplished by the reciprocating action of a shaker. The shaking speed was fixed on 130 strokes per minute in the study of D-erythroascorbic acid production. In order to change the aeration rate, a simple qualitative method was used. Different volumes of medium were distributed into test tubes or flasks; smaller volumes of medium resulting higher rate of aeration.

The primary objective of shaking is to supply the

necessary oxygen to the yeast either for growth or D-erythroacid production. A second function is to keep the yeast cells in suspension so that they could be surrounded by nutrients or oxygen uniformly.

The result in Figure 7 show the D-erythroascorbic acid production favored high aeration. It implied that the biosynthstic pathway of D-erythroascorbic acid is an oxidative process, however, the formation of the dienol structure also suggested that some reductive reactions might be involved.

The oxygen needed for this reaction is incorporated through the intermediate stage of the dissolved oxygen molecule. In other words, the yeast cells respond to the liquid phase oxygen concentration in regulation its overall metabolic activities. The solubility of oxygen is extremely limited as compared to other nutrients, it is necessary to continuously supply the broth with oxygen in order to meet the metabolic demands of the yeast cells.

2. Effect of pH

The effect of pH on D-erythroascorbic acid production was studied by changing the initial pH values in the medium from 2 to 8; the pH of the medium was adjusted with 1N NaOH or 1N HCl. Two inocula ratios of yeast cells (15% and 100%) were applied.

With a 15% washed inoculum, after the cultures were shaken for 4 days, the final pH of all this cultures dropped to pH 2. The results in Figure 8 showed peaks in both

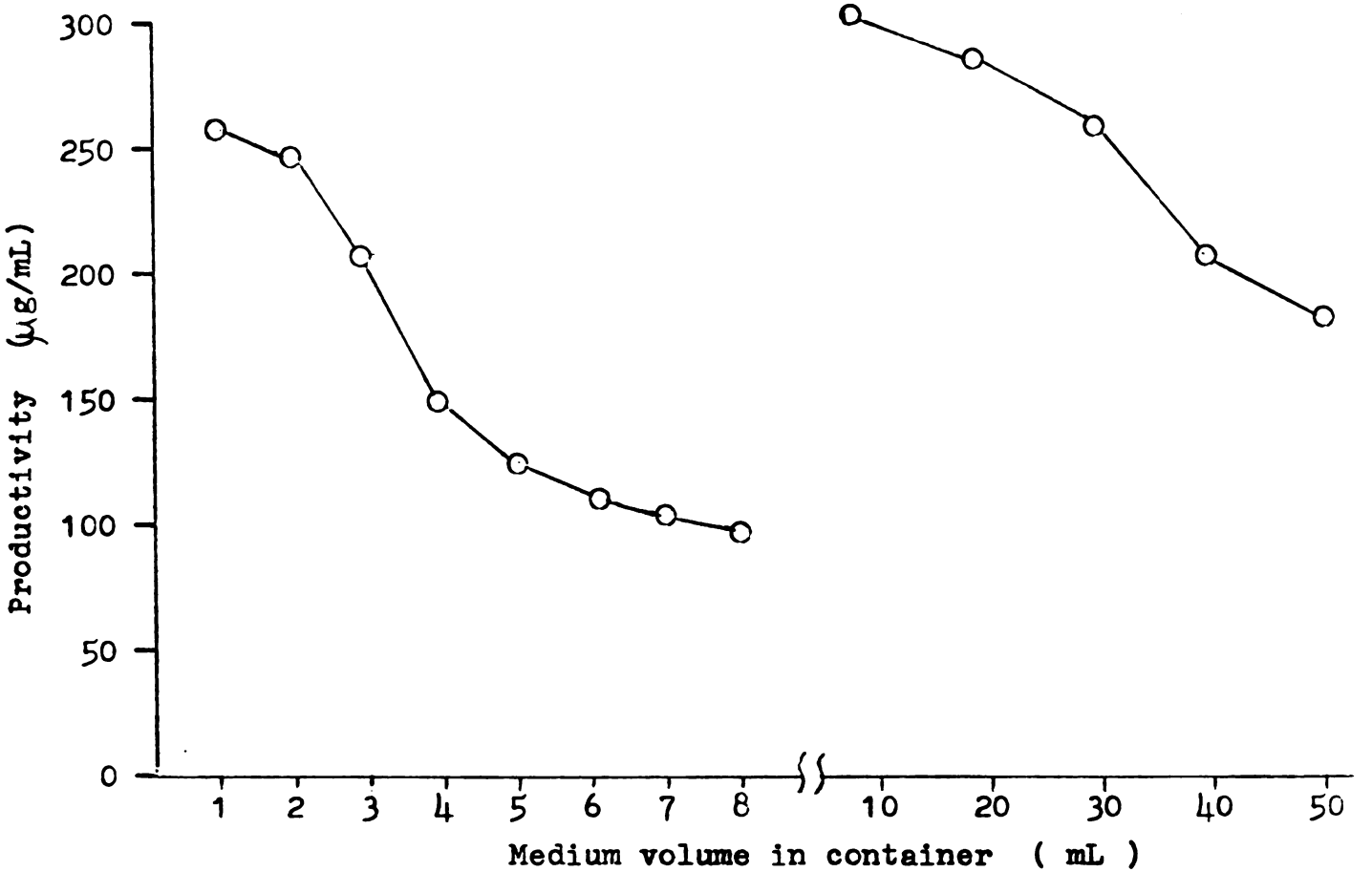


Fig. 7.--Effect of aeration on erythroascorbic acid production by C.krusei on test tube scale (left part) and flask scale (right part) fermentation.

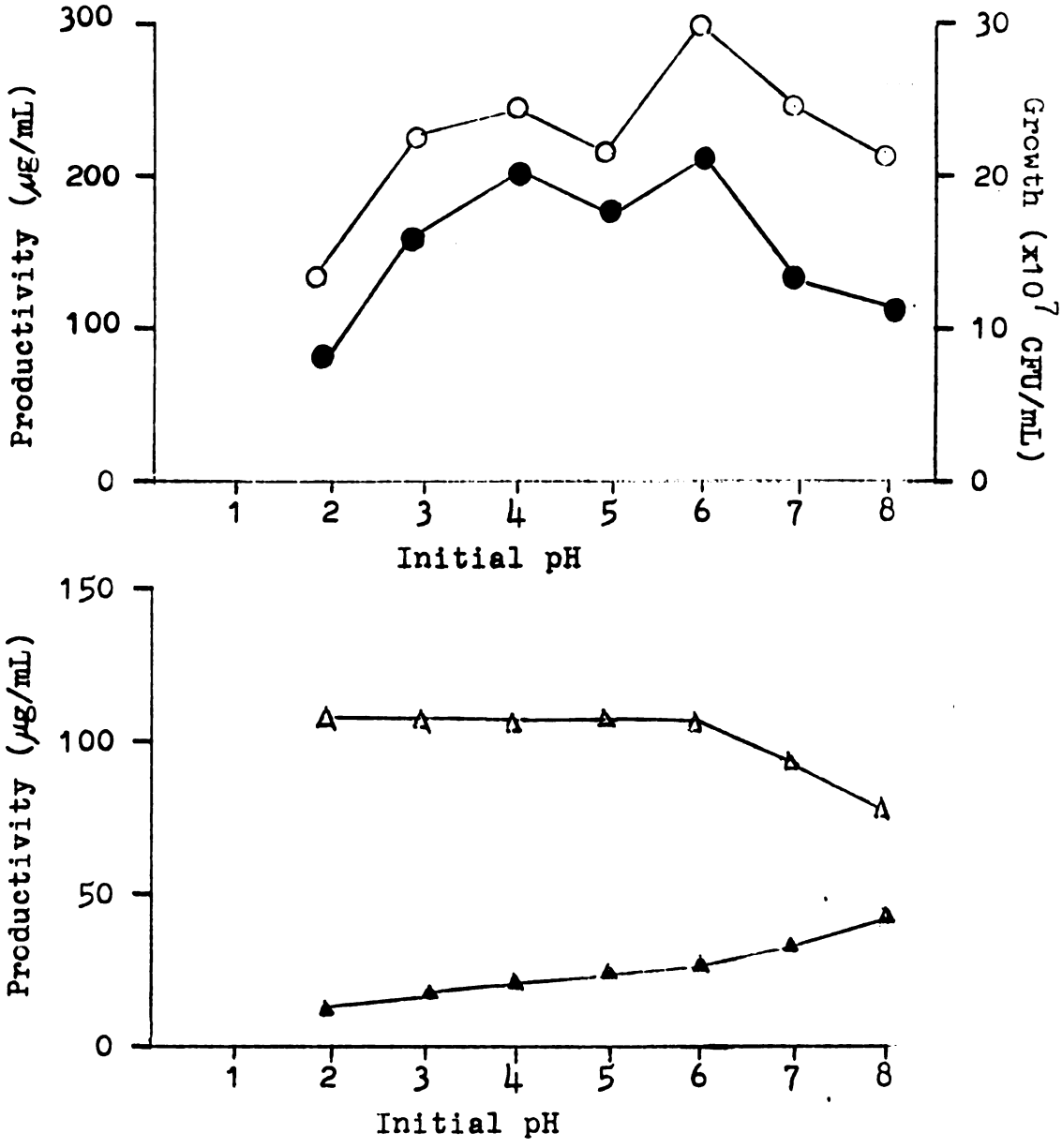


Fig. 8.--Effect of initial pH on erythroascorbic acid production by C. krusei with 15% transfer (upper part), growth (●), productivity (O); and 100% transfer (lower part), incubation time, 12 hr (▲), 60 hr (△).

growth and productivity at pH 4 and pH 6 respectively.

The 100% washed inoculum showed different results at 12 hours and 60 hours incubation. The data obtained from 1.5 hours, 3 hours, 6 hours incubation showed no difference from that obtained from 12 hours incubation. The pH of each tube dropped down to pH 2 after 60 hours incubation.

3. Effect of Temperature

Temperature can be expected to exert a profound effect on all aspects of growth, metabolism and yeast cell survial . In this study, Candida krusei was incubated at 18 °C, 23°°C, 30 °C, 37 °C or 43 °C. This yeast grew well at each temperature except 43 °C when the yeast cells coagulated and did not grow. The optimal temperature (Figure 9) for erythroascorbic acid production is 23 °C in this study.

4. Amount of Inoculum

This study was carried out on flask scale with 20 mL of minimal medium. The inoculum ratios were studied at 5%, 10% and 15%. Minimal medium with 5% glucose was used in this fermentation, the cultures reached optimal growth after 7 days cultivation at 23 °C. The higher inoculum favored the growth yield of yeast cells as well as the D-erythroascorbic acid production (Figure 10).

Time Course for D-Erythroascorbic Acid Production

The time course of the fermentation by wild type yeast cells was studied. The fermentation medium was on minimal medium with 5% glucose. 20 mL of this medium was placed in a

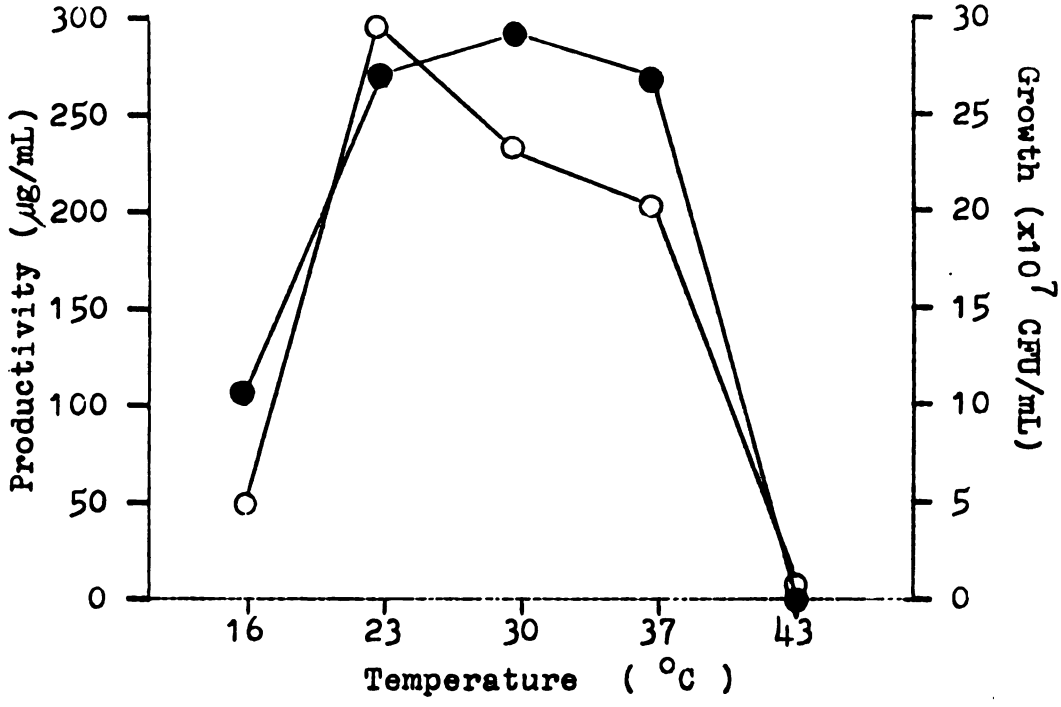


Fig. 9.--Temperature effect on erythroascorbic acid production by C. krusei, growth (●), productivity (○).

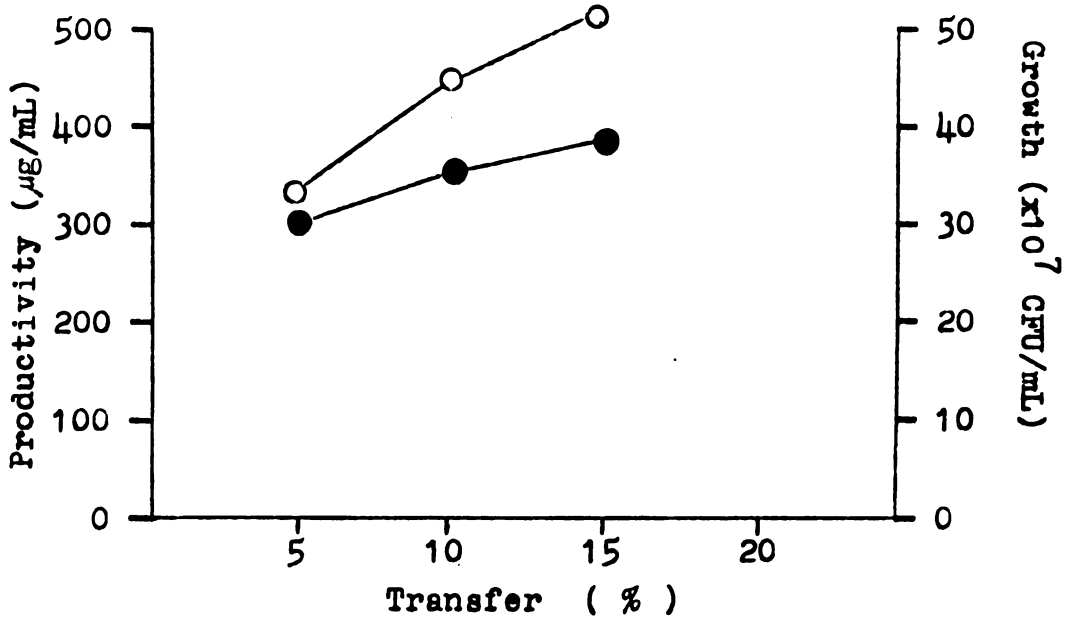


Fig. 10.--Effect of transfer ratio on erythroascorbic acid production by C. krusei, growth (●), productivity (○).

500 mL Erlenmeyer flask, with 15 % inoculum and fermented at 23 °C. The growth of yeast cells and the D-erythroascorbate concentration began to increase quickly after about 1 day; both were maximal at about 7 days. The fermentation broth was qualitatively estimated by paper chromatography to show the identity of D-erythroascorbic acid in the broth. The pH of the fermentation broth dropped very quickly and reached pH 2 within the first 48 hours and gradually decrease to pH 1.8 thereafter (Figure 11).

Genetic Improvements of D-Erythroascorbic Acid Productivity

The usual genetic manipulation required in industrial process is for yield improvement. The most common way of obtaining yield improvement after physiological variables have been optimized is by the process of mutation. In this study, ethylmethanesulfonate (EMS) was used as mutagen. The EMS mutation procedure is convenient and gives high yield of mutants. EMS is an alkylating agent that causes transition and transversion of purine and pyrimidine bases on DNA molecules, which make it possible to obtain revertants from mutant by the same process.

In this study, 3% and 6% of EMS (v/v) were applied. The killing effect of these two concentrations on wild type C. krusei were 80% and 95% respectively, the remaining 20% and 5% survivals were grown in YEPD medium and plated on YEPD agar. All the mutants that could grow on YEPD agar but not on minimal medium were picked out and transferred

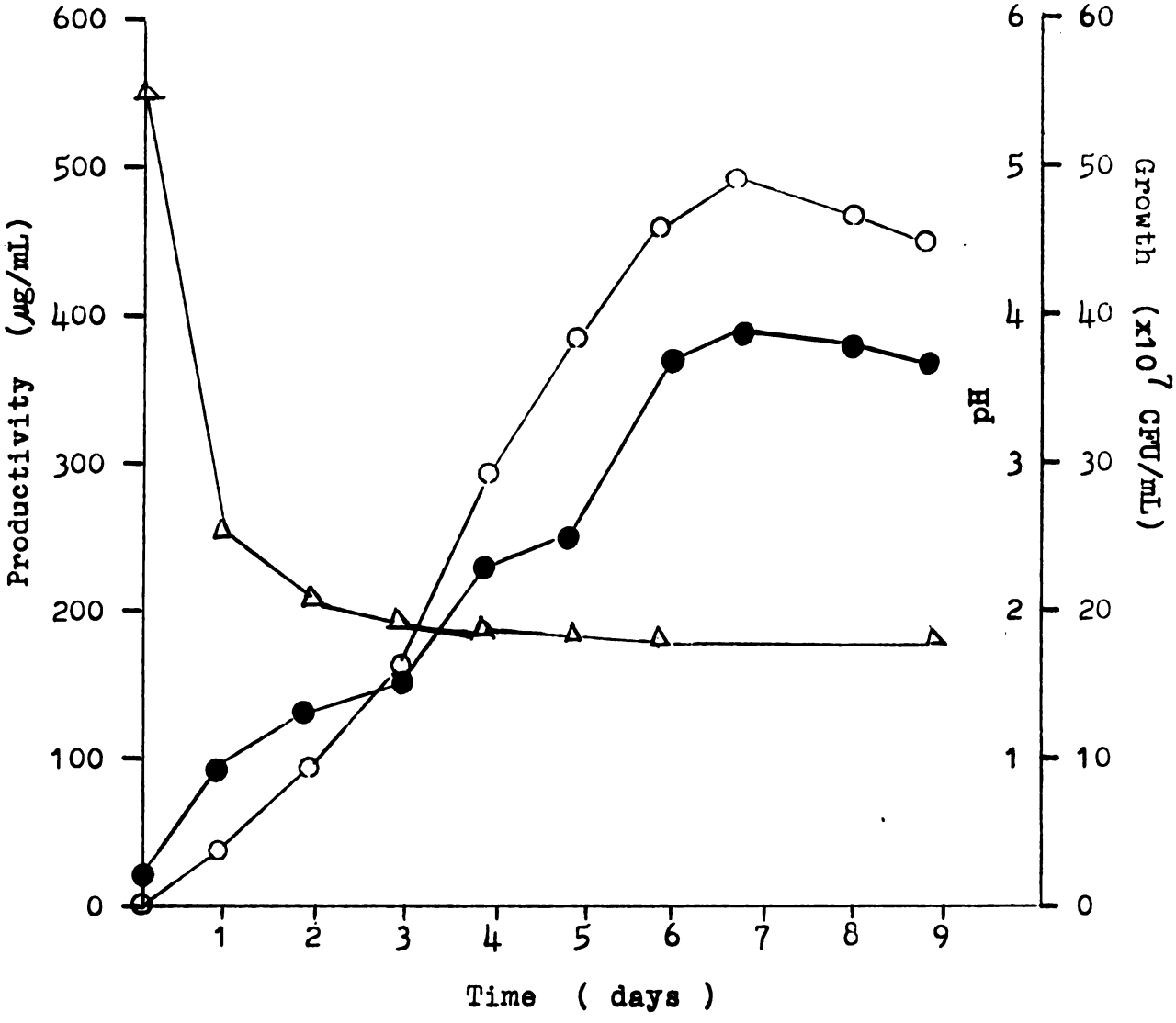


Fig. 11.--Time course of etythroascorbic acid fermentation by C. krusei, growth (●), productivity (○), pH (△).

onto YEPD agar plates. The D-erythroascorbic acid producing power of mutants was examined by growing them in minimal medium supplemented with 1% YEPD liquid medium for growth. Among two hundred mutants examined, none of them could produce increasing amount of D-erythroascorbic acid, most of them possessed between 60 to 100% of the productivity as much as the wild type cells (Table 9).

Mutants that showed less than 20% of the productivity in minimal medium supplemented with 1% YEPD liquid medium. One of these mutants did show moderate growth. This mutant was selected for another mutation to obtain revertants.

The nutritional requirements of this mutant were examined. The mutant was grown in minimal medium containing 0.1% of various amino acids in each tube and incubated for 3 days. It showed moderate growth in the medium with aspartic acid or glutamic acid (acidic amino acids), and also showed a little growth in the medium with methionine or cysteine (sulfur-containing amino acids).

The amino acids requiring mutant was mutated with 6% EMS, the master plates were imprinted onto both minimal agar plates and minimal agar supplemented with 200 μg/mL L-ascorbic acid. Of the 500 revertants growing on minimal agar plates only one could not grow on minimal medium supplemented with L-ascorbic acid; this mutant was defined as the L-ascorbic acid sensitive mutant.

The D-erythroascorbic acid productivity of these

Table 9.--Erythroascorbic acid productivity among mutants derived from wild type C. krusei

% Productivity[a]	% Mutants[b]
80 - 100	25
60 - 80	27
40 - 60	28
20 - 40	11
0 - 20	9

[a]Wild type yeast as 100%.
[b]Total number of mutants was 200.

Table 10.--Erythroascorbic acid productivity among revertants derived from amino acids requiring mutant

% Productivity	% Revertants[a]
> 100	20
80 - 100	50
60 - 80	19
40 - 60	6.5
0 - 40	0.5

[a]Total number of revertants was 500.

revertants was tested, 20% of them (Table 10) produced more D- erythroascorbic acid than wild type cells. Among these higher productivity mutants, the two highest were selected (mutant 282 and mutant 353) to study the fermentation time course along with wild type cells in the minimal medium at 23 ^{o}C (Figure 12 A&B). The revertant 353 grew faster and better than the wild type yeast, and the revertant 282 grew at about the same level as wild type yeast. D-Erythroascorbate productivity were 160% and 140% respectively. The compound in the fermentation broth of the two mutants identified by paper chromatogram showed that both mutants produced the same compound as wild type yeast.

The EMS mutation of yeast cells changed their metabolic pathways. The productivity of D-erythroascorbic acid was decreased among most of the cells either because of growth level or nutritional requirements after the first mutation. After the second mutation the productivity of most cells were restored. Some mutants even showed significant increase in both growth level and metabolic activity. Repeated mutation of the high productivity mutants could lead to better yield of D-erythroascorbic acid in future studies.

Selection of Analogue-Resistant Mutants

The selection of analogue-resistant mutants has proved to be a useful technique for increased production of vitamin (Matsui, et al., 1982) and amino acids (Tsuchida, et al., 1975) by bacteria. The over production of an essential

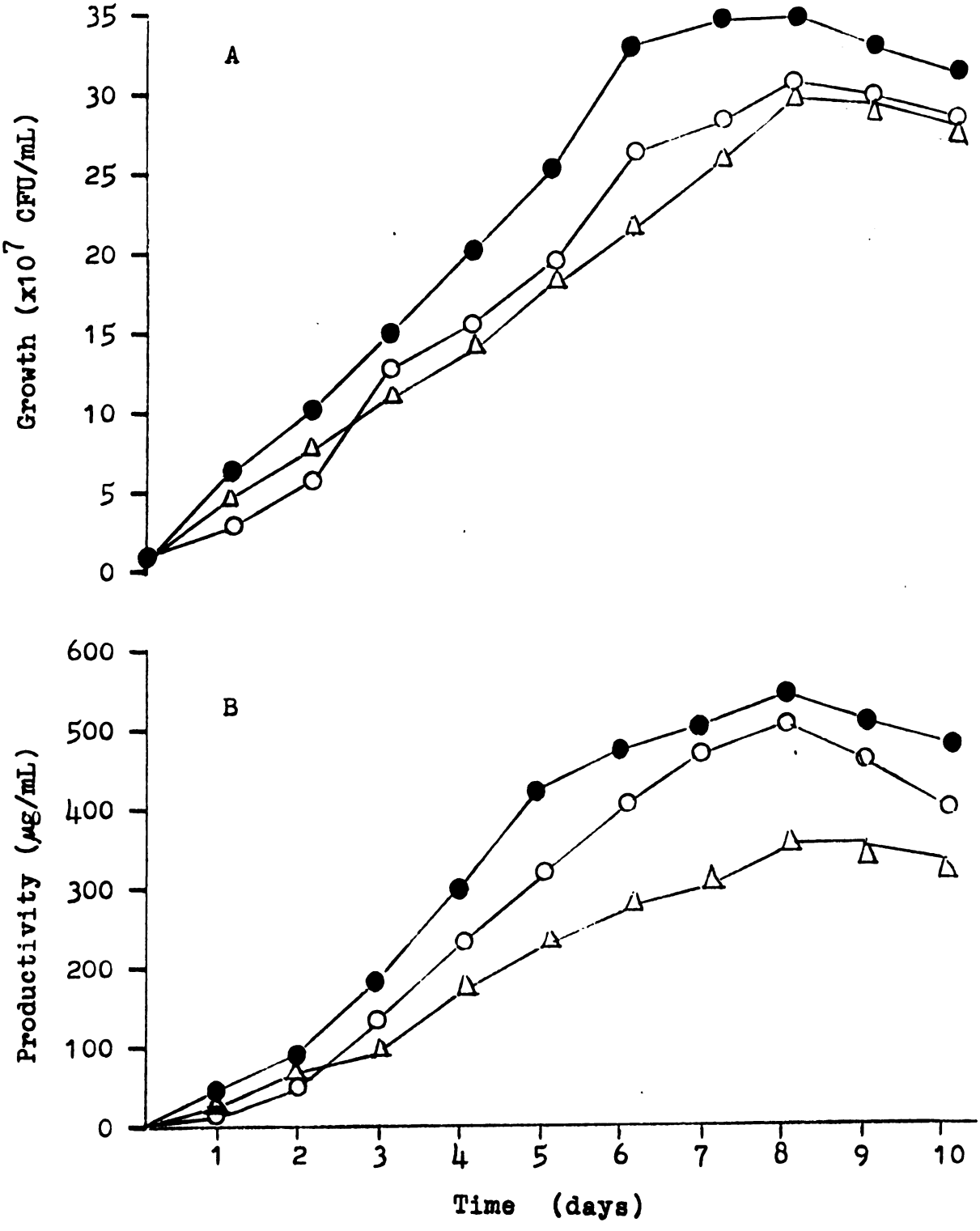


Fig. 12.--Growth curves (A) and erythroascorbic acid productivity (B) of wild type yeast (△), mutant 282 (○), and mutant 353 (●).

metabolite in a resistant bacterium in response to an antibacterial substance is already known (White and Woods, 1965). This mechanism is based on the assumption that analogues or analogue-like antibacterial substances are available for altering genetically the control mechanism of metabolites synthesis.

In this study, 7 structure related compounds were used, they were L-ascorbic acid, D-araboascorbic acid, D-glycero-L-manno-heptonic acid -γ-lactone, α,β-glucooctanoic acid lactone, α-D-glucoheptonic acid -γ-lactone, D-(+)-ribonic acid, D-glucurono-3,6-lactone. None of these compounds showed inhibitory effect on wild type yeast growth even with concentration of these compounds up to 20,000 μg/mL in the minimal agar plates. Two structure-related fungal metabolites were also used, penicilic acid and pautulin. These showed no inhibition on growth at the levels up to 250 μg/mL.

An L-ascorbic acid sensitive mutant was isolated from the back mutation of an amino acids requiring mutant. This L-ascorbic acid sensitive mutant could grow on minimal medium but not on minimal medium supplemented with L-ascorbic acid; in 500 μg/mL L-ascorbic acid concentration about 0.1% of the cells could grow, while in 1000 μg/mL concentration about 0.01% of the cells could grow.

When Ems mutation was applied to this sensitive mutants, many revertants were obtained which could grow on minimal medium plus 10,000 μg/mL L-ascorbic acid, however

no increase in D-erythroascorbic acid productivity was found.

SUMMARY AND CONCLUSION

The screening of D-erythroascorbic acid producers was carried out on a test tube scale. Of 57 yeast strains examined, 18 showed the ability to produce ascorbic acid analogues. The two most potent producers, Candida krusei and Hansenula california, were selected for further study as to the effects of carbon and nitrogen sources. A minimal medium was devised and the productivity of ascorbic acid analogues of these two strains were examined. C. krusei was selected for paper chromatography and a single ascorbic acid analogue, D-erythroascorbic acid was found in fermentation broth.

An optimal medium containing 5% glucose, 1% NH_4NO_3, 0.2% phosphate buffer and 0.05% $MgSO_4 \cdot 7H_2O$ was devised for the study of physical variables and mutation-selection process.

The study of physical variables showed that 23 ^{o}C, high aeration rate, an initial pH of 6.0, and 15% inoculum were optimal for D-erythroascorbic acid production. EMS mutation gave two mutants which showed 160% and 140% productivity respectively of that for wild type yeast cells. The selection of analogue-resistant mutants was not succesful.

The ascorbic acid analogues are widely used as

antioxidants in industry because of their strong reducing power. D-Erythroascorbic acid could be used in bath for electroplating iron alloys to give good brightness and good adherence, or as a reducing agent in acrylonitrile polymerization to give good fiber color. The direct fermentation method would be desirable if the yield of D-erythroascorbic acid could be improved dramatically through genetic techniques. This research gave the basic medium and optimal physical conditions for the investigation of yield improvement by mutants. Different mutation processes by various mutagens can be applied to the high yield mutants in future study.

LIST OF REFERENCES

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Aaronson, S., Dhawale, S.W., Patni, N.J., DeAngelis, B., Frank, O., and Baker, H., 1977. The cell content and secretion of water-soluble vitamins by several fresh-water algae. Arch. Microbiol. 112:57.

Bauernfeind, J.C., Pinkert, D.M., 1974. L-Ascorbic acid and food technology. In: Encyclopedia of Food Technology. Vol. II. edited by Johnson, A.H., Peterson, M.S. AVI Publishing Company, Inc., Westport, Connecticut.

Berensci, G., Illenyi, A., 1938. Über die ascorbinsaure-bildung des bac. prodigiosus. Biochem. Z. 298:298.

Birch, G.G., Parker, K.J., 1974. Vitamin C - Recent Aspects of Its Physiological and Technological Importance. John Wiley & Sons, New York.

Bleeg, H.S., 1966. L-Ascorbic acid in yeast and isolation of L-galactono-γ-lactone oxidase from the mitochondria. Enzymologia 31:105.

Bourne, G., Allen, R., 1935. The distribution of vitamin C in lower organisms. Austr. J. Exper. Biol. Med. Sci. 13:165.

Cameron, E., Pauling, L., 1979. Cancer and Vitamin C. Warner books, New York.

Chatterjee, I.B., 1973. Evolution and biosynthesis of ascorbic acid. Science 182:1271.

Chatterjee, I.B., Majumder, A.K., Nandi, B.K., Subramanian, N., 1975. Synthesis and some major functions of vitamin C in animals. Ann. N.Y. Acad. Sci. 258:24.

Crawford, T.C., Crawford, S.A., 1980. Synthesis of L-ascorbic acid. In: Advances in Carbohydrate Chemistry and Biochemistry. 37:79. Academic Press, New York.

Difco Manual, 1953. 9th ed. Difco Laboratories, Detroit.

Fink, G.R., 1970. The biochemical genetics of yeast. In: Methods in Enzymology. 17A:59. Academic Press, New York and London.

Geiger, H.M., Galli, H., 1945. Uber den nachweiss der l-ascorbinaure als stoffwechsel product von Aspergillus niger. Helv. Chim. Acta. 28:248.

Harris, R.S., 1967. Active compounds and ascorbic antagonists. In: Vitamins Vol. I. edited by Sebrell, W.H., Harris, R.S. Academic Press, New York and London.

Hay, G.W., Lewis, B.A., Smith, F., 1967a. Chemistry of ascorbic acid. In: Vitamins. Vol. I. edited by Sebrell, W.H., Harris, R.S. Academic Press, New York and London.

Hay, G.W., Lewis, B.A., Smith, F., 1967b. Industrial preparation of ascorbic acid. In: Vitamins. Vol. I. edited by Sebrell, W.H., Harris, R.S. Academic Press, New York and London.

Heick, H.M.C., Stewart, H.B., Graff, G.L.A., Humpers, J.Z.C., 1969. Occurrence of ascorbic acid in the yeast Lipomyces starkeyi. Can. J. Biochem. 47:751.

Heick, H.M.C., Graff, G.L.A., Humper, J.Z.C., 1972. The occurrence of ascorbic acid among yeasts. Can. J. Microb. 18:597.

Hochhauser, S.J., 1983. Bringing biotechnology to market. High Technology 3(2):55.

Hodges, R.E., 1980. Ascorbic acid. In: Modern Nutrition in Health and Disease. edited by Goodhart, R.S., Shirls, M.E. Lea and Febger, Phildephia.

Humpers, J., Graff, G., 1968. Ascorbic acid synthesis by Saccharomyces cerevisiae. Chemical Abstracts 68:112441e.

Isherwood, F.A., Chen, Y.T., Mapson, L.W., 1953. Synthesis of L-ascorbic acid in plants and animals. Nature 171:348.

Isherwood, F.A., Chen, Y.T., Mapson, L.W., 1954. Synthesis of L-ascorbic acid in plants and animals. Biochem. J. 56:1.

King, C.G., 1961. The history and present status of vitamin C. Ann. N.Y. Acad. Sci. 92:3.

King, C.G., 1973. The biological synthesis of ascorbic acid acid. World Rev. of Nut. and Diet. 18:47.

Kulhanek, M., 1970. Fermentation processes employed in vitamin C synthesis. In: Advances in Applied Microbiology. 12:11. Acedemic Press, New York and London.

Kutsky, J.R., 1981. Vitamin C. In: Vitamins, Minerals and Hormones. 2nd ed. Van Nostrand Reinhold Company, N.Y.

Lederberg, J., Lederberg, E., 1952. Replica plating and indirect selection of bacteria mutants. J. of Bact. 63:399.

Loewus, F.A., 1961. Aspects of ascorbic acid biosynthesis in plants. Ann. N.Y. Acad. Sci. 92:57.

Loewus, F., 1971. Carbohydrates interconversions. Annu. Rev. Plant Physiol. 22:337.

Mapson, L.W., Isherwood, F.A., 1956. Biological synthesis of ascorbic acid: the conversion of derivatives of D-galacturonic acid into L-ascorbic acid by plant extracts. Biochem. J. 64:13.

Matsui, K., Wang, H., Hirota, T., Mutsukawa, H., Kasai, S., Shinagawa, K., 1982. Riboflavin production by Roseoflavin resistant strains of some bacteria. Agri. Biol. Chem. 46:2003.

Miki, T., Kikuchi, N., 1962. Paper chromatographic estimation of L-ascorbic and D-araboascorbic acids. J. of Vitaminology 8:279.

Miles Laboratories, 1964. Production of D-araboascorbic acid. Chemical Abstracts 59:6508a.

Murakawa, S., SAno, S., Yamashita, H., Takahashi, T., 1977. Biosynthesis of D-erythroascorbic acid by Candida. Agri. Biol. Chem. 41:1799.

Nishikim, M., Noguchi, E., Yagi, J., 1978. Occurrence in yeast of L-galactonolactone oxidase. Arch. of Bioch. and Bioph. 191:479.

Omaye, S.T., Turnbull, J.D., Sauberlich, H.E., 1979. Selected methods for the determination of ascorbic acid in animals tissues and fluids. In: Methods in Enzymology 62:3. Academic Press, New York and London.

Prince, R., Reichstein, T., 1937. Synthesis of D-xylo-sonic acid. Chemical Abstracts 31:3008.

Reichstein, T., 1933a. Synthesis of D- and L-ascorbic acid acid. Nature 132:280.

Reichstein, T., 1933b. Die synthese der d-ascorbinsaure. Helv. Chim. Acta. 16:561.

Reichstein, T., Grussner, A., 1934. Eine ergiebige synthese der l-ascorbinsaure. Helv. Chim. Acta. 17:311.

Smith, F., 1946. Analogues of ascorbic acid. In: Advances in Carbohydrate Chemistry 2:79. Academic Press, New York and London.

Sebrell, W.H., Harris, R.S., 1967. The Vitamins. Academic press, New York and London.

Sharman, I.M., 1974. Vitamin C: Historical aspects. In: Vitamins. Vol. I. edited by Sebrell, W.H., Harris, R.S. Academic Press, New York and London.

Shigeoka, S., Yokota, A., Nakano, Y., Kitaoka, S., 1979a. The effect of illumination on the L-ascorbic acid content in Euglena gracillis Z. Agri. Biol. Chem. 43:2053.

Shigeoka, S., Nakano, Y., Kitaoka, S., 1979b. The biosynthetic pathway of L-ascorbic acid in Euglena gracillis Z. J. Nutr. Sci. Vitaminol. 25:299.

Talpasayi, E.R.S., 1967. Localization of ascorbic acid in heterocytes of blue-green algae. Current Science 36:190.

Takeshi, T., Mitsumoto, M., 1961. Studies on the pathway of D-araboascorbic acid synthesis by Penicillium. Biochim. Biophys. Acta. 51:410.

Takeshi, T., Mitsumoto, M., 1965. Production of D-araboascorbic acid by Penicillium. Chemical Abstracts 62:13549a.

Takahashi, T., Murakawa, S., Barada, M., 1976. Ascorbic acid analogues as indirect products of Serratia marcescens. Agri. Biol. Chem. 40:1255.

Theander, O., 1980. Acids and other oxidation products. In: The Carbohydrates, Chemistry and Biochemistry. edited by Pigman, W., Horton, D. Academic press, New York and London.

Tsuchida, T., 1975. Production of L-valine by 2-thiazolealanine resistant mutants. Agri. Biol. Chem. 39:1319.

White, D.J., Wood, D.D., 1965. The synthesis of p-amino-benzoic acid and folic acid by Staphylococci sensitive and resistant to sulphonamides. J. Gen. Microbiol. 40:243.

Yagi, J., 1967. Studies on erythorbic acid production by fermentation. Agri. Biol. Chem. 31:340.

Yasuda, N., 1967. Japan Pattern Sho 42-11990.