

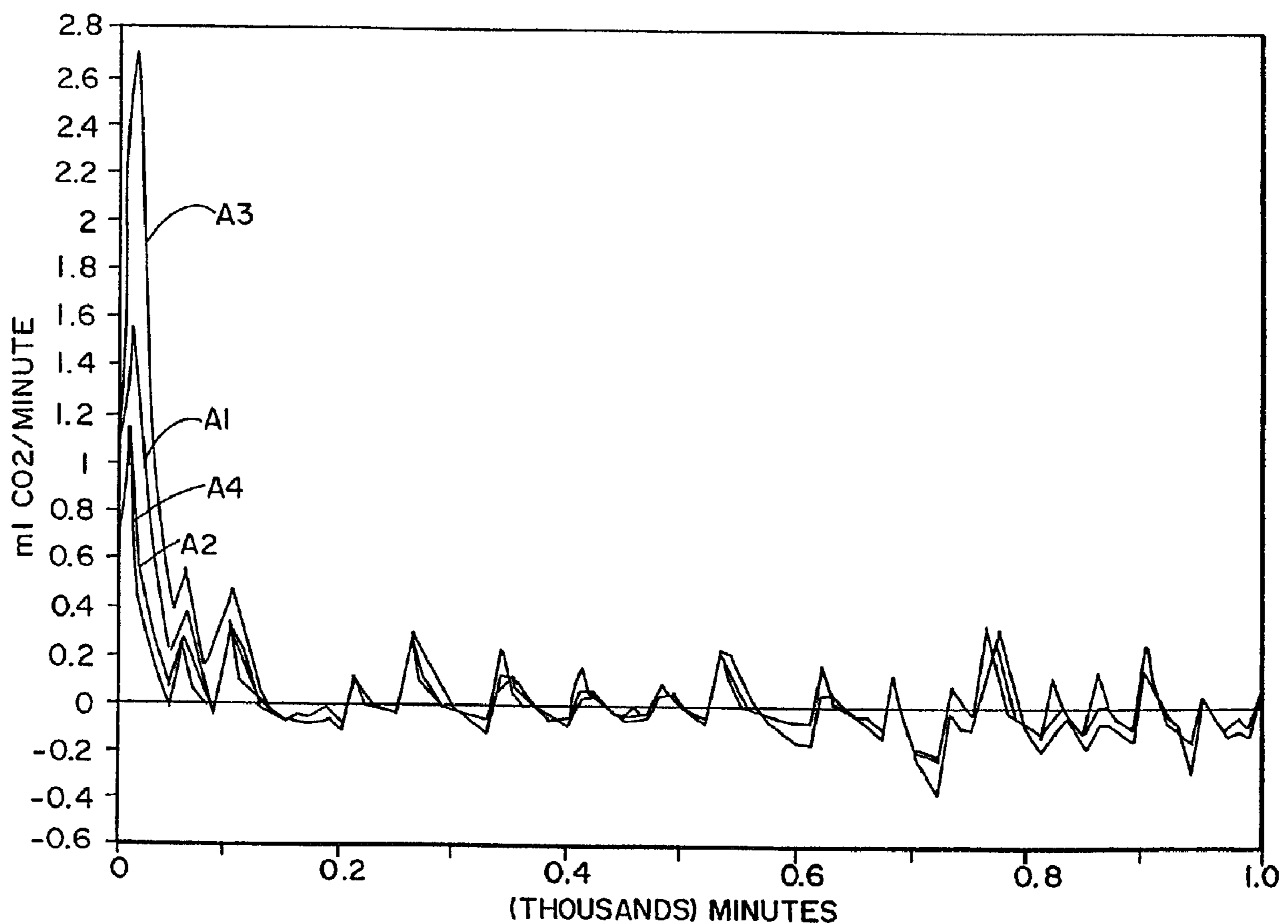


(86) Date de dépôt PCT/PCT Filing Date: 1994/03/04
(87) Date publication PCT/PCT Publication Date: 1994/09/15
(45) Date de délivrance/Issue Date: 2008/05/27
(85) Entrée phase nationale/National Entry: 1995/08/08
(86) N° demande PCT/PCT Application No.: US 1994/002333
(87) N° publication PCT/PCT Publication No.: 1994/019955
(30) Priorités/Priorities: 1993/03/05 (US08/026,927);
1993/03/19 (US08/032,219); 1993/07/02 (US08/087,616);
1993/10/27 (US08/144,236)

(51) Cl.Int./Int.Cl. *C12N 1/18* (2006.01),
A21D 10/00 (2006.01), *A21D 10/02* (2006.01),
A21D 6/00 (2006.01), *A21D 8/04* (2006.01)
(72) Inventeur/Inventor:
DOMINGUES, DAVID J., US
(73) Propriétaire/Owner:
GENERAL MILLS MARKETING, INC., US
(74) Agent: DEETH WILLIAMS WALL LLP

(54) Titre : PATE AU LEVAIN REFRIGEREE

(54) Title: YEAST-LEAVENED REFRIGERATED DOUGH PRODUCTS



(57) Abrégé/Abstract:

The present invention provides refrigeratable yeast-leavened dough compositions and methods of making such doughs. The dough's composition and the strain of yeast used therein are chosen to limit the total leavening action of the yeast by controlling the

(57) **Abrégé(suite)/Abstract(continued):**

amount of substrate in the dough fermentable by the yeast or by inactivating the yeast at high temperatures. Dough compositions made in accordance with the invention are capable of being leavened at elevated temperatures, yet stored in a sealed container at refrigeration temperatures for extended periods of time. In a preferred embodiment the yeast used in the dough is substantially incapable of fermenting carbohydrates native to the dough and a predetermined quantity of a non-native carbohydrate fermentable by the yeast (e.g. galactose) is added to the dough to provide the desired amount of proofing. A diploid yeast is further provided with the further property of being inactive at low temperature.

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ :A21D 8/04, 10/02, 10/00, C12N 1/18,
A21D 6/00

A1

(11) International Publication Number:**WO 94/19955****(43) International Publication Date:** 15 September 1994 (15.09.94)**(21) International Application Number:** PCT/US94/02333**(22) International Filing Date:** 4 March 1994 (04.03.94)**(30) Priority Data:**

08/026,927	5 March 1993 (05.03.93)	US
08/032,219	19 March 1993 (19.03.93)	US
08/087,616	2 July 1993 (02.07.93)	US
08/144,236	27 October 1993 (27.10.93)	US

(71) Applicant: THE PILLSBURY COMPANY [US/US]; 200
South Sixth Street, Minneapolis, MN 55402 (US).**(72) Inventor:** DOMINGUES, David, J.; 11520 39th Avenue North,
Plymouth, MN 55441 (US).**(74) Agents:** HOTCHKISS, Edward, S. et al.; Fredrikson & Byron,
1100 International Centre, 900 2nd Avenue S., Minneapolis,
MN 55402-3397 (US).**(81) Designated States:** CA, JP, European patent (AT, BE, CH, DE,
DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).**Published****2155675***With international search report.**Before the expiration of the time limit for amending the
claims and to be republished in the event of the receipt of
amendments.***(54) Title:** YEAST-LEAVENED REFRIGERATED DOUGH PRODUCTS**(57) Abstract**

The present invention provides refrigeratable yeast-leavened dough compositions and methods of making such doughs. The dough's composition and the strain of yeast used therein are chosen to limit the total leavening action of the yeast by controlling the amount of substrate in the dough fermentable by the yeast or by inactivating the yeast at high temperatures. Dough compositions made in accordance with the invention are capable of being leavened at elevated temperatures, yet stored in a sealed container at refrigeration temperatures for extended periods of time. In a preferred embodiment the yeast used in the dough is substantially incapable of fermenting carbohydrates native to the dough and a predetermined quantity of a non-native carbohydrate fermentable by the yeast (e.g. galactose) is added to the dough to provide the desired amount of proofing. A diploid yeast is further provided with the further property of being inactive at low temperature.

YEAST-LEAVENED
REFRIGERATED DOUGH PRODUCTS

The present invention relates to refrigeratable dough products for use in making edible baked goods. In particular, the invention provides yeast-leavened doughs which can be stored for extended periods of time at refrigeration temperatures.

A wide range of refrigeratable dough products are currently available to consumers for producing numerous different baked products. These refrigerated doughs range from doughs for biscuits and breads to sweet rolls to cornbread products. These dough products are rather popular with consumers because they are very convenient and easy to use. Most of these products are sold in a pre-proofed state so that they can be opened to remove the dough and the dough can be baked immediately. Packaging and selling doughs in a pre-proofed state omits any necessity on the part of the consumers to carefully proof the dough for an extended period of time before baking it.

In producing refrigeratable dough products, suitably sized portions of unproofed dough are placed in individual containers. The dough is then proofed within the container, such as by holding the dough at an elevated temperature, causing the dough to expand. The dough will continue to proof until a positive internal pressure of about 15-20 psi is attained; most such containers will rupture or explode if the internal pressure of the container substantially exceeds about 40 psi. Such products are desirably capable of storage at refrigeration temperatures for at least a couple of weeks, and desirably as long as a few months, without any significant degradation of the quality of the dough or any substantial likelihood of having the containers rupture.

One disadvantage of refrigeratable dough products on the market today is that these doughs generally cannot be leavened with yeast. When yeast is used in a dough, the yeast cells will tend to continue to grow, or at least continue metabolization, even at refrigeration temperatures. The yeast therefore continues to produce carbon dioxide over the entire storage time, unless the dough is stored in a frozen state. Although allowing yeast to ferment for the entire shelf life of the dough may work if the dough is intended to be used immediately, extended storage (e.g. about two weeks or more) in a sealed container generally will not work because the pressure in the container will quickly build

- 2 -

and rupture the container. If a conventional yeast-leavened dough were placed in a standard dough product container, the container may be expected to fail in no more than about two days. Additionally, continued activity of the yeast beyond the desired degree of proofing can deleteriously affect the organoleptic and rheological properties of the dough, producing unacceptable final baked products.

To date, manufacturers of refrigeratable doughs have had to replace yeast with chemical leavening agents, such as baking soda or the like. Such chemical leavening agents generally comprise a combination of leavening acid and a leavening base, with the acid and base portions reacting to generate carbon dioxide, causing the dough to rise. One of the primary advantages of such leavening agents is that their behavior is based upon a predictable chemical reaction, permitting one to readily control the volume of carbon dioxide produced to leaven the dough. Once the chemical reaction of the leavening agents has proceeded to completion, carbon dioxide production ceases.

Although a chemically leavened dough product can be stored for extended periods of time at refrigeration temperatures, the final baked product obtained by baking such a dough is noticeably inferior to a product made with a yeast-leavened dough. Products made from yeast-leavened doughs are widely acknowledged to have superior taste, aroma and texture than those made with chemical leavening agents. Commercial dough manufacturers frequently add ingredients for the sole purpose of simulating yeast-leavened doughs. For instance, these manufacturers frequently add yeast flavoring, such as inactive pasteurized yeast cultures, to the chemically leavened dough. Even with such additives, baked products made from chemically leavened doughs lack the characteristic flavor and aroma of yeast-leavened dough and continue to exhibit relatively poor texture.

Others have attempted to solve the problems associated with storage of yeast-leavened doughs by storing the doughs at freezing temperatures rather than refrigeration temperatures. Frozen yeast-leavened doughs can yield baked goods which are noticeably better than chemically leavened refrigerated doughs. Yeast becomes inactive when frozen, thereby avoiding the problems associated with continued carbon dioxide evolution at refrigeration temperatures.

However, frozen doughs simply are not as convenient as pre-proofed refrigerated dough products. Whereas such refrigerated doughs can be baked immediately after removal from the container, frozen doughs must be allowed to thaw prior to baking.

- 3 -

Also, since proofed dough does not survive freezing very well, frozen doughs generally must be proofed after thawing and prior to baking. This can further delay the baking of the dough. The consumer must spend more time monitoring the proofing process to avoid over-proofing the dough, making sure to place the dough in the oven for baking at the right time. Not only do such frozen doughs require more attention than do refrigerated dough products, it also requires the consumer to plan well in advance so the dough can be thawed and proofed to provide the baked goods at the desired time.

In a published European patent application (Published European Patent 0 442 575, published 21 August 1991), Gist-Brocades describes a dough composition which uses a substrate limitation concept. In accordance with this disclosure, a dough is leavened with a maltose negative yeast (a yeast which cannot ferment maltose) and the dough is frozen. Gist-Brocades states that the dough may be thawed, proofed and baked anytime the same day without having to carefully monitor the proofing time. However, this dough is not designed by Gist-Brocades to be stored at refrigeration temperatures for extended periods of time, e.g. two weeks or more.

Because consumers prefer the "fresh-baked" characteristics of dough products, many dough manufacturers sell pre-proofed dough in both frozen and refrigerated forms. "Pre-proofed dough" refers to dough in which the leavening agent in the dough has been allowed to generate carbon dioxide sufficient to raise the dough to a desired volume, such as by subjecting the dough to increased temperatures. Proofing the dough before distributing it to consumers eliminates the need for the consumers to carefully proof the dough for an extended period of time before baking it.

Examples of refrigerated dough compositions are described in Yong et al., U.S. Patents No. 4,381,315 and 4,383,336; and Atwell, U.S. Patent No. 4,526,801.

Refrigerated doughs are prepared by combining the dough ingredients including a leavening agent, optionally placing the dough in containers, proofing the dough, and then storing the dough at refrigeration temperatures, i.e. between about 0°C and about 12°C.

Refrigerated doughs are most commonly packaged prior to storage and may be packed prior to proofing the dough, in which case the dough is proofed in a closed container until the volume of the dough fills and seals the container. Alternatively, the dough may be packaged in flexible packaging after it has been proofed, in which case a sealing means is applied to the package in which the dough has been packed to render the

2155675

- 4 -

package substantially airtight. For purposes of this disclosure, the expression "containing means" will hereinafter be used to refer to both rigid containers and flexible packing.

Depending on the product, storage temperature and the like, the minimum acceptable shelf life of commercially produced refrigerated doughs can be as long as about 90 days. At refrigeration temperatures, conventional yeast continue to produce carbon dioxide, causing the dough to continue rising and the dough ingredients to continue reacting, even after the dough has been packaged in a sealed containing means for storage. Because conventional yeast continue to produce carbon dioxide at refrigeration temperatures, overfermentation of the dough occurs, resulting in adverse changes in the dough rheology. These changes negatively affect the taste, aroma, texture and other organoleptic qualities of the baked or cooked product prepared from the dough.

If the dough is packaged in a sealed containing means for storage at refrigeration temperatures, the continued carbon dioxide production by conventional yeast causes a continuous increase in the pressure within the containing means. Ultimately, the pressure inside the containing means increases to a point where the containing means ruptures. This rupture can occur with conventional yeast in a matter of a week or less, which is well below the minimum acceptable shelf life for most commercially produced refrigeratable doughs.

WO-A-9301724 discloses the use of a low temperature sensitive yeast in refrigeratable doughs to prevent such carbon dioxide production at refrigeration temperatures. This has, however, now been found to be unsatisfactory because the manufacturer rarely is able to control the temperatures at which the doughs are stored. Thus the problem arises of storage, by a sales outlet, of the packaged dough at temperatures high enough to activate the low temperature sensitive yeast, causing carbon dioxide production and eventual rupturing of the containers and spoiling of the contents.

Wheat flours used in most commercial dough manufacturing operations contain about 5 weight percent (wt. %) of damaged starch. Alpha- and beta-amylases (inherent in wheat flour) convert such starch into maltose, among other sugars. Maltose and some of the other sugars produced by the action of the amylase are metabolizable by many strains of yeast.

WO-A-9301724 discloses the use, in refrigeratable dough, a strain of yeast which did not ferment maltose, referred to as "maltose negative," or just "MAL-". Such yeast

can usually ferment other types of sugars, such as sucrose or dextrose. Approximately 100-200 ml of CO₂ per 100 grams of dough at 32°C is usually sufficient for proofing. The total amount of fermentable sugar in the dough was adjusted in an attempt to limit the volume of carbon dioxide gas produced by fermentation of the entire fermentable sugar supply.

It has now been found that dough products made with this dough by placing the dough in standard spirally wound refrigeratable dough containers were found to maintain acceptable internal pressures, e.g., below about 20 psi, for about 25 days. However, carbon dioxide once again began to be generated by the dough after about 25 days. This renewed activity of the yeast in the dough was projected to be sufficient to generate enough carbon dioxide to cause the containers being used, which would tend to fail at about 40 psi, to rupture after about 50-55 days.

It has not been conclusively determined why the yeast became active after apparently substantially ceasing fermentation. However, one factor which is believed to have contributed to the generation of additional carbon dioxide, and subsequent failure of the containers, is a change in the carbohydrates present in the dough. As noted above, alpha- and beta-amylases, which are inherent in wheat flours, act on carbohydrates present in the dough, and particularly in the flour. Over time, these amylases break down oligosaccharides which are not fermentable by the yeast, such as maltose and maltotriose, into sugars which can be fermented by the yeast. Accordingly, it is anticipated that, even if the yeast used in such dough composition were truly maltose negative, the changing carbohydrate profile of the dough may present sugars which are fermentable by the yeast. Accordingly, the dough could continue to generate carbon dioxide and cause containers to rupture.

Thus, a dough product made with MAL-yeast and a limited amount of initial maltose in the composition can be useful for storage at refrigeration temperatures for shorter periods of time, with a storage period on the order of about 30 days or less. If such dough products were stored for significantly longer periods of time, it is likely that the containers would begin to fail. Although a shelf life of 30 days may be suitable for some applications, current refrigerated dough products are expected to have an anticipated shelf life at refrigeration temperatures of 90 days or more. Accordingly, this MAL-embodiment of the invention may have only limited commercial application, with

2155675

- 6 -

commercial use being limited to institutional markets, such as in-store bakeries and the like, where an anticipated shelf life of 30 days may nonetheless be considered acceptable.

Hence, there has been a long-felt need in the industry for a yeast-leavened dough that can be stored at refrigeration temperatures for extended periods of time. To date, though, commercial producers have been unable to make and sell refrigeratable yeast-leavened doughs suitable for large-scale commercial production and extended shelf life, despite obvious economic potential of such a product. It appears that the problems associated with the continued generation of carbon dioxide by the yeast have precluded any such product.

SUMMARY OF THE INVENTION

The present invention provides a method of making refrigeratable yeast-containing doughs and dough products made therewith. In another aspect, the invention provides a yeast-leavened refrigeratable dough composition, a dough product comprising refrigeratable dough in a container, and a baked product made from such refrigeratable dough. In accordance with one aspect of the invention, a preselected strain of yeast is mixed with flour and water and, perhaps, other ingredients to form a dough. The yeast and the dough composition are chosen so that the total amount of carbohydrate or carbohydrates fermentable by the yeast in the dough is limited.

In one preferred embodiment, the yeast is substantially incapable of fermenting carbohydrates native to the flour used in the dough and a non-native carbohydrate, such as galactose, is added to the dough in an amount selected to provide the desired volume of carbon dioxide. By so doing, one may limit the maximum volume of carbon dioxide which the yeast can generate. This, in turn, prevents generation of sufficient carbon dioxide to rupture a sealed container of dough, even if the temperature of the dough is inadvertently elevated.

In another preferred embodiment, the yeast is capable of fermenting selected sugars native to the dough system which are naturally present in only limited amounts. Such sugars should be naturally present in amounts no more than, and desirably less than, that necessary to generate the volume of CO₂ necessary to proof the dough, with any additional sugar required to proof the dough being supplied by adding quantities of that sugar to the dough composition.

2155675

- 7 -

In one such embodiment, the yeast is substantially incapable of fermenting any carbohydrate native to the dough except fructose. Fructose concentration in wheats is initially on the order of less than 0.1 weight percent (wt. %). Through the action of various enzymes that can break down the disaccharide sucrose in the wheat into glucose and fructose monosaccharides, fructose concentration in the yeast may increase over time. Nonetheless, the concentration of fructose in most wheat-based dough systems is less than that necessary to generate the 100-200 ml CO₂ per 100 g of dough required to adequately proof the dough. Additional fructose is added to the dough to generate the desired degree of proofing.

The method may also include the additional steps of placing the resultant dough in a pressurizable container and heating the dough within the container to an elevated temperature for proofing. Once the dough in the container has been proofed, the temperature of the dough within the container is reduced to refrigeration temperatures and the dough is stored at refrigeration temperatures for an extended period of time. A method of this embodiment may further comprise the step of removing the dough from the container and baking it to produce a baked good.

A further aspect of the present invention provides a diploid yeast used in making refrigeratable yeast-containing doughs and dough products made therewith. This aspect of the invention provides a yeast-leavened refrigeratable dough composition.

The diploid yeast of this aspect of the invention, referred to below as "GAL+" yeast, is substantially incapable of fermenting carbohydrates native to wheat flour. In a dough composition of the invention, the yeast is substantially incapable of fermenting carbohydrates native to the flour used in the dough and a non-native carbohydrate, such as galactose, is added to the dough in an amount selected to provide the desired volume of carbon dioxide. By so doing, one may limit the maximum volume of carbon dioxide which the yeast can generate. This, in turn, prevents generation of sufficient carbon dioxide to rupture a sealed container of dough, even if the temperature of the dough is inadvertently elevated.

In accordance with a further embodiment of this aspect of the invention, the yeast is substantially incapable of fermenting carbohydrates native to wheat flour and is low temperature sensitive. As used herein, "low temperature sensitive" yeast (or simply "lts" yeast) is active at elevated temperatures in the presence of fermentable substrate, but

2155675

- 8 -

becomes substantially inactive, i.e. substantially ceases producing carbon dioxide, at refrigeration temperatures. Thus, the yeast of this particular embodiment of the invention can be said to be both "GAL+" and "Its".

5 A method according to the invention comprises making a dough containing flour, water, galactose and GAL+/Its or diploid GAL+ yeast and storing the dough at refrigeration temperatures for an extended period of time. The method may also include the additional steps of placing the resultant dough in a pressurizable container and heating the dough within the container to an elevated temperature for proofing. Once the dough in the container has been proofed, the temperature of the dough within the container is
10 maintained at refrigeration temperatures, preferably for an extended period of time. A method of this embodiment may also further comprise the step of removing the dough from the container and baking it to produce a baked good.

In accordance with a still further aspect of the present invention, a dough comprises flour, water and a yeast which is capable of fermenting available substrate at
15 temperatures below a threshold inactivation temperature but which will become substantially inactivated if heated to a temperature substantially equal to or greater than the threshold inactivation temperature. This threshold inactivation temperature is desirably greater than the room temperature and less than a temperature at which the dough will begin to cook or bake and become a baked product instead of dough.

20 In accordance with a method of this aspect of the invention, a dough is formed by mixing flour, water and yeast. This dough composition is allowed to proof, and is then heated to a temperature at least as high as the threshold inactivation temperature of the yeast, substantially inactivating the yeast, i.e. rendering the yeast substantially incapable of fermentation at virtually any temperature. The dough may then be stored at
25 refrigeration temperatures without any further significant proofing of the dough.

This aspect of the invention further provides a temperature-sensitive yeast which is well suited for use as the leavening agent in refrigerated dough compositions. This yeast has a threshold inactivation temperature above which the yeast is substantially inactivated, such as by being rendered an obligate aerobe. Once being heated above the threshold
30 inactivation temperature, the yeast will remain substantially inactive at virtually any temperature if maintained in a substantially anaerobic environment. In another embodiment, the yeast is sensitive to both unduly high and unduly low temperatures.

Even if a limited quantity of oxygen is present in the environment, the yeast will remain substantially inactive at refrigeration temperatures because of its sensitivity to low temperatures.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is a graph depicting the rate of carbon dioxide generation over time for a GAL+ yeast in a dough composition held at 30°C;

 Figure 2 shows the total volume of carbon dioxide generated in the sample of Figure 1;

10 Figure 3 is a graph showing the total volume of carbon dioxide generated by four dough compositions differing in the nature of non-native carbohydrates added to the dough;

 Figure 4 depicts the rate of carbon dioxide evolution for the doughs shown in Figure 3;

15 Figure 5 plots measured can pressure over time for two chemically leavened doughs, one containing a GAL+ yeast, the other without;

 Figures 6 and 7 shown can pressure over time for three dough samples having different amounts of non-native sugar incorporated in their composition;

 Figure 8 depicts the growth of D3083 and RD308.3 yeast on three different media as a function of absorbance;

20 Figure 9 is a schematic representation of the process of glycolysis, showing the reaction pathways for the utilization of various sugars in fermentation;

 Figure 10 is a graph showing the total volume of carbon dioxide generated by PGI-/G6PDH- yeast-leavened dough compositions containing different sugars;

25 Figure 11 depicts the rate of carbon dioxide evolution for the doughs shown in Figure 10;

 Figure 12 plots measured can pressure over time for two dough compositions, one dough containing fructose and the other containing sucrose;

 Figure 13 depicts measured can pressure as a function of time for doughs leavened with diploid GAL+ yeast of the invention;

30 Figure 14 depicts colony size for GAL+/lts yeast strains of the invention as a function of time at about 30°C;

2155675

- 10 -

Figure 15 depicts colony size for GAL+/lts yeast strains of the invention as a function of time at about 12°C; and

Figure 16 depicts anaerobic growth of cdc19 cells after high temperature incubation.

5 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

10 In accordance with the present invention, a dough product is prepared wherein the dough composition and the yeast used therein are chosen in a manner that effectively and controllably limits the leavening action of the yeast by controlling the amount of substrate fermentable by the yeast in the dough. Strains of yeast which do not ferment certain carbohydrates are known in the art; often, two different strains of the same species of yeast are unable to ferment the same sugars. Therefore, a strain of yeast may be utilized in a dough composition which is capable of fermenting only selected sugars. By controlling the total amount of those sugars in the dough composition, the amount of fermentation can be controlled.

15 As explained above, even at refrigeration temperatures, most yeast will generate carbon dioxide. If the sugar substrate fermentable by the yeast is limited, carbon dioxide generation will substantially cease when the sugar is exhausted. Hence, by either allowing the yeast to metabolize the fermentable sugars in the dough for a given period of time prior to canning or controlling the sugar content of the dough, carbon dioxide generation by the yeast can be substantially terminated once a certain predetermined volume has been reached, regardless of the temperature of the dough. Accordingly, the total volume of the carbon dioxide generated in the container can be prevented from reaching a level sufficient to increase internal pressure and rupture the container.

20 As discussed earlier, the problem encountered with the use of MAL-yeast in refrigeratable doughs is that the maltose is broken down by enzymes, native to the flour, into simpler sugars that are metabolized by the yeast.

25 The great majority of sugars present in most flours are metabolized by yeast by being broken down into either, or both, of glucose and fructose, and these two sugars are then further metabolized by the yeast. The present invention seeks to overcome the problem of generation of carbon dioxide by fermentation of large sugars broken down in the yeast into smaller units fermentable by the yeast, by the use of yeast that is unable to metabolize glucose. This yeast is therefore, unable to ferment one of the smallest units

30

into which a larger sugar may be broken down. In this way the yeast is substantially substrate-limited to fructose and precursors thereof, thus greatly limiting carbon dioxide production.

5 In a preferred embodiment, the yeast is incapable of metabolizing both glucose and fructose. In this way the two routes of fermenting sugars native to most flours and their by products are blocked, rendering the yeast substantially incapable of fermenting sugars native to the flour. The carbon dioxide production may then be carefully controlled by measured addition of a sugar, not native to the flour, which is metabolizable by the yeast.

10 In accordance with a preferred embodiment of the present invention suitable for significantly longer storage at refrigeration temperatures, the strain (or strains) of yeast used in the dough are substantially incapable of fermenting carbohydrates which are native to the flour. In the case of doughs using wheat flour, these native carbohydrates include sugars such as maltose, sucrose, glucose, fructose and various oligosaccharides made up of these sugars. If other flours were to be used, of course, there may be some
15 variation in the sugars native to such a flour.

Use of such a yeast has been found to effectively prevent the yeast from fermenting any carbohydrates in the dough which are either initially present in the dough composition or result from the action of alpha- and beta-amylases on the carbohydrates initially present in the dough. A predetermined quantity of a non-native carbohydrate
20 which is fermentable by the yeast may be added to the dough to provide the desired amount of proofing. Once that substrate is consumed, the fermentation activity of the yeast appears to substantially cease, preventing further carbon dioxide generation and avoiding overfermentation of the dough. It has been found that dough compositions in accordance with this embodiment of the invention can be used to make dough products
25 which can be stored for periods of time in excess of 90 days without rupturing or exploding.

The non-native carbohydrate which can be fermented by the yeast strain in the present dough can be virtually any carbohydrate which does not naturally occur in flour. This carbohydrate is preferably a sugar or an oligosaccharide, though. For instance, the
30 fermentable, non-native sugar may be galactose or lactose, a disaccharide of glucose and galactose.

In one particularly preferred embodiment, the yeast is capable of fermenting galactose, which is not native to wheat flours, but is substantially unable to ferment any sugars which are native to wheat flour; this yeast is referred to below as a “galactose positive” or “GAL+” yeast. This GAL+ yeast is mixed with flour, water and galactose to form a dough. The amount of galactose in the dough is selected to limit the activity of the yeast so that the dough is proofed no more than the desired degree. As noted above, in most circumstances about 100-200 ml of carbon dioxide per 100 grams of dough at 32°C is sufficient to proof the dough. Accordingly, the weight percentage of galactose in the dough composition should be chosen to generate no more than approximately 200 ml of carbon dioxide per 100 grams of dough at 32°C. The amount of galactose necessary to generate this volume of carbon dioxide will have to be determined on a case-by-case basis as the amount may vary for different strains of yeast.

Given the present disclosure, it will be well within the ability of those skilled in the art to make yeasts which are substantially incapable of fermenting carbohydrates native to flour but capable of fermenting other carbohydrates. Such yeasts can be made through standard methods of crossing yeast strains, isolating suitable strains having the desired properties and the like. These types of common techniques are described, for example, by Sherman *et al.* in *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1974). Of particular interest in the Sherman *et al.* publication is Section III, entitled “Making Mutants”, which appears on pages 273-369 of this reference.

Lobo and Maitra teach a method of rendering a hexokinase negative strain of *S. Cerevisiae* glucokinase negative (i.e., a method for making a GAL+ yeast strain) using standard techniques in “Physiological Role of Glucose-Phosphorylating Enzymes in *Saccharomyces Cerevisiae*,” *Archives of Biochemistry and Biophysics* 182, 639-645 (1977). In accordance with that method, the hexokinase negative strain was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine in yeast extract-peptone medium (YEP) containing 50 mM glucose-free galactose, and a glucokinase-negative mutant was isolated by replica plating from a YEP galactose plate to a YEP glucose plate as a glucose-negative colony. The genotype of the mutant, determined by independent genetic analysis, was *hxx1 hxx2 glk1*, where *hxx1*

- 13 -

and hxx2 stand for genes coding P1 and P2 hexokinases respectively, and glk1 for the genetic determinant for glucokinase synthesis.

Although Lobo and Maitra teach one suitable method of making a yeast for use in accordance with the present invention, others methods will be apparent to those skilled in the art. Those in the art will also realize that other strains of yeast which are substantially incapable of fermenting carbohydrates native to a particular flour but capable of fermenting non-native carbohydrates other than galactose can be made by known methods.

EXAMPLE 1

In order to test the ability of a GAL+ yeast to ferment carbohydrates which are native to a common dough system, a dough composition containing GAL+ yeast was prepared. This dough formula included 870.75 g (58.05 wt. %) wheat flour, 529.80 g (35.32 wt. %), water, 58.20 g (3.88 wt. %) of the wheat gluten preblend used in Example 1, 11.25 g (0.75 wt. %) salt and 28.50 (2.00 wt. %) yeast. The yeast used in this experiment was a GAL+ strain of Saccharomyces Cerevisiae designated as D308.3; this yeast was of the genotype α hxx1 hxx2 glk1 adel trp1 his2 met4. This yeast is available to the public from the Yeast Genetic Stock Center at the Donner Laboratory in the Department of Molecular and Cell Biology of the University of California, Berkeley (YGSC); in the Seventh Edition of the catalog of the YGSC dated March 15, 1991, this strain of yeast was listed under stock no. D308.3. This yeast strain was also deposited with the American Type Culture Collection of 12301 Parklawn Drive, Rockville, Maryland 20852, USA (ATCC), on 5 March 1993, under number ATCC 74211.

Isolated colonies of the D308.3 yeast from solid galactose agar plates were used to inoculate six 50 ml culture flasks containing liquid yeast extract-peptone ("YEP") and galactose. The samples were incubated for approximately 20 hours at about 30°C and then used to inoculate six one-liter flask samples, which also contained YEP and galactose. These 1 L flasks were incubated for about 24 hours at 30°C, followed by incubation at about 24°C for approximately 20 hours.

This yeast was then harvested using a GSA rotor, which is commercially available from Sorval Instruments. Sample containers for use with the GSA rotor were filled so that the total weight of the sample, lid and container was about 300 g. The sample

2155675

- 14 -

container was spun at 2500 rpm for 20 minutes, and the supernatant fluid was immediately decanted. Enough distilled water to raise the total weight of the sample, lid and container to 300 g was added to the sample container, and the container was swirled to bring the yeast pellet back into suspension. This sample container was then spun at 2500 rpm for 20 minutes again, and the supernatant fluid was again decanted.

The washed yeast paste and water were combined to form a slurry. This slurry was mixed with the other ingredients in a table-top Hobart mixer. The dough was mixed at speed 1 for 30 seconds, followed by mixing at speed 2 for between about 4 and about 5 minutes. Two 100 g samples (A1 and A2 in Figures 1 and 2) and two 50 g samples (A3 and A4 in Figures 1 and 2) were placed in the Risograph™ testing machine. The Risograph™ is commercially available from Sheldon Manufacturing, Inc. for detecting the volume of gas, e.g. carbon dioxide, generated by a sample and the rate at which the gas is generated. The samples were incubated at about 30°C for about 17 hours (1,000 minutes). The results of this Risograph testing are shown in Figures 1 and 2.

As can be seen in Figure 1, carbon dioxide was generated fairly rapidly in all of these samples for the first 40-50 minutes, after which the rate of evolution tapered off to about zero. Although the rate of carbon dioxide generation appears to have fluctuated between slight positive and negative rates, it appears as though the samples generated very little or no carbon dioxide between about 120 minutes after incubation began and the end of the experiment.

Furthermore, although the rate of carbon dioxide generation was noticeable at the beginning of the experiment, it should be noted that the total volume of carbon dioxide generated in this sample was no more than about 7 ml; this result is best seen in Figure 2. As noted above, in order to adequately proof dough, between about 100 and about 200 ml of carbon dioxide/100 g of dough is generally considered to be necessary. The volume of carbon dioxide generated in these galactose-free samples, though, fell well below those limits. The indication that about 7 ml of gas was generated in these samples may actually be attributable primarily, if not entirely, to an expansion of the headspace in the Risograph™ sample containers when the containers were heated for incubation. In other words, it appears likely that no appreciable carbon dioxide was generated by the dough samples in this experiment.

- 15 -

Accordingly, the D308.3 yeast used in this Example can be said to be substantially incapable of fermenting, or otherwise metabolizing, the carbohydrates native to this dough system. Hence, it is believed that the D308.3 strain of yeast can be accurately referred to as GAL+, as that term is used herein, and this yeast provides one example of a yeast
5 suitable for use in the present invention. As noted above, though, one of ordinary skill in the art could make other GAL+ yeasts, as well as other yeasts which are capable of fermenting only carbohydrates not native to the flour in the dough, in light of the present disclosure.

EXAMPLE 2

10 In order to test the responsiveness of the GAL+ yeast used in Example 1, four different dough compositions, with varying non-native carbohydrates, were prepared. Each of the four doughs included 290.25 g of flour, 176.60 g of water, 3.50 g of salt and 12.00 g of the D308.3 GAL+ yeast used in Example 1. The formulas of the four
15 different doughs varied in the nature of the other ingredients which were added. In a control sample, no other ingredients were added; in a second sample, 5.00 g of galactose was included; in a third sample, 10.00 g of lactose was provided; and the final sample included 20.00 g of non-fat dry milk (NFDm), which is used as a flavoring ingredient in some doughs and typically contains some lactose and may contain slight amounts of galactose.

20 Yeast paste was grown and harvested in substantially the same manner as set forth in connection with Example 1. For each of the samples, the washed yeast was slurried with the water, and this slurry was added to the other ingredients in a table-top Hobart mixer. Each sample was then mixed at speed 1 for about 30 seconds, followed by mixing at speed 2 for about 4 minutes. Two 100 g samples of each of the dough compositions
25 were placed into Risograph™ sample jars immediately after mixing and held in the Risograph™ at about 28°C for approximately 20 hours. Figures 3 and 4 show the total volume of carbon dioxide evolved and the rate of carbon dioxide evolution, respectively, for each of the samples.

30 As can be seen from Figures 3 and 4, only the dough composition which included galactose generated appreciable volumes of carbon dioxide. The control sample, the lactose-containing sample and the sample with the NFDm all generated less than about

10 ml of carbon dioxide over a period of about 20 hours. Furthermore, essentially all of the carbon dioxide generation measured for the non-galactose doughs was generated in the first one to two hours of incubation. This slight change in gas volume in the Risograph™ sample jars may be wholly attributable due to thermal expansion of the headspace in the sample jars, as explained above. Accordingly, the samples which did not contain non-native galactose quite likely did not generate any significant amount of carbon dioxide during the course of this test.

The results of this experiment show that the D308.3 yeast can metabolize galactose but it is substantially incapable of fermenting any carbohydrates which are native to flour of the dough composition. It also appears that this yeast is substantially incapable of fermenting either "straight" lactose or lactose in non-fat dry milk. During the course of this experiment, the galactose-containing dough appears to continue to generate carbon dioxide, indicating that not all of the galactose was used. Furthermore, at the end of the 20-hour incubation, the galactose dough had generated slightly more than 100 ml of carbon dioxide, with carbon dioxide generation appearing to continue beyond the end of the experiment.

The dough containing galactose was about 1.0 wt. % galactose (5.00 g galactose/487.35 g total dough). Based on the results of this experiment, it appears that about 1 wt. % galactose is more than adequate to generate the desired 100-200 ml of carbon dioxide per 100 g of dough. Additional experimentation using standard, spirally wound composite containers of about 250 cc capacity, such as are commonly used in packaging commercial refrigerated doughs, has established that about 0.5 wt. % to about 1.0 wt. % galactose is sufficient to generate enough carbon dioxide to reach an internal pressure of about 10-20 psi. Accordingly, in making a refrigeratable dough product of the invention, the dough placed in the container optimally includes between about 0.5 wt. % and about 1.0 wt. % galactose.

EXAMPLE 3

The D308.3 yeast was added to a chemically-leavened dough product in order to see if the presence of the GAL+ yeast affected the integrity of the container if no galactose was added to the dough. Two batches of a dough containing the D308.3 yeast and two separate batches of chemically leavened dough were prepared. The chemically

- 17 -

leavened doughs had the following formula: about 1590 g (56 wt. %) flour, 947 g (33.43 wt. %) water, 110 g (3.9 wt. %) of the wheat gluten pre-blend of Example 1, 89.2 g (3.15 wt. %) of yeast flavorings, 42.5 g (1.5 wt. %) glucono delta lactone (GDL), 32.0 g (1.13 wt. %) baking soda, and 21.3 g (0.75 wt. %) salt. The two batches of dough containing yeast had a very similar formula, with the approximately 947 g (33.4 wt. %) of water being replaced with about 890 g (31.4 wt. %) of water and about 56.7 g (2.00 wt. %) D308.3 yeast.

The water in each of these batches was first mixed with the flavoring ingredients before being charged with the flour and gluten pre-blend into a McDuffy mixing bowl. In the batches containing yeast, the yeast was slurried with the water before the flavoring ingredients were added to this slurry. The ingredients were mixed at speed 1 for about 30 seconds, followed by mixing at speed 2 for about 5 minutes. The salt and the leavening agents (GDL and soda) were then added to this dough and the mixture was mixed at speed 1 for approximately 30 seconds and at speed 2 for about 2.5 minutes.

Each batch of dough was sheeted to a thickness of about $\frac{1}{4}$ inch (about 0.64 cm) and rolled into a long "log" of dough. Each log of dough was divided into a series of samples weighing about 210 g and each sample was sealed into a standard, spirally wound composite can having a 250 cc capacity. These dough products were then proofed at about 32-35°C until an internal pressure of about 10-15 psi in the containers was reached. After this proofing, the dough products were transferred to refrigerated storage at about 4°C.

Figure 5 plots the measured can pressure, i.e., the internal pressure of the container, as a function of time. As can be seen in Figure 5, there does not appear to be any significant difference between the pressure in the dough product containing the standard chemically leavened dough and the dough product containing the chemically leavened dough with the GAL+ yeast.

A variety of other physical measurements were made on the different samples to compare the standard chemically leavened dough with the yeast-doped dough. Among the physical measurements compared were water retention, pH, and sugar content. Samples of the doughs were also baked at approximately 375°F (163°C) for about 20 minutes. The specific volume, as well as the appearance, aroma and other sensory properties, of the resulting baked goods were compared. Aside from a slightly lower specific volume

2155675

- 18 -

for the sample containing the GAL+ yeast, there did not appear to be any significant differences between these two dough compositions.

EXAMPLE 4

The relationship between galactose content of the dough and the resultant internal pressures of dough products containing dough in accordance with the invention was tested. Four different batches were prepared, with the batches differing only in the amount of galactose added. Each dough composition contained about 870.75 g (58.05 wt. %) wheat flour, 529.80 g (35.32 wt. %), water, 58.20 g (3.88 wt. %) of the wheat gluten preblend used in Example 1, 11.25 g (0.75 wt. %) salt and 28.50 g (2.00 wt. %) D308.3 yeast. Additionally, one batch contained about 5.92 g (0.5 wt. %) galactose, another contained about 7.40 g (0.63 wt. %) galactose, a third contained about 8.87 g (0.75 wt. %) galactose, and the final batch contained about 11.83 g (1.00 wt. %) galactose.

The D308.3 yeast was grown and harvested in substantially the same manner as that detailed above in Example 2. In forming batches of dough containing the 0.5 wt. % and 1.0 wt. % galactose, the yeast paste was then mixed with the water and the galactose in a 1 L culture flask and incubated in the flask for about 1 hour at about 30°C while the flask was agitated. This slurry was then added to a McDuffy mixing bowl and mixed with the other ingredients at speed 1 for about 30 seconds, followed by mixing at speed 2 for about 7 minutes. The 0.63 wt. % and 0.75 wt. % galactose batches were prepared slightly differently in that the yeast, water and galactose were not incubated prior to being mixed with the other ingredients. Instead, these three ingredients were slurried in a table-top Hobart and were mixed at speed 2 for only about 4 minutes with a dough hook.

After the doughs were mixed, two 50-gram samples from each batch of dough were placed in Risograph sample jars and incubated in the Risograph at about 28-30°C. The dough was then rolled, divided into 210-gram samples, and packaged in a standard refrigeratable dough container, as outlined above in Example 3. The resultant dough product was incubated at about 35°C for about three hours and subsequently stored at about 4°C.

Figures 6 and 7 illustrate the can pressures of the samples as a function of time, with the can pressures for samples from each batch being averaged together to generate

these plots. It can be seen that the ultimate can pressure of the sample is generally proportional to the amount of galactose in the dough. Whereas the sample containing 0.63 wt. % galactose had a can pressure of about 5-6.5 psi, the 0.75 wt. % dough had can pressures of about 9-10.5 and the pressure in the dough with 3 wt. % yeast and 1 wt. % galactose generated a maximum pressure of just under 16 psi. Accordingly, it appears as though the desired pressure in a container of the invention can be fairly readily controlled as a simple function of the amount of galactose added to the dough - once the galactose is exhausted, the dough will substantially cease producing carbon dioxide.

EXAMPLE 5

The D308.3 yeast perhaps adversely affected the sensory appeal of baked doughs containing such yeast in that the final baked product exhibited a slightly off-white color. Although all of the other organoleptic qualities of the dough were exemplary, doughs which would not exhibit this slight discoloration would probably be more appealing to consumers. It was determined that the discoloration of the dough was most likely due to inability of the D308.3 yeast to make adenine, causing the yeast to develop a pinkish or reddish hue when it is grown in a medium without adenine supplementation. This discoloration of the yeast is presumed attributable to a build up of metabolites which are toxic to the yeast (but not to humans).

Spontaneous revertant strains of the D308.3 yeast which do not require adenine for metabolization, referred to herein as RD308.3 yeast, were isolated. First, a concentrated paste of the D308.3 yeast was formed by spinning down the yeast in a rotor, as outlined in Example 1. This yeast paste was then diluted with a potassium phosphate monobasic buffer (about 43 mg KH_2PO_4 added to a liter of distilled water, with the Ph adjusted to about 7.2 with NaOH) and spread on an "adenine drop out" (ADO) medium, i.e. a medium which does not contain any supplemental adenine, at a concentration of about 1×10^7 colony forming units (CFU)/ml. The ADO medium contained, for each liter of distilled water, about: 6.7 g of bacto-yeast nitrogen base without amino acids, 20 g galactose, and 20 g of bacto-agar, 2 g of a "drop out mix" which contained alanine, argenine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, inositol, isoleucine, leucine, lysine, methionine, *para*-aminobenzoic acid, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, uracil, and valine. (Substantially the

same formula is taught by Rose et al. in Appendix A of *Methods in Yeast Genetics, A Laboratory Course Manual* (1990), at pages 179-180, but that formula used glucose rather than galactose.)

These ADO plates were incubated at about 25°C for approximately 4 days and colonies of the yeast which did not require adenine were isolated. Identifying these colonies was greatly
5 simplified by the fact that the non-revertant strains tended to be pinkish or reddish in hue while the revertant colonies were whitish. The isolated yeast was then once again plated onto a fresh ADO medium and incubated under substantially the same conditions. Colonies of revertant strains of the yeast were once again isolated from any strains inadvertently carried over in the first isolation and the plating and incubation were repeated one final time. Although it is believed
10 that one skilled in the art could readily make such a yeast in light of the present disclosure, this resulting strain of RD308.3 yeast has been deposited with the ATCC on 5 March 1993, under number ATCC 74212 and this strain is available to the public from the ATCC.

Two samples were prepared, with one sample containing the original D308.3 yeast and the other containing the RD308.3 yeast. These samples were prepared by mixing an isolated
15 colony (about one loop) of the desired yeast with about 5 ml of YEP/galactose (which contained about 10 g of bacto-yeast extract, 20 g of bacto-peptone, and about 20 g of galactose per 1 liter of distilled water) and incubating for about 12-15 hours at about 30°C. (The formula for the YEP/galactose medium is substantially the same as the YEP/glucose formula taught on page 177 of Appendix A of *Methods in Yeast Genetics*, noted above, except that the glucose in that formula
20 was replaced with galactose in the present medium.) Titer results indicated a population of approximately $48 \pm 2 \times 10^5$ CFU/ml for each strain. For each of the resulting samples, about 100 µl of the sample was added to three separate 5 ml portions of media, with one medium comprising just YEP, another comprising YEP and glucose and the third comprising YEP and galactose.

The absorbance of each resulting sample was measured over time and is graphically
25 illustrated in Figure 8. The growth behavior of the D308.3 and RD308.3 yeasts appeared to be essentially the same for all three of these growth media. Furthermore, both of these yeasts appear able to readily metabolize galactose, but can only grow slightly on YEP or YEP/glucose. It is also interesting to note that both the D308.3 strain and the RD308.3 strain grew slightly less on the YEP/glucose than on YEP

alone. This further demonstrates the substantial inability of these yeasts to metabolize glucose.

The auxotrophic markers adenine, histidine, methionine and tryptophan as growth supplements for the D308.3 and RD 308.3 strains were compared by standard techniques. The D308.3 yeast was not able to grow on galactose minimal media unless all four of these growth supplements were present, but the RD 308.3 yeast was able to grow if only the histidine, methionine and tryptophan were added.

Thus, the only significant difference noted between the auxotrophic markers of these two strains was that the D308.3 yeast requires adenine supplementation while the RD308.3 yeast does not. Accordingly, it is believed that the RD308.3 yeast will behave substantially as described above in connection with the D308.3 yeast when added to dough, but the slight discoloration of baked goods associated with doughs containing the D308.3 yeast should be substantially eliminated.

Figure 9 is a schematic representation of the process of glycolysis. As is well known in the art, various sugars are broken down by glycolysis into pyruvic acid via glycolysis and the resulting pyruvic acid can be utilized by yeast to generate carbon dioxide through fermentation in an anaerobic environment.

As schematically illustrated in Figure 9, glucose can be converted into glucose 6-phosphate by either hexokinase (HXK) or glucokinase (GLK). This glucose 6-phosphate can then be converted into fructose 6-phosphate by one of two pathways. In the normal glycolytic pathway, glucose 6-phosphate is converted into fructose 6-phosphate by the action of phosphoglucoisomerase.

In an alternative pathway for converting glucose 6-phosphate to fructose 6-phosphate, the glucose 6-phosphate is first converted into 6-phosphogluconolactone by the action of glucose 6-phosphate dehydrogenase (G6PDH), also known as zwischenferment (ZWF). Through the pentose phosphate pathway, also called the pentose phosphate shunt, this 6-phosphogluconolactone can be converted into fructose 6-phosphate.

In the embodiment described above wherein the yeast is substantially incapable of fermenting any sugars native to the dough systems, the yeast mutation was substantially incapable of generating either hexokinase (HXK) or glucokinase (GLK). As can be seen from the schematic illustration of Figure 9A, this prevents glucose or fructose from being converted into fructose 6-phosphate. Since the yeast is substantially incapable of

2155675

- 22 -

converting either glucose or fructose into fructose 6-phosphate, glucose and fructose generally cannot be converted into pyruvic acid and therefore cannot be utilized effectively by the yeast to produce CO₂.

As can be seen in the schematic representation of Figure 9A, galactose can be converted into glucose 1-phosphate by the action of galactokinase. This glucose 1-phosphate can then be converted into glucose 6-phosphate through the action of phosphoglucomutase. This glucose 6-phosphate can then be converted into fructose 6-phosphate, and thence to pyruvic acid as outlined above, by either phosphoglucoisomerase (PGI) or glucose 6-phosphate dehydrogenase (G6PDH).

Accordingly, the yeast in the embodiment set forth above can utilize galactose through the action of galactokinase, but the pathways for the glycolysis of glucose and fructose are disabled due to a lack of hexokinase and glucokinase.

As noted above, in an alternative embodiment, the present invention does not utilize a non-native sugar, but instead ferments a sugar which is native to the dough system, but is present only in a limited quantity. In one particularly preferred embodiment of this invention, the native sugar is fructose. Fructose is commonly present in wheat flours, but in a concentration of less than 0.1 wt. %, with concentrations on the order of 0.02-0.08 wt. % being common for most wheat flours. Once a wheat flour is combined with water and yeast, this relatively low concentration of fructose will be diluted even further. As doughs leavened with haploid yeasts commonly require at least about 1.0 wt. % of a fermentable substrate to generate the 100-200 ml of CO₂ necessary to proof about 100 g of dough, the quantity of native fructose generally will be less than that necessary to adequately proof a dough.

In accordance with the present invention, a yeast which is capable of phosphorylating only selected sugars to produce fructose 6-phosphate is mixed with flour and water to form a dough composition. In one preferred embodiment, the yeast is capable of fermenting fructose but is substantially incapable of fermenting any sugar naturally occurring in the flour in significant concentrations. An additional quantity of fructose is added to the dough composition to provide additional fermentable substrate for the yeast. The quantity of fructose added to the dough should be sufficient to allow the yeast to generate about 100-200 ml CO₂/100 g of dough as measured at about 30°C. The amount of fructose necessary to generate these quantities of carbon dioxide should be

determined on a case-by-case basis for different strains of yeast to yield the desired degree of proofing.

As described above and illustrated in Figure 9, fructose requires hexokinase in order to be converted into fructose 6-phosphate through phosphorylation. Accordingly, the yeast used in accordance with the present invention must be capable of generating hexokinase. However, hexokinase also breaks glucose down into glucose 6-phosphate, which can then be converted into fructose by either PGI or G6PDH. In order to allow the yeast of the present invention to utilize fructose but not glucose, the two pathways for the conversion of glucose 6-phosphate into fructose 6-phosphate are blocked.

As schematically depicted in Figure 9B, this is accomplished in accordance with the instant invention by using a yeast which is both phosphoglucoisomerase negative ("PGI-") and glucose 6-phosphate dehydrogenase negative ("G6PDH-"), i.e. the yeast lacks PGI and G6PDH. As such a yeast cannot generate either PGI or G6PDH, it is substantially incapable of utilizing glucose 6-phosphate by converting it into fructose 6-phosphate by phosphorylation. Accordingly, even if glucose is converted to glucose 6-phosphate by the action of hexokinase or glucokinase, the process of glycolysis is substantially blocked at that point and the glucose cannot be converted to pyruvic acid.

As illustrated schematically in Figure 9B, this leaves only one pathway through the glycolysis process, that being the conversion of fructose to fructose 6-phosphate by hexokinase. Thus, the amount of fructose in a dough of the present invention will determine the volume of carbon dioxide which is generated.

In accordance with one embodiment of the present invention, a yeast which is both PGI- and G6PDH- is mixed with flour, water and an amount of fructose sufficient to provide the desired degree of proofing of the dough. The amount of fructose added to the dough is optimally selected to produce a volume of 100-200 ml CO₂/100 g of dough as this is usually considered necessary to proof the dough, as noted above. The precise amount of fructose added to the dough will depend on a number of factors, including the specific strain of yeast being used and the amount of native fructose in the flour.

Accordingly, the amount of fructose added to a given dough composition should be determined on a case-by-case basis for different dough formulations. However, once a suitable formula has been determined for a given composition, the volume of carbon

2155675

- 24 -

dioxide produced can be predictably varied as a function of the amount of fructose added to the dough.

Although there do not appear to be any PGI-/G6PDH- yeasts currently commercially available, a skilled artisan in the field will be able to make such a yeast using known techniques, such as those outlined by Sherman *et al.*, *supra*. In making such a yeast, one can cross a PGI- yeast with a G6PDH- yeast through known techniques.

Once the two yeasts have been crossed and sporulated to yield haploid strains, the strains should be tested to ensure that they are indeed PGI-/G6PDH-, i.e. they behave as though they have substantially no phosphoglucose isomerase and substantially no glucose 6-phosphate dehydrogenase activity. Once the PGI-/G6PDH- nature of the yeast is confirmed (e.g. by confirming substantially zero growth on glucose media), the yeast can be use to make a dough of the invention. For example, PGI-/G6PDH- yeast of the invention was made as follows.

Example 6

A number of putatively PGI- yeast strains and a number of putatively G6PDH- yeast strains were obtained from publicly available sources. In this experiment, the following strains of *Saccharomyces cerevisiae* yeast were used:

Yeast Strain Genotype

N543-9D	α pgi1 leu2 can ^r cyh ^r SUC2 mal mel gal2 CUP1
N548-8A	a pgk1 leu2 can ^r cyh ^r SUC2 mal mel gal2 CUP1
9520T4C	α pgi1 ade1 trp1 ura3 his2 met14
YM3269	a zwf1 URA3 ura3-52 his3-200 ade2-101 lys2-801 try1-501 met-

The YM3269 yeast's genotype includes the designation zwf1, indicating that the yeast is deficient in ZWF, i.e. glucose 6-phosphate dehydrogenase (G6PDH). The first three strains of yeast were all obtained from the YGSC at UC Berkeley, noted above; the last strain (YM3269) was obtained from Dr. M. Johnston, Department of Genetics, Washington University School of Medicine, St. Louis, Missouri, USA.

Although these particular yeast strains were selected for the present experiment, it is well within the ability of those skilled in the art to select or develop other suitable PGI- and G6PDH- strains of yeast. For instance, in "Identification of the Structural Gene for Glucose-6-phosphate Dehydrogenase in Yeast. Inactivation Leads to a Nutritional Requirement for Organic Sulfur", *The EMBO Journal*, vol. 10 no. 3 pp. 547-553 (1991),

Thomas et al. teach that a defect in the MET19 gene of *S. Cerevisiae* will cause such a yeast to be G6PDH- because the MET19 gene encodes glucose-6-phosphate dehydrogenase from yeast. Thomas et al. also describes methods by which such a defect can be cloned into other yeast strains.

5 The ability of each of these yeasts to utilize different substrates was tested by inoculating a sample of each of a number of different media with a sample of each yeast and measuring the absorbance of the samples over time at 25°C. The different media were all employed liquid YEP as a base and varied in the addition of sugars to the media, with one medium having no additional substrate, a second including glucose, a third including fructose, a fourth including sucrose, and a
10 fifth including maltose. This process of testing the ability of yeast to grow on different media is well known in the art and need not be discussed in great detail here.

By using such known testing protocols, it was determined that the N543-9D yeast grew readily on fructose, sucrose and glucose but not on maltose. The ability of this yeast to grow on glucose indicates that it is not actually PGI-, as reported.

15 The 9520T4C yeast strain also grew readily on fructose and sucrose, but grew poorly on maltose and initially could not grow well on glucose. After about 6 days of incubation in liquid glucose media, this strain did adapt to growth on glucose, though. It has been surmised that this strain is most likely at least partially PGI-, but that the yeast may be able to process glucose by shunting the glucose through the pentose phosphate pathway (noted above in connection with
20 Figure 9) or the PGI- mutation may be "leaky", i.e. PGI is not fully disabled.

Strain YM3269 was able to grow readily on glucose, fructose and sucrose, although the yeast seemed to have difficulty growing on maltose. As can be seen in Figures 9, one would expect the yeast to be able to use glucose, fructose and sucrose despite a lack of G6PDH because the glucose 6-phosphate can proceed through the normal glycolysis pathway without having to go
25 through the pentose phosphate pathway disabled by the lack of G6PDH.

The N548-8A yeast grew slowly on glucose, fructose and sucrose and was generally unable to grow on maltose. This is consistent with an understanding from the literature that this yeast has a "leaky" phosphoglucokinase (PGK) mutation.

Thus, the YM3269 yeast was apparently G6PDH negative and the 9520T4C yeast appeared to be PGI negative. Accordingly, these two yeast strains were selected for crossing to yield PGI-/G6PDH- haploids. In mating these two strains to make the desired haploids, a protocol was derived from Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, pp. 53-59 (1990).

In accordance with this method, YM3269 yeast and 9520T4C yeast were plated onto separate YEP+fructose plates using a sterile loop to apply the strains on their respective plates in a series of parallel lines about 7 mm apart. These plates were allowed to incubate at approximately 30°C for about one day.

An impression of the G6PDH- YM3269 strain was made on a replicate plate pad. This impression was imprinted onto a fresh plate pad including a YEP+fructose medium (about 1 wt. % bacto-yeast extract, about 2 wt. % bacto-peptone, about 2 wt. % bacto agar, and about 2 wt. % fructose, with the balance being distilled water) supplemented with adenine, histidine, methionine and uracil. Using a fresh replicate plate pad, an impression of the 9520T4C PGI- strain was made. The second replicate pad was imprinted on the same YEP+fructose plate used for the previous imprinting, but at an orientation generally perpendicular to the first imprint, resulting in a pattern of yeast strains resembling a checkerboard. This doubly imprinted YEP+fructose plate was incubated at approximately 30°C overnight (i.e. about 12-15 hours).

The YEP+fructose plate thus prepared was imprinted on a fructose minimal media plate containing adenine, histidine, methionine and uracil. The fructose minimal media included about 6.7 g of bacto-yeast nitrogen base without amino acids, about 20 g of bacto-agar and about 20 g of fructose in about 1 liter of distilled water; the adenine, histidine, methionine and uracil were added to this formula in aqueous form. Such a minimal medium, utilizing dextrose instead of fructose, as well as the formulas for the supplements are taught in Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, pp. 178-179 (1990).

These fructose minimal media plates were incubated for about two days at about 30°C. Growth at the intersections of the "checkerboard" pattern was scored and plated onto a fresh fructose minimal media plate to isolate the diploid (crossed) colonies from

the haploid colonies. The diploid colonies isolated on the fructose minimal media plate were streaked onto a plate of sporulation media and incubated for about 4-5 days at about 25°C. The sporulation media contained about 10 g (1 wt. %) potassium acetate, about 1.0 g (0.1 wt. %) bacto-yeast extract, about 0.5 g (0.05 wt. %) fructose, about 20 g (2.0 wt. %) bacto-agar, with the balance being about 1000 ml distilled water.

About one loopful of yeast cells was taken from the sporulation plate and combined with about 300 microliters distilled water and approximately 15 microliters glucosylase in a Eppendorf™ microfuge tube. This solution was mixed by vortex and incubated at around 30°C for approximately 30 minutes. The incubated sample was briefly sonicated to separate spore clusters. Within about 20 minutes of being made, serial dilutions of about 10^{-4} , 10^{-5} and 10^{-6} of the sonicated sample were plated onto YEP+fructose plates.

These serial dilutions were then exposed to ethyl ether fumes in a manner adapted from "Guide to Yeast Genetics and Molecular Biology", Guthrie and Fink editors, in *Methods of Enzymology*, vol. 194, pp. 146-147 (1991). In this process, a 4 mm X 4 mm piece of filter paper was placed into the inverted lid of each petri dish containing one of the serial dilutions. In a ventilated hood, 0.75 ml of ethyl ether was added to each filter paper and the lids and dilutions were placed in a glass chamber along with a beaker containing 10 ml of ethyl ether to maintain the vapor pressure in the chamber elevated.

The chamber was sealed and the samples were incubated for about 15 minutes at room temperature, at which time an additional 0.75 ml portion of ethyl ether was added to each filter paper square. These samples were again incubated in the glass chamber at room temperature for about 15 minutes, following which the samples were removed from the chamber and allowed to sit in the open atmosphere with the lid of each sample ajar for about 30 minutes.

Isolated yeast colonies from each of the 10^{-4} , 10^{-5} and 10^{-6} dilution plates and a sample of each of the YM3269 and 9520T4C parent stains were grid plated onto YEP+fructose plates and incubated at about 25°C for approximately 24 hours. Each of these samples was then replicate plated onto one plate of YEP+fructose and one plate of YEP+glucose. These plates were then incubated at about 30°C for about 1-2 days to

2155675

- 28 -

determine which of the isolated putative YM3269 x 9520T4C strains were able to grow on the fructose-enriched medium but not the glucose-containing medium.

The growth rate of each isolated strain was then evaluated by inoculating a 5ml volume of YEP+fructose with one loop of the yeast. Control samples for each of the YM3269 and 9520T4C parent strains were also prepared by inoculating similar media with a loop of the parent yeast. These samples were incubated at about 30°C for about 24 hours and 100 microliter samples of each resultant yeast were used to inoculate separate 5 ml samples of YEP, YEP+fructose and YEP+glucose, with one such set of three samples being prepared from each isolated strain and each of the parent strains.

The absorbency of each sample was measured at 600 nm and the samples were incubated at about 25°C for about 2 weeks, with absorbency measurements being taken about twice a week for each sample.

Approximately 200 colonies of putative YM3269 x 9520T4C haploid strains were initially obtained from the "checkerboard" plating. Of these 200 colonies 47 were found to grow on the fructose medium but substantially unable to grow on the YEP or YEP+glucose media in the initial stages of the incubation. As noted above, though, the 9520T4C parent strain was found to be able to adapt to the glucose media after about 6 days in incubation. Of the 47 strains which did not initially grow in the glucose medium, all but 15 demonstrated an ability to adapt to the glucose like the parent yeast.

Accordingly, only 15 of the 200 initially isolated colonies were evaluated as being truly PGI-/G6PDH- in that they were able to grow on fructose but not on glucose. These 15 colonies are referred to herein as PGI-/G6PDH- 1 through PGI-/G6PDH- 15.

This experimental example therefore readily provided some 15 colonies of yeast which appear to be useful in the present invention. Although this experimental example illustrates one straightforward method of making yeast of the present invention, it is to be understood that other variations of this method or other procedures for making such yeasts will be apparent to those skilled in this field.

The PGI-/G6PDH- 1 yeast was deposited with the ATCC on July 2, 1993 under designation number ATCC 74230. This strain has been deposited simply as an example of a suitable yeast strain in accordance with the invention; it is to be understood that any one or more of the 15 isolated PGI-/G6PDH- strains are believed likely to work as well

- 29 -

and that other strains of yeast in accordance can be readily produced by those skilled in the art in accordance with the present disclosure.

In accordance with a further embodiment of the present invention, such a yeast of the invention is incorporated into a dough composition. In accordance with this
5 embodiment, a yeast of the invention is mixed with flour, water and a substrate fermentable by the yeast. The substrate fermentable by the yeast may occur naturally in the dough system, but if so the quantity of such substrate in the dough should be no more than that necessary to generate 200 ml of CO₂ per 100 g of dough, and is desirably substantially less than that amount. As used herein, a substrate is said to be naturally
10 occurring in the dough if it is either native to the flour or is generated over time by the action of enzymes in the flour on other carbohydrates initially present in the flour.

For instance, fructose occurs naturally in wheat and will generally comprise between about 0.02 wt. % and about 0.08 wt. % of wheat flour. Such fructose can be said to be native to the flour as it is present in the flour in its natural state. Wheat flours also
15 include sucrose, which is a disaccharide of glucose and fructose, and sucrose can be broken down by the action of commonly occurring enzymes into its constituent monosaccharides, giving rise to additional fructose in the dough over time. The concentration of sucrose in most wheats is generally on the order of about 0.2 wt. %, which if completely broken down would yield approximately 0.1 wt. % additional fructose
20 to the initial 0.02-0.08 wt. % fructose in the flour. Also, various wheat flour oligosaccharides (e.g. glucofructose) contain varying amounts of fructose which could possibly also contribute the total amount of fructose in a wheat flour dough system over time. Both the native fructose and that produced by the degradation of native sucrose and other native oligosaccharides is considered to be "naturally occurring" as that term is used
25 herein.

Continuing with the example of fructose, it should be noted that the total naturally occurring fructose in most wheat flours will be no more than about 0.2 wt. %, even if the sucrose is completely degraded into glucose and fructose. By the time the flour is mixed with water and the other ingredients of the dough, the weight percentage of naturally
30 occurring fructose in the dough composition will be even less, frequently on the order of about 0.12 wt. % of the dough. However, a concentration more on the order of 1 wt. % of a fermentable substrate is usually necessary to generate the 100-200 ml CO₂/100 g of

dough necessary to adequately proof the dough. Accordingly, the naturally occurring fructose is substantially less than that which one would expect to be required to properly proof a dough made with the flour.

5 Fructose would therefore serve as a suitable substrate fermentable by yeast of the present invention, particularly where the yeast is to be used with wheat flours. Naturally occurring glucose, on the other hand, while initially present at relatively low concentrations in a refrigerated wheat flour dough (e.g. native concentrations on the order of about 0.2 wt. %) will, over time, increase in concentration to as much as 1 wt. % or more by the end of about 90 days of refrigerated storage. Thus, the "naturally occurring" 10 glucose in wheat flour doughs can be as much or more than that necessary to suitably proof the dough.

Without further treatment of the wheat to limit the amount of naturally occurring glucose, the additional amount of glucose generated in the dough system over the anticipated 90-day shelf life of the product could very well generate more than the 100- 15 200 ml of CO₂/100 g of dough desired for proper proofing of the dough. Furthermore, when dough is proofed in a standard container, the expansion of the dough during proofing is used to flush the container of air initially present in the container and to substantially seal the container. The amount of native glucose in wheat flours generally will not be sufficient to adequately flush and seal standard containers in commercial use 20 today.

Inadequate flushing and sealing would permit some quantities of air to remain in the container and, perhaps, enter the container before it is sealed. Significant oxygen concentrations in the container can have a number of adverse effects on the dough, such as graying of the dough and promoting growth of deleterious bacteria. Thus, the native 25 glucose may be insufficient to generate the 15-20 psi internal pressure desired in dough packages, but yet the total volume of CO₂ generated in the dough from the total naturally occurring glucose may well exceed the desired volumes. Accordingly, glucose would not be a good candidate for a fermentable substrate for a yeast-leavened dough of the invention.

30 By utilizing a yeast which is capable of fermenting only a substrate (most commonly a sugar) which is present in the dough in naturally occurring amounts no more than that necessary to proof a dough, the total volume of carbon dioxide which the yeast

can generate will also be limited. Hence, by selecting the concentration of fermentable substrate in the dough and using a yeast of the present invention, the proofing of a dough with yeast can be controlled on a commercial basis. This is critical, as outlined above, in that it can permit the dough to be stored for extended periods of time, e.g. on the order of 90 days or more, without rupturing the container in which it is placed.

Example 7

In order to test the ability to use the yeast produced in Example 6 to proof a dough composition and to survive extended refrigerated storage, such a yeast was mixed with flour and water to form a dough. In particular, yeast strain PGI-/G6PDH- 1 (ATCC designation number ATCC 74230) was used in four different dough compositions, with each dough composition including about: 428.3 g (57.7 wt. %) wheat flour; 259.7 g (35.0 wt. %) distilled water; 26.5 g (3.56 wt. %) wheat gluten preblend of a formula substantially the same as outlined in Example 1; 5.63 g (0.76 wt. %) salt; and 15.0 g (2.02 wt. %) PGI-/G6PDH- 1 yeast. The four dough compositions differed in the amount and type of substrate added to the dough, with the first composition having no added sugars, the second having about 7.5 g (1.0 wt %) fructose, the third dough having about 7.5 g (1.0 wt %) glucose, and the fourth dough having about 7.5 g (1.0 wt %) sucrose.

The doughs were made by slurring the PGI-/G6PDH- 1 yeast with the water and the sugar, if any, and mixing this slurry with the remaining ingredients in a table top Hobart mixer. The dough was mixed at speed 1 for about 30 seconds, followed by mixing at speed 2 for approximately 4 minutes.

After the doughs were mixed, two 100 g samples of each dough composition were placed in separate Risograph™ sample jars. These sample jars were then held in the Risograph™ at about 30°C for about 24 hours and the gas evolution of these samples was monitored by the Risograph™. Figure 10 is a graph of the total volume of CO₂ generated over time for each sample while Figure 11 is a graph of the rate of CO₂ generation for the same samples. (The data collected from the two samples from each batch of dough was averaged together to yield the data for that sample shown in these drawings.)

Additionally, two 210 g samples of each of the fructose-containing and sucrose-containing doughs were placed in standard, commercially available spirally wound cans (about 2.25" diameter by about 4" in length). The canned dough samples were then

incubated at about 97°F (36°C) for about 3.5 hours and stored at about 4°C for about 90 days. Figure 12 is a graph of the pressure in the sealed container for each of these doughs over time. (The pressure measurements were begun after the initial proofing incubation at 97°F and the data for the two samples of each dough were averaged together to yield the illustrated data for that dough.) The data of Figures 10 and 11 indicate that the dough containing fructose generated significantly more CO₂ than the other three samples. This is much as one would expect for a dough leavened with a yeast which is both phosphoglucose isomerase (PGI) negative and glucose 6-phosphate dehydrogenase (G6PDH) negative. As illustrated in Figures 9 and 9B, such a yeast should be able to readily utilize fructose in the dough, but not other sugars.

The dough which did not include any additional sugar did generate some CO₂, although it was substantially less than that generated by the fructose-supplemented dough. The yeast may have been able to utilize the naturally occurring fructose in the dough as a substrate in generating carbon dioxide. It is interesting to note that the 100 g control sample containing no non-naturally occurring fructose (i.e. no added fructose in the composition) generated somewhat more than 100 ml CO₂.

The reliability of this result is not certain, though. In some circumstances, various bacteria in a dough will generate CO₂ or other gases if the dough is proofed for an extended period of time, such as more than 10 hours. As the doughs of Figures 10 and 11 were held at about 30°C for much longer than 10 hours, it is believed that at least some of the volume of gas generated by the control sample may be attributable to the action of bacteria, i.e. to a source other than the yeast. It is well known that the growth of bacteria in yeast can not only yield undesirable byproducts which can adversely affect the dough. Accordingly, commercially proofed doughs generally are proofed for as short a period of time as possible and one should not rely on the generation of CO₂ from sources other than the yeast in these doughs for leavening purposes.

Accordingly, it is believed that the volume of CO₂ generated by the yeast is less than 100 ml for this 100 g sample of dough. Since this falls below the level believed to adequately proof the dough, it appears as though the dough lacks sufficient naturally occurring fructose to proof the dough to the desired degree.

The dough containing 1 wt. % sucrose actually generated slightly less CO₂ than the control sample, which did not contain any additional sugars. At first, this may seem

anomalous in that the additional sucrose could be broken down into glucose and fructose, adding to the fermentable substrate in the dough. This is not completely understood, but it is believed that this may be attributable either to an inability of the PGI-/G6PDH- 1 yeast to cleave sucrose into its constituent monosaccharides or to metabolic suppression of yeast due to the abundance of unconsumed glucose in the dough. The fact that the glucose-supplemented formulation produced even less gas than the sucrose dough would seem to bear out that the presence of glucose, which cannot be fermented by the yeast, is suppressing the metabolism of the yeast.

As noted above, Figure 12 shows the pressure in the canned dough stability test. The fructose-supplemented dough reached an internal pressure of about 18 psi in the first 10 days of storage, with the pressure gradually creeping up to about 24 psi by the end of the 90 days. As explained above, it is desirable to have an internal can pressure of at least about 15-20 psi, but that the internal pressure of the can should not exceed about 40 psi or the container may rupture. Accordingly, the fructose-supplemented dough of Figure 12 appears to readily meet the desired operational parameters of standard canned doughs and can be stored for extended periods of time without rupturing the container. In this case, the dough was stored for 90 days at refrigeration temperatures without any adverse effects on can pressure, which meets the requirements for most current commercially produce refrigeratable dough products, as noted above.

The sucrose-supplemented dough did not generate as much internal pressure as the fructose-supplemented dough. As seen in Figure 12, the sucrose dough reached a pressure of less than about 10 psi in the first 10 days of refrigerated storage and gradually crept up to an internal pressure of about 14 psi by the end of the 90 days shown in this graph. It is interesting to note that this product did not reach the desired 15-20 psi of internal pressure even after more than two months of storage. Accordingly, by varying the concentration of non-fermentable sugar (e.g. glucose) in the dough product system, one can effectively limit or control the gas production of the dough, and hence internal pressure of a package containing the dough, during the dough's shelf life.

Another embodiment of the present invention provides a method of forming a dough which can be stored at refrigeration temperatures for extended periods of time without generating significant volumes of carbon dioxide. This method may further

- 34 -

include the steps of packaging the dough, proofing the dough in the package, and storing the dough for an extended period of time at refrigeration temperatures.

In making a dough of the invention, flour, water, a yeast substantially incapable of fermenting carbohydrates native to the flour, and a quantity of a carbohydrate fermentable by the yeast are mixed together, as outlined above. The amount of the fermentable carbohydrate added to the dough is desirably sufficient to provide only the necessary degree of proofing of the dough; adding too much fermentable substrate could cause adverse changes in dough rheology due to overfermentation. This amount is optimally determined on a case-by-case basis for a given strain of yeast as different strains of yeast may utilize the fermentable substrate more efficiently than others.

In a particularly preferred embodiment of the method of the invention, the yeast used in making the dough is a GAL+ yeast and a predetermined quantity of galactose is added to the dough to provide the desired degree of proofing. This GAL+ may be the D308.3 yeast or the RD308.3 yeast described above, but it is to be understood that other GAL+ yeasts can be made in accordance with the present disclosure which will also work in accordance with the invention.

As noted above, the method may further include the steps of packaging the dough, proofing the dough in the container, and storing the dough at refrigeration temperatures for an extended period of time. Virtually any known refrigeratable dough package known in the art may be used in this method. For instance, spirally wound dough containers such as those currently used in commercially manufactured refrigeratable dough products should suffice. A quantity of dough somewhat less than that necessary to fill the container is placed in the container, leaving a headspace in the container when it is sealed.

The dough may then be proofed in the container, expanding to fill the container and flush out any air in the headspace. The proofing is continued until substantially all of the fermentable carbohydrate is consumed by the yeast, at which point an internal pressure of about 15 to about 20 psi is attained in the container. This proofing may be advantageously carried out at an elevated temperature, e.g. about 30°C to about 40°C, to allow the yeast to ferment, and thus proof the dough, more rapidly.

This proofed dough may then be placed in refrigerated storage for extended periods of time, desirably up to at least about two weeks. The dough of the invention is

- 35 -

optimally capable of storage at refrigeration temperatures for at least about 90 days, the anticipated shelf life of current doughs, as explained above. By "refrigerated storage", storage at temperatures between about 12°C and about 0°C, and preferably between about 4° and bout 7.2°C, is intended. Such temperatures are referred to in the present
5 specification as "refrigeration temperatures".

As noted above, a preferred embodiment of the invention provides a yeast which is substantially incapable of fermenting carbohydrates native to a flour but capable of fermenting a non-native carbohydrate. In a first particularly preferred embodiment, this yeast comprises a diploid yeast which is substantially incapable of fermenting
10 carbohydrates native to wheat flour, but is capable of fermenting a non-native sugar such as galactose. This yeast is referred to herein as a diploid GAL+ yeast.

The GAL+ yeast described and tested in the above description is perfectly suitable for producing refrigeratable yeast-leavened doughs. In a commercial production setting, though, it may be desirable to provide such a yeast which is more stable and robust. In
15 commercial operations, there is the possibility that the strain of yeast used in producing doughs could come into contact with other strains of yeast which are not GAL+. If the GAL+ yeast being used is a haploid, the possibility exists that the GAL+ yeast will mate with a contaminating strain of yeast, affecting the integrity of the yeast being used. Similarly, there is the possibility that some of the haploid GAL+ yeast strain may revert
20 to the wild-type and become capable of freely metabolizing glucose or other carbohydrates native to the flour.

Some contamination of the yeast should not be problematic in commercial dough production. If the yeast strain becomes too contaminated, though, the yeast added to the dough composition may be capable of metabolizing native carbohydrates to an appreciable
25 extent. This could yield yeasts which adapt to the carbohydrate profile in the dough being produced and continue to produce significant amounts of carbon dioxide even after the predetermined supply of the non-native carbohydrate is exhausted. This, in turn, could produce doughs having unacceptable rheology after extended shelf storage and could conceivably cause pressures high enough to cause the containers in which the dough
30 is packaged for storage to rupture.

Accordingly, it is desirable to have a yeast which is less sensitive to contamination and less likely to revert to the glucose-utilizing wild type. It is believed that a diploid

- 36 -

GAL+ yeast of the invention is more robust and less sensitive to contamination and less likely to revert to wild type.

EXAMPLE-7

In an attempt to provide a more robust yeast strain for use in commercial dough manufacturing operations, a diploid strain of GAL+ yeast was created. The D308.3 and RD308.3 strains are both mating type " α " haploid GAL+ yeasts and therefore cannot mate with one another to form a suitable diploid strain. A mating type "a" GAL+ yeast strain was therefore created to mate with either or both of the D308.3 and RD308.3 yeasts.

The following strains of yeast were used in creating the desired GAL+ diploid yeast:

Strain	Genotype
XA83-5B	a lts8 lys2 leu1
D308.3 (trp+)	α hxx1 hxx2 glk1 ade1 his2 met14*
RD308.3	α hxx1 hxx2 glk1 trp1 his2 met14
YM3270	α zwf1:: URA3 ura3-52 his3-200 ade2-101 lys2-801 try1-501 met-

The XA83-5B strain of yeast is available to the public from the YGSC under the same designation. The YM3270 was acquired from Dr. Mark Johnston of the Washington University University of Medicine in Saint Louis, Missouri, U.S.A. These strains of yeast were found to be useful in the present mating protocol in that the XA83-5B yeast allowed the generation of a GAL+ haploid having a mating type a and the YM3270 yeast was useful in testing to confirm that the resultant yeast was indeed mating type a. It is believed and should be understood, though, that other yeasts having an a mating type could have been used instead. This is particularly true in the case of the YM3270 yeast, which was simply used to determine the mating type of a yeast generated as outlined below.

The D308.3 (trp+) yeast used in this experiment was a spontaneous revertant of the D308.3 yeast detailed above and available to the public from the ATCC under number ATCC 74211. The deposited D308.3 yeast was determined to need tryptophan

supplementation in order to grow at a suitable rate. This D308.3 (trp+) yeast (also referred to below as D308.3' yeast) is a spontaneous revertant of the deposited D308.3 which does not require tryptophan supplementation for suitable growth. The D308.3' yeast was isolated in a manner analogous to the procedure outlined above in Example 5 for isolating the RD308.3 yeast.

5 In particular, a concentrated paste of the D308.3 yeast was obtained and spread on a "tryptophan drop out" medium (i.e. a medium which does not contain any supplemental tryptophan) at a concentration on the order of about 1×10^7 to about 1×10^8 CFU/ml. The formula of the tryptophan drop out medium was substantially the same as that for the adenine drip out medium of Example 5, but the tryptophan in that formulation was replaced with adenine so that there was
10 substantially no tryptophan in the medium.

These inoculated drop out plates were incubated and colonies of the yeast which grew on the drop out medium, and therefore must not require tryptophan supplementation for growth, were isolated. These isolated revertant strains were once again plated onto a tryptophan drop out medium and incubated and growing colonies were isolated from that plate. Samples of these
15 isolated colonies were once again plated onto Tryptophan drop out medium, incubated and isolated one last time to remove substantially all non-revertant yeast from the isolated colonies. These isolated colonies are the D308.3' yeast used in the present Example 8.

The mating type of GAL+ haploid yeast was created by crossing the XA83-5B yeast, which is a mating type a yeast, and the RD308.3 yeast. The crossing was carried out under a
20 protocol derived from Methods in Yeast Genetics, A Laboratory Course Manual, referred to above, at pp. 53-59, as follows:

XA83-5B yeast and RD308.3 yeast were plated onto separate YEP+galactose agar plates using a sterile loop to apply the strains on their respective plates in a series of parallel lines about 7 mm apart. These plates were allowed to incubate at approximately 30°C for about 24 hours.

25 An impression of the mating type a XA83-5B strain was made on a replicate plate pad. This impression was imprinted onto a fresh plate including a YEP+galactose medium (about 1 wt. % bacto-yeast extract, about 2 wt. % bacto-peptone, about 2 wt. % bacto agar, and about 2 wt. % galactose, with the balance being distilled water). Using a fresh replicate plate pad, an impression of the mating type α RD308.3 strain was made.

The second replicate pad was imprinted on the same YEP+galactose plate used for the previous imprinting, but at an orientation generally perpendicular to the first imprint, resulting in a pattern of yeast strains resembling a checkerboard. This doubly imprinted YEP+galactose plate was incubated at approximately 30°C for about 24 hours.

5 The YEP+galactose plate thus prepared was imprinted on a synthetic dextrose minimal media plate. The synthetic dextrose minimal media included about 6.7 g of bacto-yeast nitrogen base without amino acids, about 20 g of bacto-agar and about 20 g of glucose in about 1 liter of distilled water. Such a minimal medium is taught in Methods in Yeast Genetics, A Laboratory Course Manual, noted above, at pp. 178-179. These synthetic dextrose minimal media plates
10 were incubated for about 24 hours at about 30°C.

Growth at the intersections of the "checkerboard" pattern was scored and plated onto a fresh synthetic dextrose minimal media plate to isolate the diploid (crossed) colonies from the haploid colonies. The diploid colonies isolated on the synthetic dextrose minimal media plate were streaked onto a plate of sporulation media and incubated for about 4-5 days at about 30°C.
15 The sporulation media contained about 10 g (1 wt. %) potassium acetate, about 1.0 g (0.1 wt. %) bacto-yeast extract, about 0.5 g (0.05 wt. %) galactose, about 20 g (2.0 wt. %) bacto-agar, with the balance being about 1000 ml distilled water.

About one loopful of yeast cells was taken from the sporulation plate and combined with about 300 microliters distilled water and approximately 15 microliters glucylase in an
20 Eppendorf™ microfuge tube. This solution was mixed by vortex and incubated at about 30°C for approximately 30 minutes. The incubated sample was briefly sonicated to separate spore clusters. Serial dilutions of about 10^{-4} , 10^{-5} and 10^{-6} of the sonicated sample were plated onto YEP+galactose glass petri plates.

These serial dilutions were then exposed to ethyl ether fumes in a manner adapted from
25 "Guide to Yeast Genetics and Molecular Biology", Guthrie and Fink editors, in *Methods of Enzymology*, vol. 194, pp. 146-147 (1991). In this process, a 4 mm x 4 mm piece of filter paper was placed into the inverted lid of each petri dish containing one of the serial dilutions. In a ventilated hood, 0.75 ml of ethyl ether was added to each filter paper and the lids and dilutions were placed in a glass chamber along with a beaker containing 10 ml of ethyl ether to maintain
30 the vapor pressure in the chamber elevated.

- 39 -

The chamber was sealed and the samples were incubated for about 15 minutes at room temperature, at which time an additional 0.75 ml portion of ethyl ether was added to each filter paper square. These samples were again incubated in the glass chamber at room temperature for about 15 minutes, following which the samples were removed from the chamber and allowed to sit in the open atmosphere with the lid of each sample ajar for about 30 minutes.

Some 287 putative haploid GAL+ yeast colonies were isolated from these 10^{-4} , 10^{-5} and 10^{-6} dilution plates. Each of these putative haploids were grid plated onto YEP+galactose plates and onto YEP+glucose plates and incubated at about 30°C for approximately 48 hours to determine which of the isolated putative XA83-5B x RD308.3 strains were able to grow on the galactose-enriched medium but not the glucose-containing medium. 50 of these 287 colonies were determined to be GAL+ by their ability to grow well on galactose but general inability to grow on glucose. These GAL+ haploids were then isolated by streaking them onto fresh YEP+galactose plates.

The mating type of each of these 50 GAL+ haploids was determined by attempting to mate samples of these yeasts with the YM3270 yeast noted above. Since the YM3270 yeast is mating type α , only mating type a strains of the isolated GAL+ yeasts will be able to mate with the YM3270 yeast. This therefore identifies those GAL+ strains capable of mating with the D308.3' and RD308.3 yeasts, both of which are mating type α , to produce the desired diploid of the invention.

In determining ability to mate, the procedure utilized was analogous to the mating procedure, outlined above, used to cross the RD308.3 and XA83-5B strains. In mating the isolated GAL+ strains and the YM3270 yeast, three generally parallel lines of each of three different strains of the GAL+ yeasts were streaked onto a single YEP+galactose petri dish (for a total of nine streaks per petri dish). A number of such petri dishes were prepared so that samples of each of the isolated GAL+ strains were plated onto a petri dish. On a separate plate, six generally parallel lines of the YM3270 yeast were streaked onto YEP+galactose medium. Both the GAL+ samples and the YM3270 plate were incubated at about 30°C for about 24 hours.

The GAL+ strains and the YM3270 strain were replica plated onto a series of plates in a generally perpendicular orientation to yield a checkerboard pattern, as outlined above in the earlier mating protocol. These strains were plated onto synthetic dextrose

2155675

- 40 -

complete uracil dropout media rather than the synthetic dextrose minimal media utilized in the earlier mating. The synthetic dextrose complete uracil dropout media contained about 6.7 g of a bacto-yeast nitrogen base substantially without amino acids, about 20 g of glucose, about 20 g of bacto-agar, and about 2 g of a "drop out mix", with the balance being about 1000 ml of distilled water. The "drop out mix" contained about 0.5 g of adenine, about 4.0 g of leucine, about 0.2 g of *para*-aminobenzoic acid, and about 2.0 g of each of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, inositol, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

These checkerboard plates were incubated at about 30°C for about 24 hours and scored for growth. Since the YM3270 yeast strain has been determined to require uracil supplementation for growth, plating the yeast onto the uracil drop out medium effectively permits mated diploid yeasts to be separated from the haploid colonies. Of the 50 isolated GAL+ colonies, only six were found to be viable haploid strains of mating type α based on their ability to mate with mating type α YM3270 yeast via auxotrophic complementation on the uracil dropout media.

The auxotrophic markers of the six GAL+ strains identified as being mating type α and the D308.3' and RD308.3 strains were then determined using known techniques. In particular, samples of each of these six strains were plated onto a series of plates having different media. The media for all of the plates included about 6.7 g of a bacto-yeast nitrogen base substantially without amino acids, about 20 g of glucose, about 20 g of bacto-agar, and about 2 g of a "drop out mix", with the balance being about 1000 ml of distilled water. The general formula of the "drop out mix" was about 0.5 g of adenine, about 4.0 g of leucine, about 0.2 g of *para*-aminobenzoic acid, and about 2.0 g of each of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, inositol, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, uracil and valine.

The media formulations differed from one another in that each "drop out mix" added to the medium omitted one of isoleucine, tryptophan, lysine, adenine, histidine, or methionine, but included all of the other ingredients of the formula. These series of plates were incubated at about 30°C for about 24 hours and visually inspected for growth. Although drop-out media were used in this protocol, it should be understood that any

recognized means for determining auxotrophic markers could have been used. The following auxotrophic markers were determined for each of the six mating type a GAL+ strains:

Yeast Strain	Drop Out Media					
	ade	met	his	lys	leu	trp
a GAL+ #6	-	+	-	-	+	+
a GAL+ #11	+	+/-	+	+	+	-
a GAL+ #33	+	-	-	-	+	+
a GAL+ #44	+	+/-	-	+	-	+
a GAL+ #46	-	+/-	-	+	+	+
a GAL+ #50	-	+	-	+	+	-

In this table, a "+" designation indicates that the strain grew on the identified drop out media, a "-" designation indicates that the strain did not appear to grow well on the identified drop out media, and a "+/-" designation indicates that this strain appeared to grow on the identified drop out media but required the addition of met when mated to strains of either RD308.3 or D308.3' via auxotrophic complementation as outlined below. Although one of ordinary skill in the art will be able to readily make other GAL+/Its yeast strains in accordance with the present disclosure, for purposes of convenience, the GAL+ #33 yeast strain was deposited with the ATCC on 4 March 1994 and is available to the public under the designation number ATCC 74272.

Based on these auxotrophic determinations, mating type a GAL+ strain numbers 6, 33, 44, 46 and 50 were mated to parent strain RD308.3 while mating type a GAL+ strain number 11 was mated to strain D308.3'. Substantially the same mating protocol as that outlined above for mating the RD308.3 and XA83-5B strains was used in mating the present strains. However, in this mating protocol, the synthetic galactose minimal media used to isolate the resulting diploid colonies from their parent haploids were supplemented with amino acids as follows:

2155675

- 42 -

Mating Pair	Synthetic Galactose Minimal Media Amino Acid Supplement(s)
α RD308.3 x a GAL+ #6	his
α D308.3' x a GAL+ #11	met*
α RD308.3 x a GAL+ #33	his, met
α RD308.3 x a GAL+ #44	his, met*
α RD308.3 x a GAL+ #46	his, met*
α RD308.3 x a GAL+ #50	his, trp

*diploids appeared to require met supplementation for growth although the strain was seemingly heterozygous with respect to the met-requiring mutation.

Each of these six diploid strains were tested to see if they did indeed remain GAL+ in the sense that they were able to grow on galactose but were substantially unable to grow on glucose. A heavy inoculum (e.g. on the order of about 10^7 CFU/ml) of each diploid and the RD308.3 and D308.3' strains were plated onto a YEP+galactose plate and a YEP+glucose plate. All six of the diploids and both the RD308.3 and D308.3' haploids grew well on the galactose medium but did not exhibit any significant growth on the glucose medium.

Given the present disclosure, those skilled in the art can clearly make a GAL+ diploid yeast of the invention. It should be understood that the experimental procedure outlined above is just one of a wide variety of possible methods of accomplishing this end and other effective means for making GAL+ diploids of the invention will be obvious to those skilled in the art in light of the present teaching. For instance, one could select other starting yeasts than those used in the present example, the mating of yeast strains may be conducted in other known manners, and auxotrophic markers could be determined by other known means.

It is believed that the diploid GAL+ yeast strain of the present invention will be both more active and more resistant to reversion and contamination than either of the RD308.3 and D308.3' haploid strains. Such improved activity and resistance should yield a GAL+ yeast strain which is more valuable in commercial dough manufacturing operations than either the RD308.3 or D308.3' haploid yeasts.

EXAMPLE 9

The properties of the GAL+ diploids produced in Example 8 relevant to use of the yeast in leavening refrigeratable dough in accordance with the invention were tested. The genotypes of the six GAL+ diploids produced in Example 8, as well as those of the RD308.3 and D308.3' strains, are believed to be as follows, based on the genotypes of the parent strains:

Yeast Strain	Genotype
a/ α GAL+ #6	a/ α h _{xk1} /h _{xk1} , h _{xk2} /h _{xk2} , glk1/glk1, his2/his2
a/ α GAL+ #11	a/ α h _{xk1} /h _{xk1} , h _{xk2} /h _{xk2} , glk1/glk1, met14/met14
a/ α GAL+ #33	a/ α h _{xk1} /h _{xk1} , h _{xk2} /h _{xk2} , glk1/glk1, met14/met14, his2/his2
a/ α GAL+ #44	a/ α h _{xk1} /h _{xk1} , h _{xk2} /h _{xk2} , glk1/glk1, met14/met14, his2/his2
a/ α GAL+ #46	a/ α h _{xk1} /h _{xk1} , h _{xk2} /h _{xk2} , glk1/glk1, met14/met14, his2/his2
a/ α GAL+ #50	a/ α h _{xk1} /h _{xk1} , h _{xk2} /h _{xk2} , glk1/glk1, his2/his2, trp5/trp5
α RD308.3	α h _{xk1} h _{xk2} glk1 trp5 his2 met14
α D308.3'	α h _{xk1} h _{xk2} glk1 his2 met14

First, the ability of each of the above-listed strains of yeast to utilize certain carbohydrates was tested. One loop of each yeast strain was added to a separate 300 ml flask containing about 50 ml of YEP+galactose liquid media having about the same formula as outlined above. These flasks were incubated at about 30°C for about 18 hours while being shaken. Three 10 ml test tubes for each of the incubated samples were provided with about 100 μ l of the incubated sample, with one test tube for each sample containing about 5 ml YEP, another test tube containing about 5 ml YEP+dextrose and the third test tube containing about 5 ml YEP+galactose. The formulas of these media were also substantially the same as set forth above for like media. A set of three control test tubes was prepared by placing about 5 ml of YEP, YEP+dextrose or YEP+galactose in each of three test tubes without any added yeast.

The absorbency of each of these resulting test tubes was measured at 600 nm prior to and during incubation at about 30°C. The absorbance of the test tubes containing YEP or YEP+dextrose media were measured over a period of about two weeks while the

2155675

- 44 -

absorbance of the test tubes containing YEP+galactose was measured for about 100 hours of incubation both with shaking and without shaking of the test tubes.

It was found that each of the diploid a/α GAL+ strains except a/α GAL+ #50 grew noticeably more quickly than the RD308.3 yeast strain on the galactose medium.

5 The D308.3', RD308.3 and a/α GAL+ strain numbers 6, 11, 33 and 44 grew rather poorly on both YEP and YEP+glucose media. The a/α GAL+ #50 yeast strain grew well on the galactose medium, but not appreciably better than the RD308.3 strain. The a/α GAL+ #44 and a/α GAL+ #50 strains also appeared to begin growing in the YEP+glucose medium after about 12 days of incubation. Although the reason for this
10 apparent ability to utilize glucose is not clearly understood, it has been surmised that either these strains spontaneously reverted to glucose utilization or the samples used in the test were contaminated with another organism.

The ability of the six diploid a/α GAL+ strains to revert to glucose utilization was tested by first adding a loop of the yeast strains to separate 10 ml volumes of
15 YEP+galactose and incubating these samples at about 30°C for about 20 hours while shaking. Three separate YEP+glucose plates for each sample were prepared by spread plating 100 μ l of the sample on each of the sample's three plates. In addition, 10^{-4} , 10^{-5} and 10^{-6} serial dilutions of each sample were spread plated onto similar YEP+galactose plates.

20 These plates were incubated and the number of observed revertant colonies for each of the six strains on their respective YEP+glucose plates were recorded. These rates were then compared to the total number of actual colonies plated onto the plates by comparison to the serial dilutions on the YEP+galactose plates. This technique for determining reversion frequency is well known in the art. The reversion rates of these
25 samples was so determined to be as follows:

Yeast Strain	Reversion Frequency (# of revertants per 10 ⁶ CFU)
a/ α GAL+ #6	0.10
a/ α GAL+ #11	0.04
a/ α GAL+ #33	0.03
a/ α GAL+ #44	0.03
a/ α GAL+ #46	0.09
a/ α GAL+ #50	0.01
α RD308.3	0.23

Thus, the frequency with which the diploid yeast strains of the invention reverted to glucose utilization was significantly lower than the reversion frequency of the parent RD308.3 haploid. Accordingly, it appears as though the diploid yeasts of the invention are probably more stable than the RD308.3 yeast of the invention, at least with respect to reversion to glucose utilization.

These diploid strains were evaluated in dough systems to determine their utility in leavening a refrigeratable dough in accordance with the invention. First, the yeast strains were grown by inoculating 300 ml culture flasks containing about 50 ml YEP+galactose liquid media with one isolated yeast colony, with two such inoculated flasks being prepared for each strain of yeast. These flasks were incubated at about 30°C for about 48 hours while shaking the flasks. These incubated samples were then added to separate 2 liter flasks containing about 1000 ml YEP+galactose liquid media and the larger flasks were incubated at about 30°C for about 24 hours prior to being harvested in paste form as outlined above in Example 1.

A dough was prepared from each strain of the diploid yeasts as well as the RD308.3 yeast. Each dough contained: about 758 g (56.1 wt%) wheat flour, about 49.0 g (3.5 wt%) wheat gluten preblend, about 498 g (35.6 wt%) water, about 14 g (1.0 wt%) salt, 35 g (2.5 wt%) of the yeast paste, about 14 g (1.0 wt%) galactose and about 4.2 g (0.3 wt%) yeast food. The yeast food used in this formula is commercially available from Red Star Universal Foods Corporation of Milwaukee, Wisconsin, USA under the designation "Regular Yeast Food". The water, galactose and yeast food were combined

and mixed with the yeast paste in a McDuffy™ mixing bowl at speed 1 for about 30 seconds, followed by mixing at speed 2 for about 6 minutes.

50-gram samples of the doughs so produced were placed into a Risograph™ sample jar and gas evolution data was collected for the samples as they were incubated at about 30°C for about 42 hours. The 50 g dough samples leavened with diploid strain numbers 33, 46 and 50 and the sample leavened with the RD308.3 yeast each generated about 90-100 ml of CO₂ over the course of the test, which is within the 100-200 ml CO₂/100 g of dough deemed necessary to properly proof the dough. The sample leavened with the a/α GAL+ #44 yeast generated only about 65 ml CO₂. The diploid strain numbers 33 and 46 appeared to generate CO₂ a little more rapidly than the haploid RD308.3 yeast. Diploid strain numbers 6 and 11 generated gas at a lower rate than the RD308.3, but the reason for this lower rate is not understood at this time in light of the liquid culture testing. The #44 and #50 strains appeared to generate gas slightly more slowly than the haploid parent strain.

The dough remaining after the Risograph samples were taken was rolled into sheets about one quarter of an inch thick and cut into rectangular slabs weighing about 250 g. These slabs were rolled into a log shape and placed into standard, 250 g-capacity spirally wound dough cans. Similar slabs of a standard chemically leavened dough such as is used in current commercial refrigerated dough operations was also placed into such cans. The canned doughs were proofed at about 100°F (about 38°C) until pressure in the can reached about 15-20 psi. These proofed dough samples were then stored in the cans at about 4°C and the internal pressure of the cans was monitored over time.

The dough leavened with RD308.3 yeast took about three hours to proof within the can to the desired degree. All of the doughs leavened with the diploid yeasts, with the exception of the a/α GAL+ #6 yeast, proofed somewhat more quickly than the RD308.3 dough, with the dough containing a/α GAL+ #33 taking only about 2.5 hours and the dough leavened with the a/α GAL+ #46 taking only about two and a quarter hours. The a/α GAL+ #6 yeast-leavened dough took significantly longer, with a proof time of over 5 hours.

Figure 13 is a plot of the measured pressures in canned dough samples as a function of time. The upper line illustrates the can pressures for the sample leavened with the a/α GAL+ #33 yeast, while the lower line (having data points illustrated by

hollow boxes) represents measured pressures for the chemically leavened dough sample. Measurements of can pressure for both of the samples were started after the dough was proofed in the cans, so the initial internal pressure is between 15 and 20 psi for both samples.

The pressure in both cans remained substantially constant over the month-long time period illustrated in Figure 13 and the behavior of the dough leavened with the yeast of the invention compares favorably with that of the chemically leavened sample. Since the plots of both of these samples remained generally flat over the four weeks during which measurements were collected, there is no reason to believe that the pressures in the containers would reach any critical stage within the anticipated 90-day shelf life of commercial refrigerated doughs. Hence, it appears that the diploid GAL+ yeast of the present invention is well suited for making doughs which can be stored at refrigeration temperatures for extended periods of time.

As noted above, a yeast according to a further embodiment of the invention is both substantially incapable of fermenting carbohydrates native to wheat flour and low temperature sensitive. Low temperature sensitive yeast ("lts" yeasts) are characterized by the fact that they behave essentially "normally" at elevated temperatures but become essentially dormant or inactive at refrigeration temperatures.

In producing a GAL+/lts yeast of the invention, a GAL+ yeast of the invention as outlined above and a low temperature sensitive yeast are mated to produce a GAL+/lts yeast. Low temperature sensitive yeasts desirably comprise genetic mutations of normal strains of yeast. Normal strains of yeast are believed to contain a certain percentage of such yeast cells, and these lts mutants of the yeast may be isolated in any of a variety of methods.

For instance, cold sensitive mutants of the yeast may be isolated by tritium suicide enrichment as described by Littlewood and Davies in "Enrichment for Temperature – Sensitive and Auxotrophic Mutants in Saccharomyces cerevisiae by Tritium Suicide", Mutat. Res. Vol. 17, pp. 315-322 (1973). In this tritium suicide enrichment process, a strain of yeast, which is preferably Saccharomyces. cerevisiae, is placed in a growth medium at normal temperatures and the

temperature is then reduced to refrigeration temperatures. Once the yeast has reached the lower temperature, tritiated uridine or tritiated amino acids may be supplied to the culture. Those cells which continue to remain active at these lower temperatures incorporate these precursors and are killed off by the tritium. Low temperature sensitive mutants present in the yeast sample, though,
5 will not incorporate the uridine or the amino acids because they remain substantially inactive at the lower temperature. Accordingly, the lts mutants preferentially survive the reduced temperature storage.

Some researchers in the field of genetics have investigated certain properties of these yeasts. For instance, Ursic and Davies reported the results of certain studies in "A Cold-Sensitive
10 Mutant of Saccharomyces cerevisiae Defective in Ribosome Processing", Molec. Gen. Genet. 175, 313-323 (1979), and Singh and Manney discuss the results of their testing in "Genetic Analysis of Mutations Affecting Growth of Saccharomyces cerevisiae at Low Temperature", Genetics, 77:651-659 (August 1974).

There appear to be a relatively large number of genes in yeast which can mutate to prevent
15 the growth of the yeast at low temperatures. For purposes of the present invention, though, it does not appear to be critical which of these genes is affected in the mutant which is utilized. The import factor in selecting a yeast is that the yeast should remain active at elevated temperatures, such as room temperature, yet become substantially inactive and substantially cease carbon dioxide production at refrigeration temperatures. Eight suitable examples of such lts yeasts are
20 available from the ATCC under deposit numbers ATCC 74124 through ATCC 74131.

The substantial inactivity of the lts yeast at refrigeration temperatures permits one to predictably proof or leaven the dough to the desired degree at elevated temperatures, then hold the leavened dough at refrigeration temperatures for extended periods of time. Such extended storage will significantly change the volume of the lts yeast-leavened dough because the yeast is inactive
25 and does not generate any significant volume of additional carbon dioxide. This allows a commercial dough manufacturer to controllably leaven or proof dough and place it in a sealed container for sale to consumers at a later date. So long as the dough is stored at refrigeration temperatures until it is sold, the pressure in the container should not substantially increase over time. Even if the dough is temporarily warmed above refrigeration temperatures, as during
30 improper transportation

or storage, if it is chilled back down, leavening action brought on by the elevated temperatures should be arrested and the yeast should once again become inactive.

GAL+/lts yeasts of the invention are believed to be superior to at least the haploid GAL+ strain explained in detail above for use in a refrigerated dough system. The low temperature sensitive characteristics of the GAL+/lts yeast provides a back-up mechanism for limiting excess carbon dioxide production if the strain does become contaminated. More particularly, it is believed that the low temperature sensitive nature of the yeast will render the yeast substantially inactive at refrigeration temperatures even if the yeast would otherwise continue to produce carbon dioxide.

For example, if some of the yeast reverts and more readily utilizes glucose, the low temperature sensitive nature of the yeast should limit any adverse effects from this contamination by substantially halting carbon dioxide when a dough leavened with this yeast is stored at refrigeration temperatures. Alternatively, if the integrity of the yeast strain is not compromised but an excess of a fermentable substrate (e.g. excess galactose) is inadvertently used in the dough composition, GAL+/lts yeast should substantially cease carbon dioxide production during refrigerated storage of the dough even though excess fermentable substrate may still be present.

EXAMPLE 10

In example 8, yeast strain XA83-5B was mated with yeast strain RD308.3 to produce diploid yeasts. While yeast strain RD308.3 is a GAL+ yeast, it has been previously determined that the XA83-5B yeast is a low temperature sensitive strain.

As noted above in Example 8, of the 287 putative GAL+ haploid yeast colonies isolated from the serial dilution plates, 50 strains were actually identified as GAL+ by their ability to grow on galactose-enriched medium, but substantially unable to grow on the glucose-containing medium. In Example 8, six of these strains determined to be mating type α were mated with parent strain RD308.3 or strain D308.3'.

In the present experiment, one loop of each of the fifty isolated GAL+ haploids of Example 8 were added to separate 10 ml sterile test tubes containing about 5 ml YEP+galactose. These inoculated samples were incubated at about 30°C while shaking for about 24 hours to grow the strains. Two samples (about 100 μ l per sample) of each of these 24-hour incubated specimens were then used to inoculate separate sterile 10 ml

test tubes containing about 5 ml of YEP+galactose. This yielded 50 pairs of inoculated test tubes.

One inoculated test tube from each pair was incubated at about 30°C while the other test tube from each pair was incubated at about 12°C. Absorbance measurements at about 600 nm were taken for each of the test tubes incubated at 30°C for several days (i.e. until all of the samples appeared to reach log phase). Absorbance of the samples incubated at 12°C at about 600 nm was also measured, with measurements being taken after about 10 and about 30 days of incubation.

All of the yeast strains appeared to grow well, as indicated by the rate of increase in absorbance, when incubated at about 30°C. Of the 50 GAL+ yeast strains tested, though, five strains appeared to be able to grow poorly or not at all, as indicated by little or no appreciable increase in absorbance, when incubated at about 12°C. These five strains therefore appear to be low temperature sensitive, as that term is used herein. These apparent GAL+/Its strains were assigned the designations GAL+/Its #8, GAL+/Its #17, GAL+/Its #21, GAL+/Its #26, and GAL+/Its #48.

One of the ordinary skill in the art will be able to readily make other GAL+/Its yeast strains in accordance with the present disclosure. For purposes of convenience, though, the GAL+/Its #8 yeast strain was deposited with the ATCC on 4 March 1994 and is available to the public under the designation number ATCC 74271.

In order to confirm the low temperature sensitivity of these five strains of yeast, the growth rates of colonies of these yeasts at 30°C and 12°C were compared to the parent RD308.3 and XA83-5B strains. Seven generally parallel rows, each row having four colonies of one of these seven strains, were grid plated onto duplicate YEP+galactose agar plates. In forming the rows, sterile tooth picks were used to transfer a colony of the yeast. Two such plates were made and one plate was incubated at about 30°C while the other was incubated at about 12°C.

The colonies resulting from this plating had an initial diameter about equal to that of the tooth pick with which they were transferred and measurements of the diameters of the colonies on the two differently incubated plates were measured over time. Figures 14 and 15 illustrate the colony diameter data of yeast strains GAL+/Its #8, GAL+/Its #17, GAL+/Its #21, GAL+/Its #26, GAL+/Its #48, RD308.3 and XA83-5B for the plate incubated at about 30°C and the plate incubated at about 12°C, respectively,

- 51 -

The diameters were measured visually with a digital micrometer and, since the tooth pick used to transfer the samples created a slight indentation in the agar plates, it was rather difficult to obtain reliable measurements below about 1.0 mm in diameter. Measurements for all four of the colonies one the row dedicated to one strain of yeast were averaged together to yield the measurements shown in these two graphs.

Figure 14 shows that all six of the illustrated strains of the yeast grew reasonably well over the course of the test, with colonies ranging from about 3.5 mm to about 7.5 mm by the end of about 9 days of incubation at about 30°C. The RD308.3 yeast grew the most rapidly while all of the low temperature strains grew a little more slowly. The GAL+/lts #21 and GAL+/lts #17 strains grew at about the same rate as the XA83-5B yeast, with the GAL+/lts #8 and GAL+/lts #48 strains growing a little more slowly.

Figure 15 represents the growth rates of the same yeasts at about 12°C. The growth rate of the RD308.3 yeast was slower than that illustrated in Figure 12 for 30°C incubation, but this strain of yeast nonetheless seemed to grow fairly well at the lower temperature. The XA83-5B yeast and the GAL+/lts #8 and GAL+/lts #26 strains all showed some measurable growth over the 20-day test, but the maximum colony size for any of these three yeasts was still no more than about 2 mm. There appears to be a rather sudden jump in colony diameter after about a week of incubation, but as noted above, it was relatively difficult to read colony diameters accurately below about 1.0 mm. It is believed that these colonies grew at a relatively steady, though quite slow, rate rather than experiencing a sudden growth spurt between 6 days incubation and the next measurement after about 9 days of incubation.

It is believed that the 12°C temperature at which these yeasts were incubated is about at the upper limit of the XA83-5B yeast strain's low temperature sensitivity threshold and this might explain the slight, but measurable, growth of this strain at 12°C.

The other three strains of the GAL+/lts yeast of the invention, though, did not exhibit any measured growth when incubated at 12°C. This indicates that the GAL+/lts #17, GAL+/lts #21 and GAL+/lts #48 yeasts are, surprisingly, even more low-temperature sensitive than the XA83-5B parent strain. These three strains of the yeast also grew about as well as their low temperature sensitive parent at about 30°C.

Given that these strains of yeast also appear to be substantially unable to ferment any carbohydrates native to wheat flour, it appears that the GAL+/lts #17, GAL+/lts

2155675

- 52 -

#21 and GAL+/lts #48 yeasts are particularly well suited for use in a refrigeratable dough composition of the invention. Accordingly, based on the evaluation outlined above, these strains would be the most preferred strains of those obtained by the present mating.

5 The present disclosure teaches how to select or isolate GAL+ and lts strains of yeast, at least one method of mating such yeasts, and methods for testing the resultant strains of yeast to isolate and evaluate GAL+/lts strains so obtained. Given the present teaching, it is well within the ability of one skilled in the art to make and test any number of GAL+/lts yeasts.

10 Another embodiment of the present invention provides a method of forming a dough which can be stored at refrigeration temperatures for extended periods of time without generating significant volumes of carbon dioxide. This method may further include the steps of packaging the dough, proofing the dough in the package, and storing the dough for an extended period of time at refrigeration temperatures.

15 In making a dough of the invention, flour, water, a yeast substantially incapable of fermenting carbohydrates native to the flour, and a quantity of a carbohydrate fermentable by the yeast are mixed together, as outlined above. The amount of the fermentable carbohydrate added to the dough is desirably sufficient to provide only the necessary degree of proofing of the dough; adding too much fermentable substrate could cause
20 adverse changes in dough rheology due to overfermentation. This amount is optimally determined on a case-by-case basis for a given strain of yeast as different strains of yeast may utilize the fermentable substrate more efficiently than others. If so desired, additional flavoring ingredients, such as salt, additional quantities of sugars native to the flour, or wheat gluten, could be added to the dough to achieve a desired flavor in the
25 final baked good produced from the dough.

 In a particularly preferred embodiment of the method of the invention, the yeast used in making the dough is a GAL+ yeast and a predetermined quantity of galactose is added to the dough to provide the desired degree of proofing. This GAL+ may be the D308.3, D308.3' yeast or the RD308.3 yeast described above, but it is to be understood
30 that other GAL+ yeasts can be made in accordance with the present disclosure which will also work in accordance with the invention.

As noted above, the method may further include the steps of packaging the dough, proofing the dough in the container, and storing the dough at refrigeration temperatures for an extended period of time. Virtually any known refrigeratable dough package known in the art may be used in this method. For instance, spirally wound dough containers
5 such as those currently used in commercially manufactured refrigeratable dough products should suffice. A quantity of dough somewhat less than that necessary to fill the container is placed in the container, leaving a headspace in the container when it is sealed.

The dough may then be proofed in the container, expanding to fill the container
10 and flush out any air in the headspace. The proofing is continued until substantially all of the fermentable carbohydrate is consumed by the yeast, at which point an internal pressure of about 15 to about 20 psi is attained in the container. This proofing may be advantageously carried out at an elevated temperature, e.g. about 30°C to about 40°C, to allow the yeast to ferment, and thus proof the dough, more rapidly.

This proofed dough may then be placed in refrigerated storage for extended
15 periods of time, desirably up to at least about two weeks. The dough of the invention is optimally capable of storage at refrigeration temperatures for at least about 90 days, the anticipated shelf life of current doughs, as explained above. By "refrigerated storage", storage at temperatures between about 12°C and about 0°C, and preferably between about
20 4° and about 7.2°C, is intended. Such temperatures are referred to in the present specification as "refrigeration temperatures".

The present invention further provides dough compositions in which the yeast is temperature sensitive. If they are heated above a threshold inactivation temperature of the yeast, the yeast's carbon dioxide producing capabilities in substantially anaerobic
25 environments are substantially inactivated at essentially all temperatures. By effectively controlling the production parameters and the yeast's carbon dioxide producing capabilities, the proofing of dough compositions by yeast can be regulated.

As explained above, even at refrigeration temperatures, conventional yeast can continue to produce carbon dioxide. If carbon dioxide production is substantially
30 inactivated by heating the dough composition above the yeast's threshold inactivation temperature, the amount of proofing can be controlled once the dough has reached a

predetermined volume, regardless of the subsequent temperature conditions to which the dough composition is exposed.

Refrigerated dough compositions of the present invention employ temperature sensitive strains of yeast which function normally at temperatures below their threshold inactivation
5 temperatures. For instance, the yeast may be capable of fermenting substrate in the dough at about room temperature and can continue to ferment substrate at temperatures below the threshold inactivation temperature. However, once the yeast is heated above the threshold inactivation temperature it loses its ability to produce carbon dioxide required for proofing the dough. It has been surprisingly discovered that by using these temperature sensitive yeast, the dough
10 compositions may be anaerobically proofed to a predetermined volume, then heated to at least the threshold inactivation temperature to substantially inactivate the yeast's carbon dioxide producing capabilities. In some dough compositions, the proofing of the dough may be carried dough while heating the dough up to the threshold inactivation temperature. The dough composition may then be stored, e.g. at refrigeration temperatures, for extended periods of time without the disadvantages
15 associated with excess carbon dioxide production and degradation of dough rheology by conventional yeast during storage.

The refrigeratable dough composition of the invention may optionally be placed in containing means for storage at refrigeration temperatures. Such containing means are know to those skilled in the art and include spiral wound composite cans. Unproofed dough is placed in the
20 can which is then closed, proofed to an internal pressure to about 15-20 p.s.i. thereby filling the entire volume of the can with dough. The dough creates a seal in said containing means by filling vents and thus rendering the internal environment of the can substantially anaerobic. Examples of such spiral wound composite cans suitable for refrigerated dough include those described in the following patents: Culley et al., U.S. Patent No. 3,510,050; Reid, U.S. Patent No. 3,972,468; and
25 Thornhill et al., U.S. Patent No. 3,981,433.

An alternate containing means is formed of a flexible packaging material, such a polymeric film, and the internal environment of the containing means is substantially anaerobic. On method for making the internal environment substantially anaerobic is by hermetically sealing the containing means under a vacuum or in an inert gas environment.

- 55 -

Alternatively, an inert gas may be added to the package, substantially flushing oxygen from the containing means, prior to sealing.

The refrigerated dough composition of the present invention may alternatively be stored without a containing means at refrigeration temperatures directly after cessation of proofing.

The formulation of the dough composition is not critical; virtually any dough comprising flour, water and a yeast in accordance with the invention may be used. An example of one dough composition formulation suitable for use in the present invention is provided in Table I:

Table I

<u>Ingredient</u>	<u>Weight Percent of Dough</u>
flour	54.53-57.93
water	34.71-35.45
gluten pre-blend*	3.88-3.91
dextrose	0.99
salt	0.75
temperature sensitive yeast	1.00

*The gluten pre-blend comprises about 75% vital wheat gluten; 21.9% hard, high gluten; enriched ingredient flour; 2.5% xanthan gum; and 0.616% azodicarbonamide premix.

The temperature sensitive yeast used in one embodiment of the present invention may be any member of the species Saccharomyces cerevisiae which have a threshold inactivation temperature, i.e., which are substantially inactivated if heated to a temperature above some threshold. The threshold inactivation temperature of the yeast is optimally no more than about 43°C, at which temperature conventional dough made with wheat flour will usually begin to bake. (As used herein, the term "dough" or "dough composition" refers to an unbaked dough, while a dough which has been baked is referred to as a "baked dough", "baked product" or the like.)

On the other hand, if the threshold inactivation temperature of the yeast is below room temperature, making the dough in a commercial operation may be unduly difficult because the entire production area would likely have to be maintained below that temperature in order to permit the dough to be proofed by the yeast before it is inactivated. Accordingly, in a preferred embodiment the threshold inactivation

2155675

- 56 -

temperature of a yeast used in a dough of the invention is between about 25°C and about 43°C.

It has also been found that heating a dough above about 40°C for a significant period of time can be harmful to the dough due to possible deactivation and denaturation of wheat flour gluten, as well as promotion of the growth of spoilage microorganisms native to wheat flour, at higher temperatures. As explained more fully below, the yeast is allowed to proof the dough before it is heated above the threshold inactivation temperature. Proofing takes place more quickly at elevated temperatures, e.g. about 30-40°C, than it does at room temperature or below; the activity of the yeast, and hence the rate of proofing, can be said to be roughly positively correlated with temperature.

It would therefore be advantageous in a commercial production environment to be able to heat the dough above room temperature, e.g. to about 30° or more, without exceeding the threshold temperature of the yeast and rendering it substantially inactive. Hence, in a particularly preferred embodiment of the invention the threshold inactivation temperature of the yeast is desirably between about 25°C and about 40°C, and optimally between about 30°C and about 40°C.

Such temperature sensitive yeast desirably enter cell cycle arrest when exposed to temperatures above their threshold inactivation temperatures. Applicants have discovered that once the temperature-induced cell cycle arrest occurs, the yeast tend to become obligate aerobes and are rendered substantially incapable of fermentative anaerobic growth. Applicants have further discovered that these characteristics can be used to effectively control dough proofing and that dough containing such temperature sensitive yeast may be stored under refrigeration conditions for extended periods of time.

By using temperature sensitive yeast in dough compositions, the dough can be proofed to the desired volume at temperatures below the threshold inactivation temperature, following which the yeast can be substantially inactivated by subjecting the dough, and hence the yeast therein, to temperatures above the yeast's threshold inactivation temperature. The dough thus prepared can be refrigerated for extended periods of time without the risks of overfermentation and rupture of optional containing means by excess proofing of the dough composition. Following the inactivation of the yeast, allowing the dough composition temperatures to change, even to temperatures

below the threshold levels, has no measured effect on the substantial inactivation of the yeast's fermentative capabilities. These characteristics of the dough make it especially suitable for transportation and storage where large temperature fluctuations are possible, which could have deleterious effects on dough leavened with conventional, non-temperature sensitive yeast. The product obtained by baking or cooking this refrigerated dough composition can be defined as a bread product as it is leavened with yeast and has the desired organoleptic qualities associated with the yeast-leavened dough products.

Given the present disclosure, it will be well within the ability of those skilled in the art to make yeasts which can be substantially inactivated at by heat treatment at elevated temperatures. Such yeasts can be made through standard methods of crossing yeast strains, isolating suitable strains having the desired properties and the like. These types of common techniques are described, for example, by Sherman *et al.* in *Methods in Yeast Genetics*. Of particular interest in the Sherman *et al.* is Section III, entitled "Making Mutants", which appears on pages 273-369 of this reference.

One process for creating and isolating such mutants has been taught by Hartwell *et al.* in "Genetic Control of the Cell Division Cycle Mutant in Yeast: V. Genetic Analysis of *cdc* Mutants". *Genetics* 74: 367-286 (June, 1973). As disclosed in that article, Hartwell *et al.* treated a strain of yeast having the genotype *a ade1 ade2 ura1 tyr1 his7 lys2 gal1*, identified as strain A364A, with a known mutagen, namely either N-methyl-N'-nitro-N-nitrosoguanadine or ethylmethane sulfonate. Resulting temperature-sensitive mutants having a permissive temperature of about 23°C and a restrictive growth temperature of about 36°C were isolated. Of these temperature-sensitive mutants, cell division cycle mutants were isolated by identifying morphological criteria for these mutants and selecting those mutant colonies in which 80% or more of the cells exhibited a uniform morphology.

Following this process, Hartwell *et al.* identified nearly 150 different *cdc* mutants. Complementation studies demonstrated that these mutants defined 32 complementation groups, with 30 of those groups defined by single mutations in nuclear genes (as determined by standard genetic analysis techniques). One such strain which has been found to work well exhibited a structural mutation in the pyruvate kinase gene, but it is to

2155675

- 58 -

be understood that there may be other thermally sensitive mutations which can work well in the present invention.

One possible mechanism for thermal inactivation has been elucidated for the S. cerevisiae strain cdc19, available from the Yeast Genetic Stock center at the Donner Laboratory in the Department of Molecular and cell Biology at the University of California, Berkeley (YGSC) and deposited with the American Type Culture Collection, of 12301 Parklawn Drive, Rockville, MD (ATCC) on 5 March 1993 under the number ATCC 74213. The fermentation pathway of cdc19 yeast strain is substantially inactivated following exposure to temperatures above the threshold inactivation temperature due to a mutation in the yeast that results in a thermally labile pyruvate kinase enzyme. Mutants having a mutation in the pyruvate kinase gene can be identified by known morphological or genetic analysis techniques.

If the dough composition containing the temperature sensitive yeast is subjected to temperatures above the threshold inactivation temperature, pyruvate kinase is rendered structurally inactive and the yeast become obligate aerobes, incapable of virtually any significant anaerobic fermentation until pyruvate kinase biosynthesis resumes. In the cdc19 strain, pyruvate kinase biosynthesis resumes when i) oxygen is present and *de novo* biosynthesis of pyruvate kinase can occur, or ii) the genetic mutation reverts to the wild type. Such reversions generally may occur only when the yeast are growing. As seen in Figure 16, wherein the anaerobic growth of such temperature sensitive yeast was observed at 25°C following 0-4 hours of incubation at 40°C, once the cdc19 cells have been exposed to temperatures above their threshold inactivation temperatures, under anaerobic conditions the yeast lose the ability to grow and thereby revert to the wild type.

The thermal inactivation of cdc19 yeast substantially eliminates any further carbon dioxide production under anaerobic conditions at all relevant temperatures (e.g. between about 0°C, when the dough is frozen, and about 45°C, when the dough is baking). This makes the yeast particularly suitable for refrigerated dough compositions in accordance with the present invention.

In a second embodiment of the present invention, the temperature sensitive yeast used in the dough compositions as outlined above may be any member of the species S. cerevisiae having an upper threshold inactivation temperature and which becomes substantially inactive at refrigeration temperatures. Such a yeast could be obtained by

Mating low temperature sensitive yeast to high temperature sensitive yeast, for example. There are numerous yeast strains that carry low temperature sensitive mutations. For purposes of the present invention, the choice of which of these mutant strains are utilized is not believed to be critical as long as the requisite characteristics of substantial inactivity as refrigeration temperatures and substantially normal fermentative activity at elevated temperatures, i.e. above refrigeration temperatures, are observed in the particular strain chosen.

Low temperature sensitive mutants are sometimes found in normal strains. Isolation of these low temperature sensitive mutants may be accomplished by a variety of methods known to those skilled in the art. One isolation method is the "tritium suicide" enrichment protocol described by Littlewood and Davies in "Enrichment for Temperature Sensitive and Auxotrophic Mutants in Saccharomyces cerevisiae by Tritium Suicide," Mutation Research Volume 17, pp. 315-322 (1973).

In this protocol, a strain of yeast is first placed in a growth medium at normal permissive temperature followed by reduction of the temperature to refrigeration (non-permissive) temperature. Tritiated uridine or tritiated amino acids are supplied to the culture. Yeast cells remaining active at these temperatures incorporate the tritiated precursors and are killed by the tritium. Those cells that are inactive at these lower temperatures do not incorporate the toxic precursors and are able to survive the low temperatures by virtue of their low temperature sensitivity.

One skilled in the art could readily make a low temperature sensitive yeast in accordance with the tritium suicide process. However, the following strains of yeast which become substantially inactive at refrigeration temperatures are available to the public from the ATCC: "Its1" S. cerevisiae (Designation No. ATCC 74124), "Its2" S. cerevisiae (Designation No. ATCC 74125), "Its3" S. cerevisiae (Designation No. ATCC 74126), "Its4" S. cerevisiae (Designation No. ATCC 74127), "Its5" S. cerevisiae (Designation No. ATCC 74128), "Its6" S. cerevisiae (Designation No. ATCC 74129), "Its7" S. cerevisiae (Designation No. ATCC 74130), and "Its8" S. cerevisiae (Designation No. ATCC 74131).

Mating a high temperature sensitive yeast strain with a low temperature sensitive yeast strain can be performed by any means known to produce haploid mutants in yeast.

One such protocol is derived from Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, pp. 53-59 (1990).

Applicants have discovered that combining the properties of a high temperature sensitive yeast strain with a low temperature sensitive yeast strain results in a mutant yeast strain with characteristics that make it excellent for use as a leavening agent in refrigerated dough compositions. By combining the high and low temperature sensitivities in the yeast, not only can the yeast be inactivated by raising the temperature of the dough composition above the threshold inactivation temperature, but the remote possibility of some yeast cells not becoming inactivated and continuing to generate carbon dioxide at lower temperatures such as refrigeration temperatures is also eliminated by introducing low temperature growth sensitivity into the yeast strain.

One possible mechanism for the low temperature growth sensitivity in low temperature sensitive yeast has been elucidated for "Its 8" *S. cerevisiae* (noted above). The mutation carried by this strain renders the yeast incapable of any protein synthesis at lower temperatures such as refrigeration temperatures. These yeast, therefore, cannot grow below their threshold inactivation temperatures.

The combination of the characteristics of the high and low temperature sensitive yeast makes pyruvate kinase biosynthesis highly unlikely after the temperature of the yeast in the dough composition has been raised above its threshold inactivation temperature followed by refrigeration, which substantially inactivates virtually all protein synthesis as a result of the low temperature sensitive mutation. Since pyruvate kinase is substantially inactivated, and pyruvate kinase cannot be synthesized de novo by the yeast in any significant quantities, virtually all anaerobic fermentative growth is eliminated and no significant volume of carbon dioxide will be produced by the yeast. The low-temperature sensitive mutation of the yeast thus serves a safety function – if some oxygen is inadvertently permitted to come into contact with the yeast (e.g. if a container of dough leavened with this yeast allows air to leak in), the yeast still will be substantially unable to produce carbon dioxide.

In accordance with a method for making dough composition in accordance with the present invention, the temperature sensitive yeast is mixed with water and a flour product, such as ground wheat, in suitable proportions to form a dough which is suitable

for baking. Additional ingredients necessary to achieve a desired texture or taste in the final, cooked dough product may be added during this mixing as well. Such ingredients are commonly known in the art and include salt, sugars, wheat gluten, dough conditioners and other flavorings. All of these ingredients should be thoroughly mixed together to ensure a uniform dough composition; a wide variety of means for mixing doughs are well known in the art and need not be discussed in detail here.

Once the dough is mixed, the yeast should be allowed to generate carbon dioxide to proof the dough. This proofing may be carried out at any suitable temperature. However, if the above-described yeast having low-temperature sensitivity is used, the proofing should be carried out at temperatures greater than refrigeration temperatures as the yeast remains substantially inactive at such temperatures. In order to reduce production time in a commercial operation, it may be advantageous to heat the dough to a temperature greater than room temperature to speed up carbon dioxide production. The dough may therefore be heated for proofing, but care should be taken to remain below the threshold inactivation temperature throughout most of the dough in order to avoid inactivating the yeast before proofing is completed. In one embodiment which has been found to work well using the cdc19 yeast described below, which has a threshold inactivation temperature of about 36°C, proofing is carried out at a temperature between about 30°C and about 35°C.

Once the dough has been proofed to a predetermined degree, the temperature of the dough may be elevated up to, or desirably above, the threshold inactivation temperature. The dough will not heat to a uniform temperature instantly; there will tend to be a temperature gradient in the dough due to the low coefficient of thermal transfer of doughs, with the outer portion of the dough being at a temperature closer to the ambient temperature than the inner portion of the dough. The dough is optimally heated slowly enough or held at a temperature at or above the threshold inactivation temperature long enough to ensure that most, and preferably substantially all, of the yeast in the dough reaches the threshold and is substantially inactivated.

The resulting yeast can be then stored at refrigeration temperatures for an extended period of time without generating any significant additional volume of carbon dioxide. As the term is used herein, refrigeration temperatures are between about 0°C and about 12°C, with a temperature range of about 4°C to about 7.2°C being preferred. The dough

2155675

- 62 -

may be stored at such temperatures for upwards of about 90 days, which is the minimum acceptable shelf life for most commercially produced refrigeratable doughs, without any undue deterioration in quality.

5 In accordance with a further embodiment of the present method, the dough is packaged in a container means such as that described above. It is preferred that the dough be placed in the container means prior to proofing and be proofed in the container means. In current commercial packaging operations using spirally wound cans, the dough is placed in the container and proofed until the internal pressure in the can reaches about 15-20 psi. In accordance with the present invention, the dough is placed in the container
10 and is allowed to proof until the internal pressure of the container means reaches about 15-20 psi, at which point the dough is heated above the threshold inactivation temperature to substantially inactivate the yeast. The packaged dough product may then be stored at refrigeration temperatures for an extended period of time.

15 As noted above, there may be temperature gradients in the dough which can produce a lag time between an ambient temperature change and a change in the temperature of the center of the dough. The temperature profile of the proofing and inactivation process should be designed to take this lag time into account. In some instances, the lag time may be sufficient to enable the dough to be proofed at an elevated temperature at the same time as the dough is being heated up to the threshold inactivation
20 temperature of the yeast. In such an embodiment of the present method, the temperature profile of the heat treatment may be smooth, i.e. not exhibit a sharp demarcation between the proofing and the inactivation steps.

Example 11: Preparation of High and Low Temperature Sensitive Yeast

25 A strain of yeast having a sensitivity to high temperatures, i.e. which is substantially inactivated at elevated temperatures, was crossed with a low temperature sensitive yeast, i.e. a yeast which becomes substantially inactive at low temperatures, but may retain the ability to ferment substrate at higher temperatures. The yeast sensitive to high temperatures was a mutant strain of Saccharomyces cerevisiae designated cdc19 and deposited with the ATCC on 5 March 1993 under the number ATCC 74213. This
30 particular strain of yeast has a threshold inactivation temperature of approximately 36°C and is rendered an obligate aerobe if heated at or above that temperature.

The low temperature sensitive yeast strain used is the *lts8* yeast deposited with the ATCC under deposit number ATCC 74131, as mentioned above. Yeast mutant strain *lts8* is representative of many known low temperature sensitive yeast strains which behave substantially normally at elevated temperatures, e.g. can ferment available substrate at room temperature or greater, but become substantially inactive at refrigeration temperatures.

The high and low temperature sensitive mutant yeast strain was obtained by mating the high temperature sensitive mutant strain *cdc19* with the low temperature sensitive mutant strain *lts8*. The protocol used to mate these strains was derived from Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, pp. 53-59 (1990), as follows:

Six substantially parallel lines were drawn on a white sheet of cardboard. For each of the *cdc19* and *lts8* strains, a YEPD (YEP plus dextrose) plate was placed over the striped pattern. Using a sterile loop, the strains were streaked onto their respective plates using the parallel lines as a guide and allowed to incubate at approximately 25°C for about 24 hours.

An impression of the *cdc19* strain was made on a replicate plate pad. This impression was imprinted onto a fresh YEPD plate. Using a fresh replicate plate pad, an impression of the low temperature sensitive *lts8* strain was made. The second replicate pad was imprinted on the same YEPD plate used for the previous imprinting, but at an orientation generally perpendicular to the first imprint, resulting in a pattern of yeast strains resembling a checkerboard. This doubly imprinted YEPD plate was incubated at approximately 25°C overnight (i.e about 12-15 hours).

The YEPD plate thus prepared was imprinted on a synthetic dextrose minimal media (SD) plate containing about 6.7 g of bacto-yeast nitrogen base without amino acids, about 20 g glucose and about 20 g bacto-agar per liter of distilled water. The SD plate was incubated for about two days at about 25°C. Growth at the intersections of the "checkerboard" pattern was scored and plated onto a SD fresh plate to isolate the diploid (crossed) colonies from the haploid colonies. The diploid colonies isolated on the SD plate were streaked onto a plate of sporulation media (as formulated below) and incubated for days at about 25°C: about 10 g (1 wt. %) potassium acetate, about 1.0 g (0.1 wt. %)

2155675

- 64 -

bacto-yeast extract, about 0.5 g (0.05 wt. %) glucose, about 20 g (2.0 wt. %) bacto-agar, with the balance being about 1000 ml distilled water.

About one loopful of yeast cells was taken from the sporulation plate and combined with about 300 microliters distilled water and approximately 15 microliters
5 glusulase in an Eppendorf™ microfuge tube. This solution was mixed by vortex and incubated at around 30°C for approximately 30 minutes. The incubated sample was briefly sonicated to separate spore clusters. Serial dilutions of about 10⁻⁴, 10⁻⁵ and 10⁻⁶ of the sonicated sample were plated onto YEPD and stored at approximately 25°C for about two days.

10 Three replicate plates were prepared from each of the 10⁻⁵ and 10⁻⁶ dilution plates, with one of the three plates from each dilution being stored at about 12°C, another at about 25°C, and the third plate from each dilution being stored at about 38°C. These plates are incubated for 4 days at their respective temperatures. cdc19xlt8 haploid colonies should only grow at 25°C as their high temperature sensitivity substantially
15 prevents growth at 38°C and the low temperature sensitivity will reduce the yeast's growth rate to a very low rate at about 12°C.

The growth rate of the cdc19 strain, the lts8 strain and the cdc19 x lts8 strain produced as outlined above were compared at about 12°C, about 25°C and about 38°C. The cdc19 yeast was able to grow well at both about 12°C and about 25°C, but was
20 substantially unable to grow at about 38°C. The lts8 yeast was able to grow at all three temperatures, but this is to be expected because the lts8 strain does not substantially cease activity until the temperature drops below about 10°C. However, the growth of the lts8 yeast at about 12°C is less vigorous than that of the cdc19 strain. The cdc19 x lts8 yeast grew very poorly at about 12°C, grew fairly well at about 25°C, and was substantially
25 unable to grow at about 38°C.

Although the present invention has been described with reference to preferred embodiments, the invention is not to be limited to those embodiments described herein except to the extent that such limitations are found in the appended claims.

WHAT IS CLAIMED IS:

1. A yeast-leavened refrigeratable dough product comprising flour, water, yeast of the genus-species *Saccharomyces cerevisiae* substantially incapable of fermenting any carbohydrates native to the flour, and a carbohydrate additive
5 fermentable by said yeast.
2. The dough product of claim 1 wherein the amount of carbohydrate additive fermentable by the yeast is chosen to provide no more than 200 ml CO₂ per 100g of dough when substantially all of the carbohydrate additive has been metabolized by the yeast.
- 10 3. The dough product of claim 1 or 2 further comprising a container having a sealed cavity, the dough being disposed within said cavity.
4. A yeast of the genus-species *Saccharomyces cerevisiae* for leavening a refrigeratable dough including a wheat flour, comprising a yeast exhibiting the following characteristics:
15
 - a) substantially incapable of fermenting carbohydrates native to wheat flour;
 - b) capable of fermenting galactose without adenine supplementation.
5. The yeast according to claim 4 wherein the yeast is a diploid yeast.
6. The yeast according to claim 4 or 5 further exhibiting the following
20 characteristic:
 - c) becomes substantially inactive at temperatures between 0°C and 12°C.

7. The yeast of claim 6 wherein the yeast become substantially inactive at temperatures between 4°C and 7.2°C.

8. A yeast leavened refrigeratable dough product comprising wheat flour, water and a yeast according to any one of Claims 4 to 7.

5 9. A method of producing a refrigeratable dough product comprising the steps of:

a) mixing flour, water, yeast of the genus-species *Saccharomyces cerevisiae* substantially incapable of fermenting any carbohydrates native to the flour, and a carbohydrate additive fermentable by said yeast;

b) placing said dough in a container and proofing the dough in the container; and

c) storing said proofed dough in said container at refrigeration temperatures for at least two weeks.

15 10. The method of claim 9 wherein the amount of yeast and carbohydrate additive fermentable by the yeast mixed to form the dough are chosen to provide no more than 200 ml CO₂ per 100g of dough.

11. The method of claim 9 or 10 wherein the dough is proofed by heating the dough in the container at an elevated temperature.

20 12. The method of claim 9, 10 or 11 wherein the dough is proofed until an internal pressure of between 69 and 128 KPa (10 and 20 psi) developed in the container.

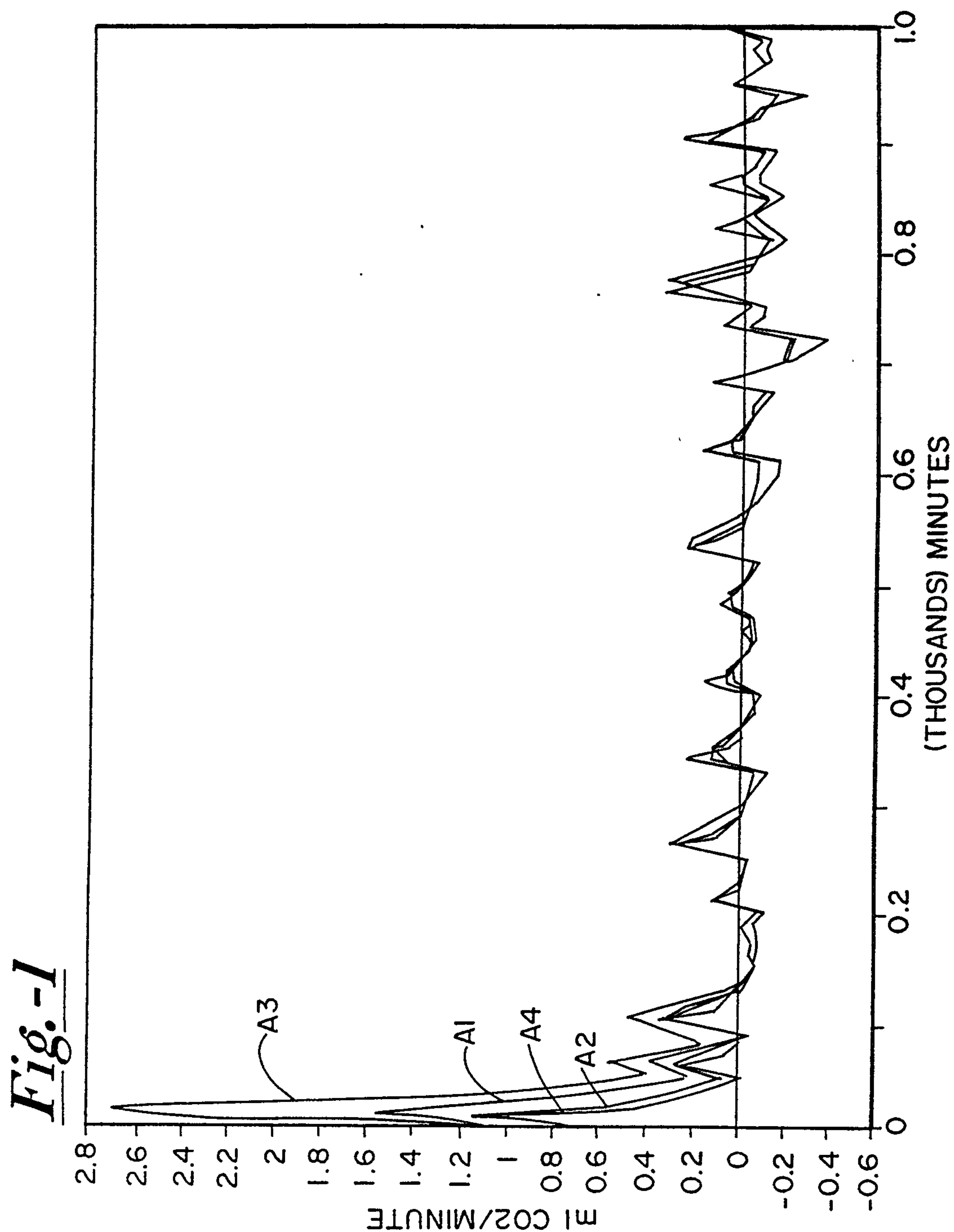
13. The yeast leavened refrigeratable dough product according to claim 8, wherein the yeast is of the genus-species *Saccharomyces cerevisiae*.

14. The yeast according to claim 4, 5, 6 or 7, wherein the yeast is of the genus-species *Saccharomyces cerevisiae*.

5 15. The dough product of claim 1, 2 or 3 wherein the yeast is capable of fermenting galactose without adenine supplementation and the carbohydrate additive fermentable by said yeast comprises galactose.

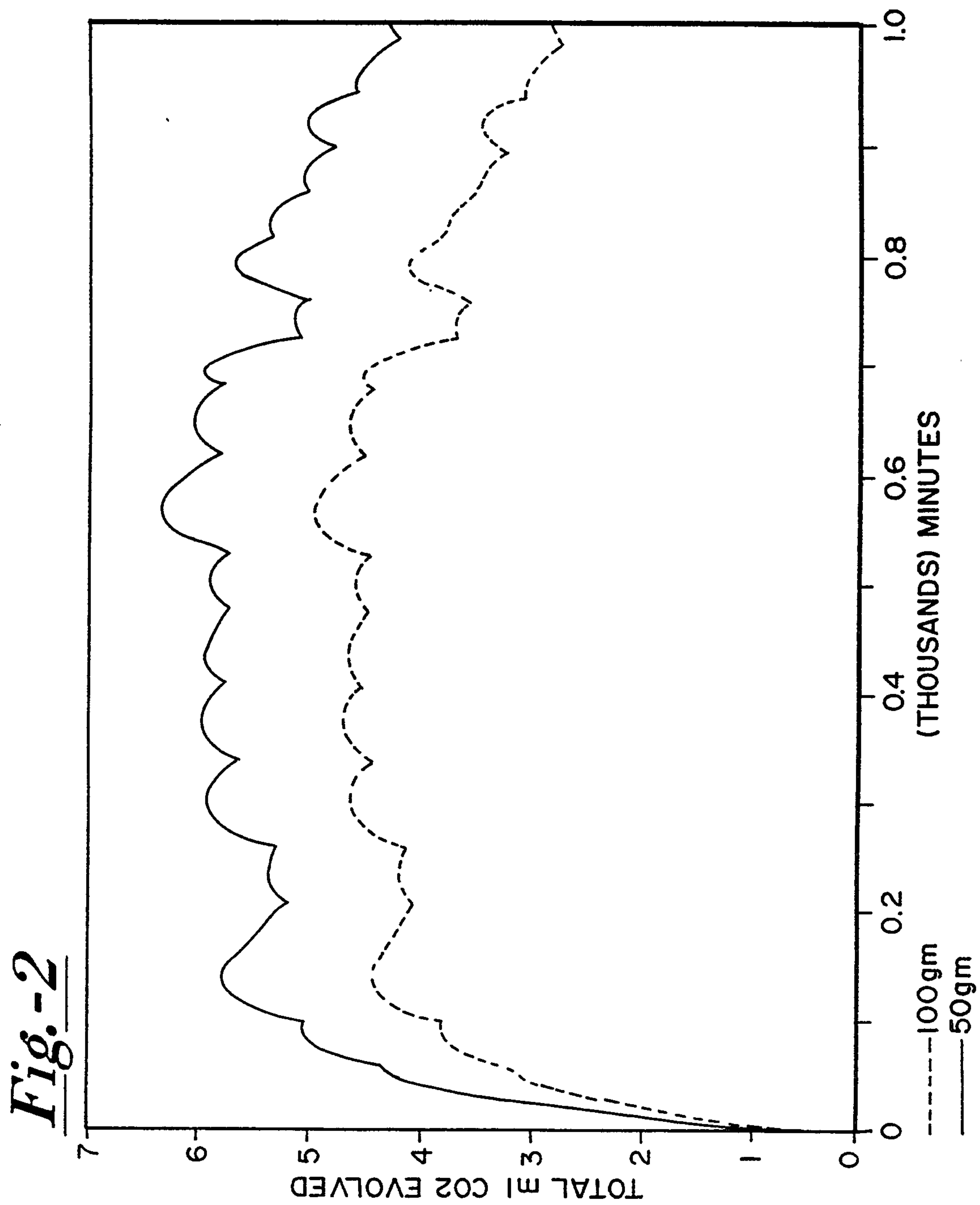
16. The method of claim 9, 10, 11 or 12 wherein the yeast is capable of fermenting galactose without adenine supplementation and the carbohydrate
10 additive fermentable by said yeast comprises galactose.

1/18



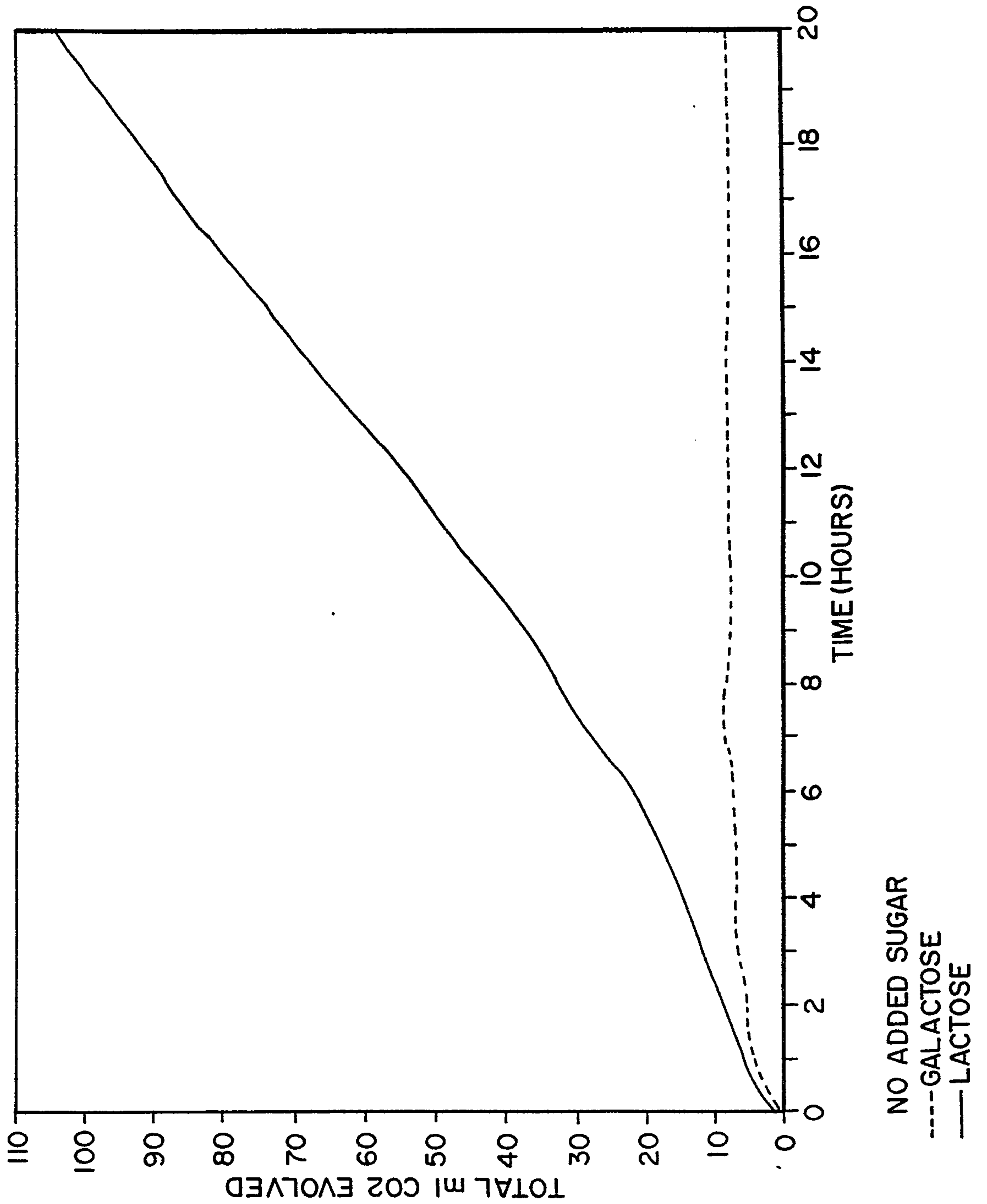
2155675

2/18

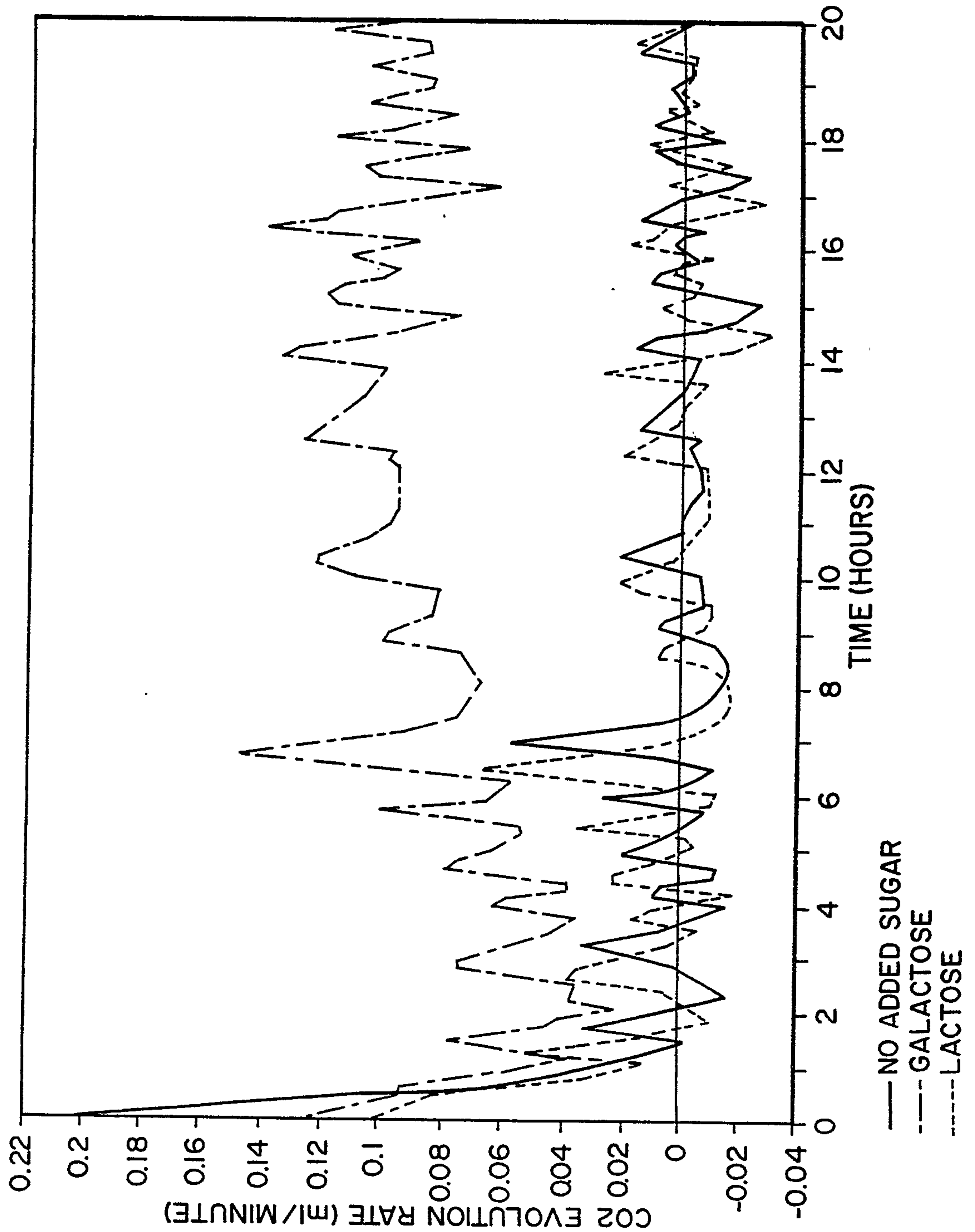


3/18

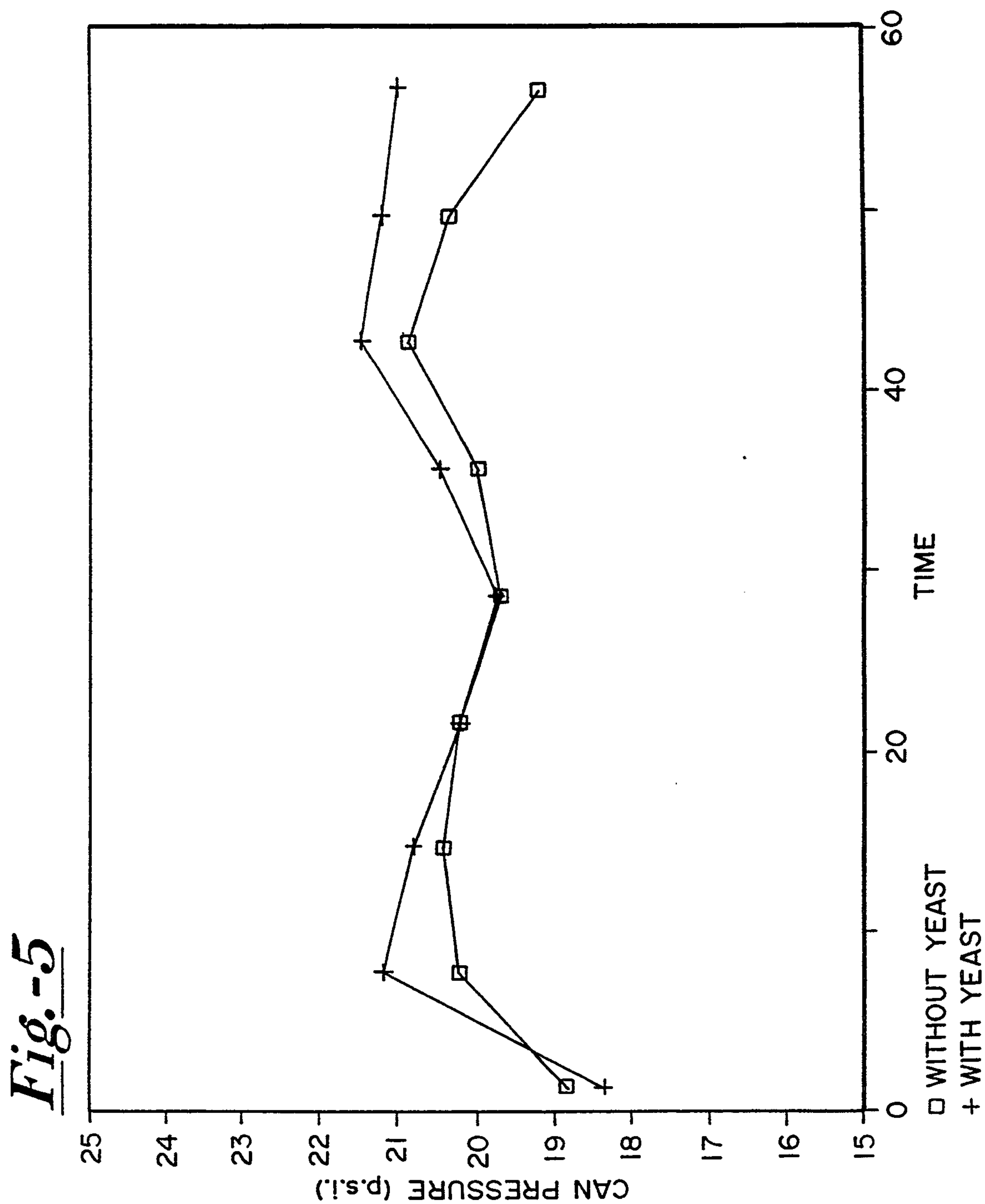
Fig.-3



4/18

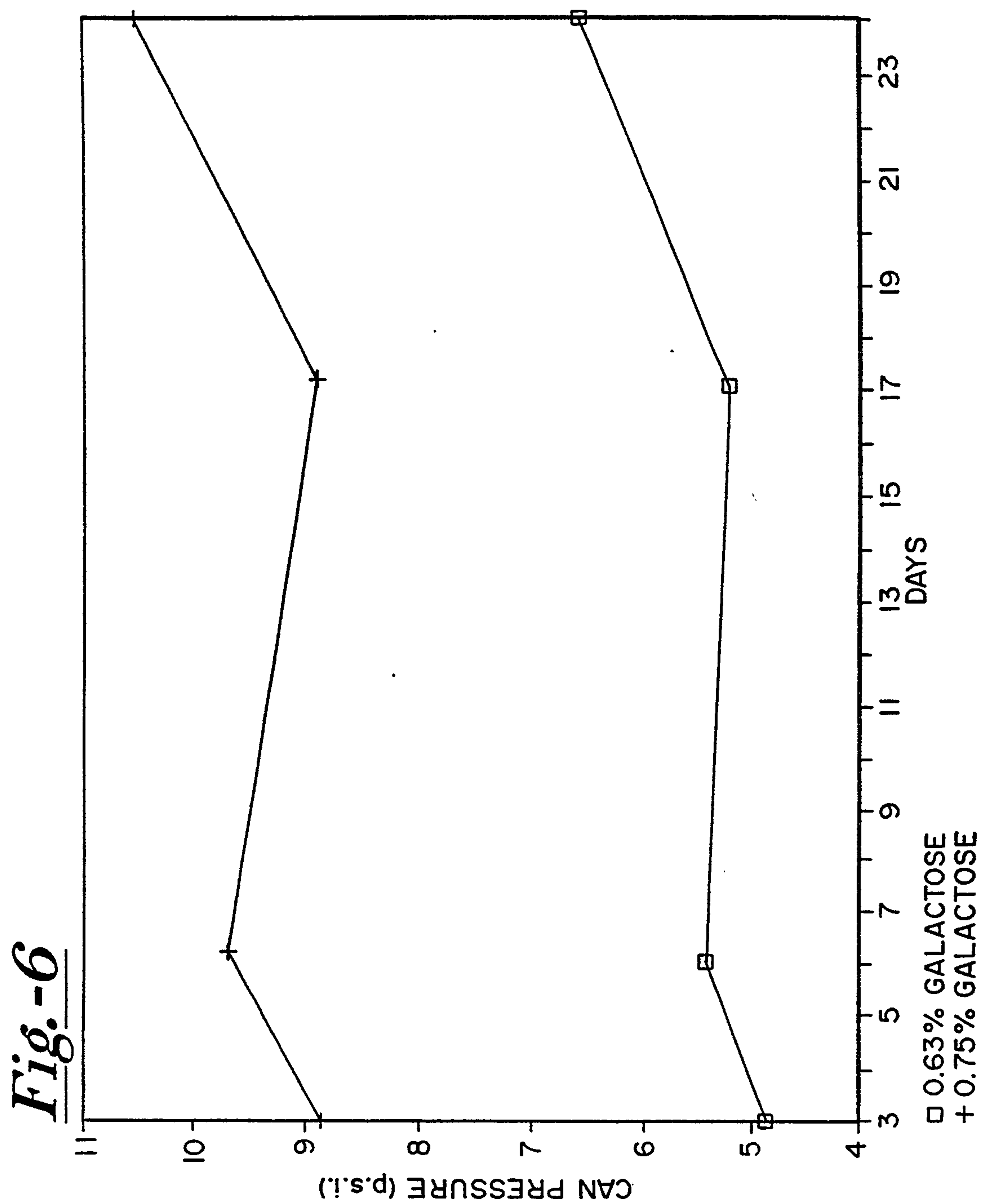
Fig.-4

5/18

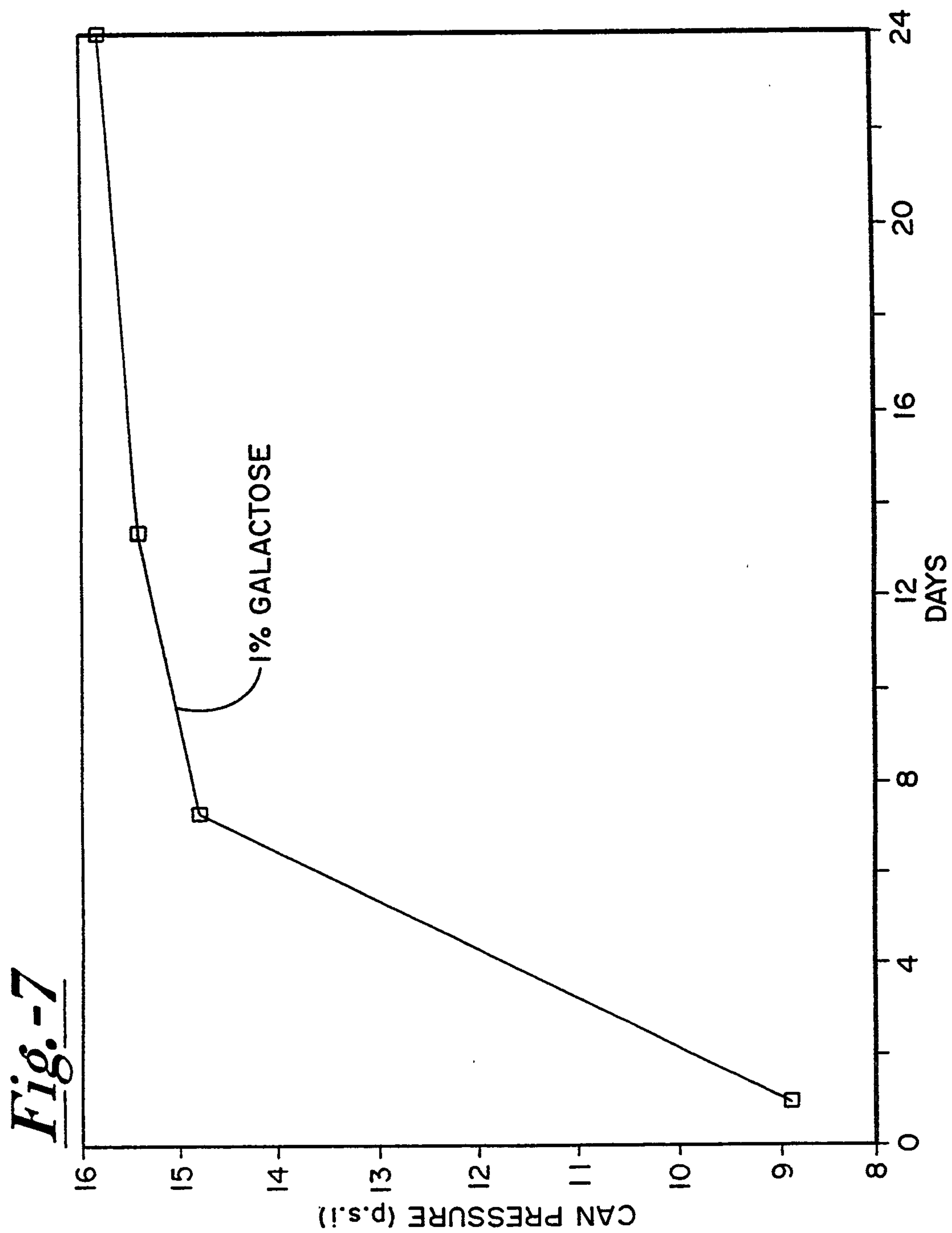


2155675

6/18



7/18



8/18

Fig.-8

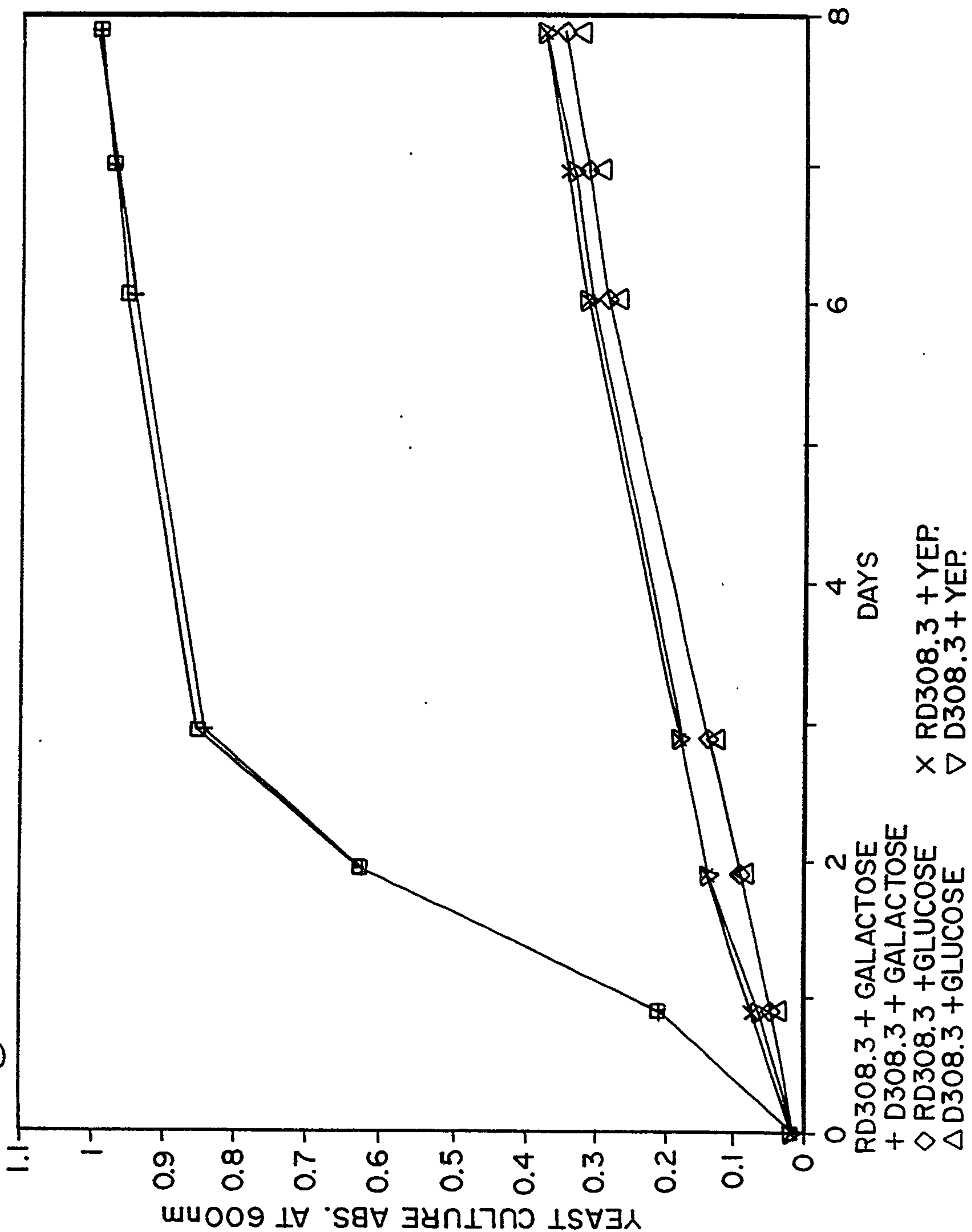
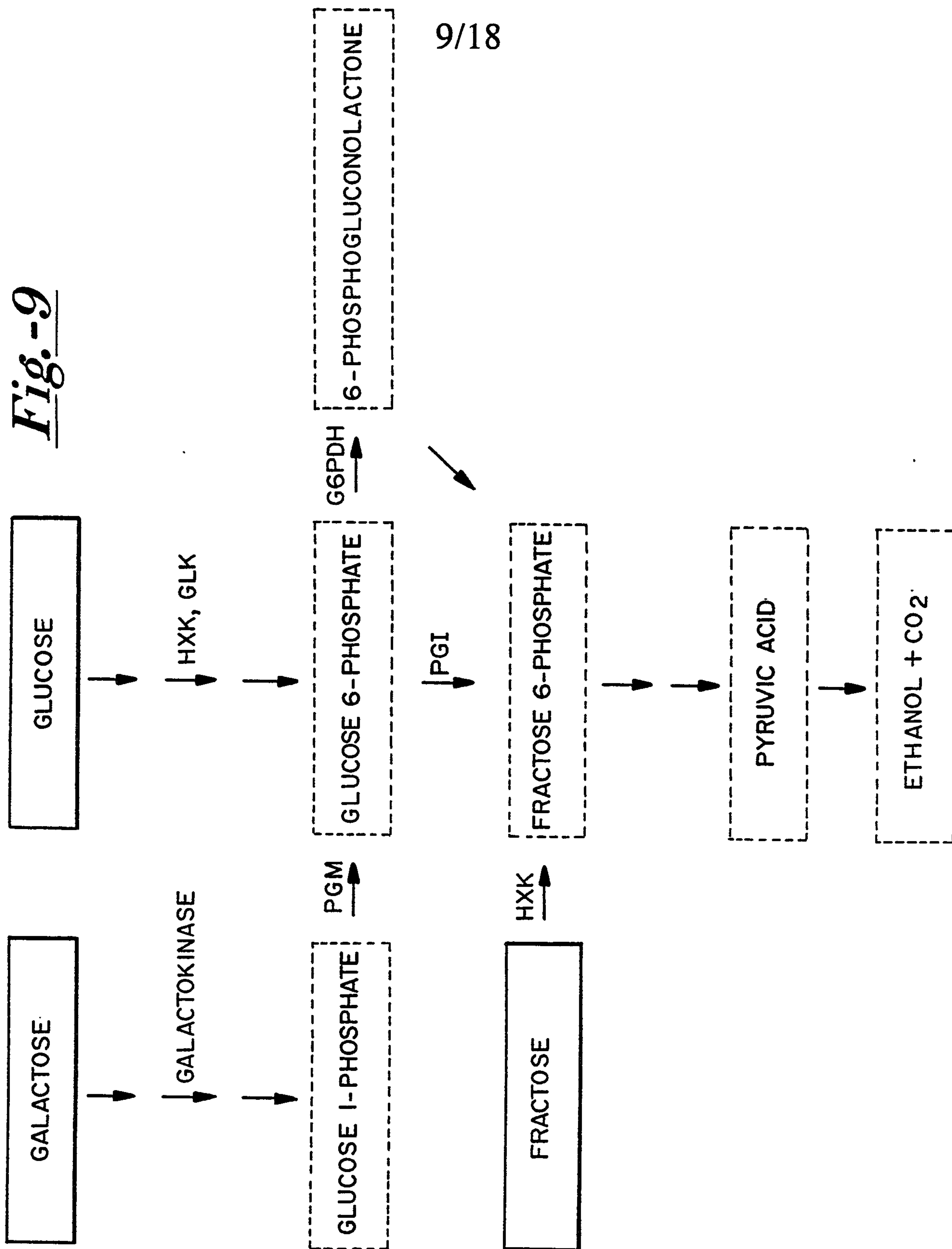
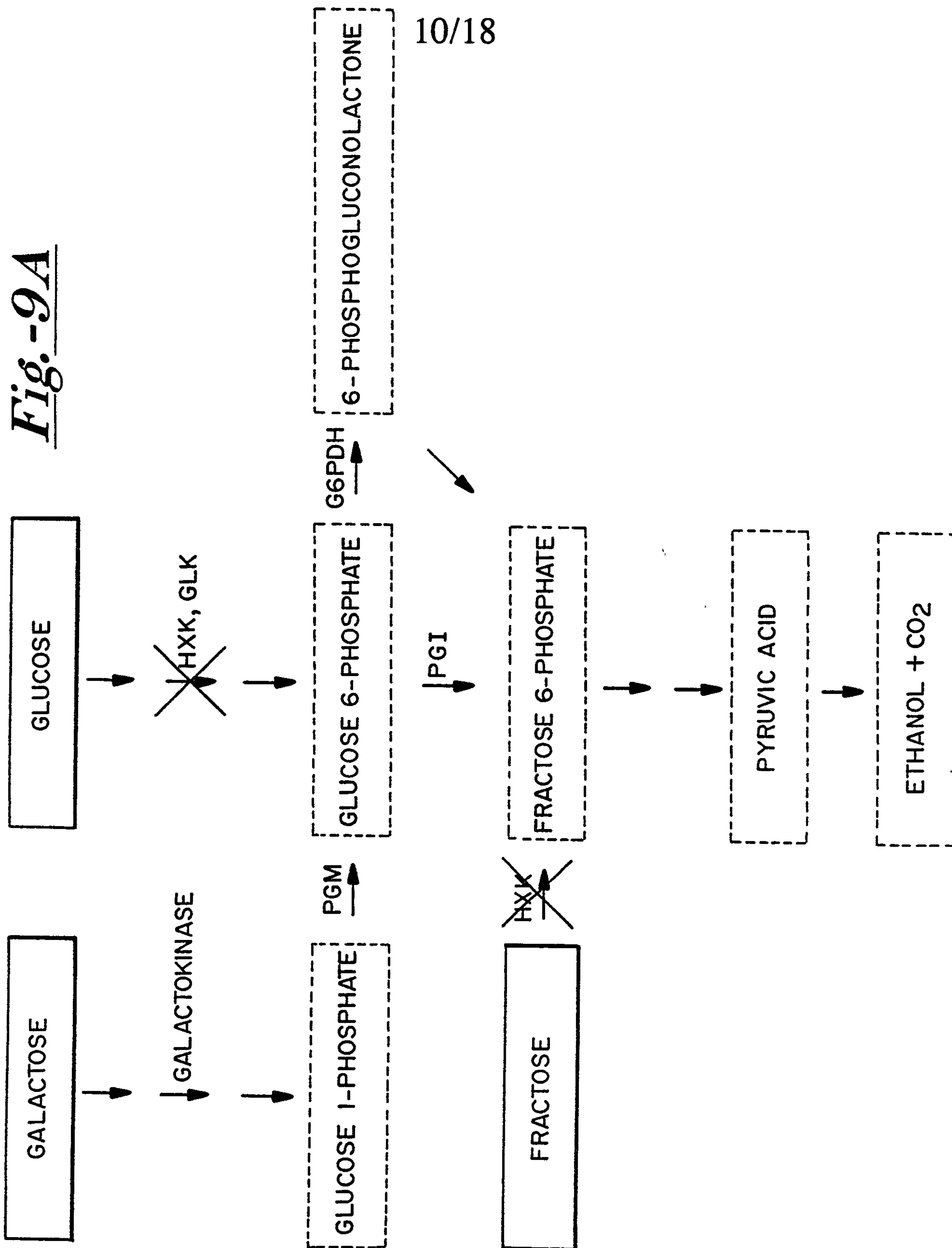


Fig.-9



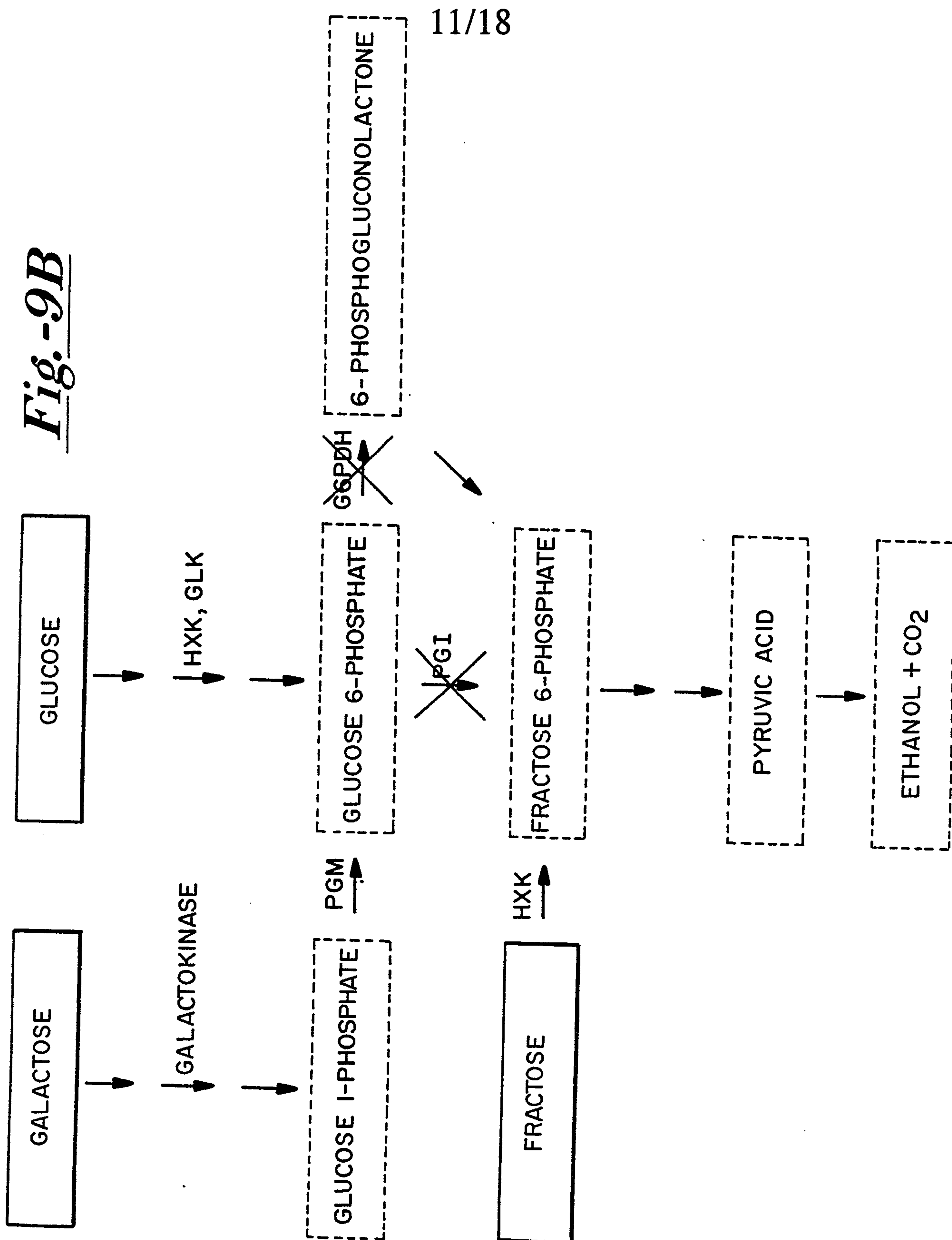
9/18

Fig.-9A



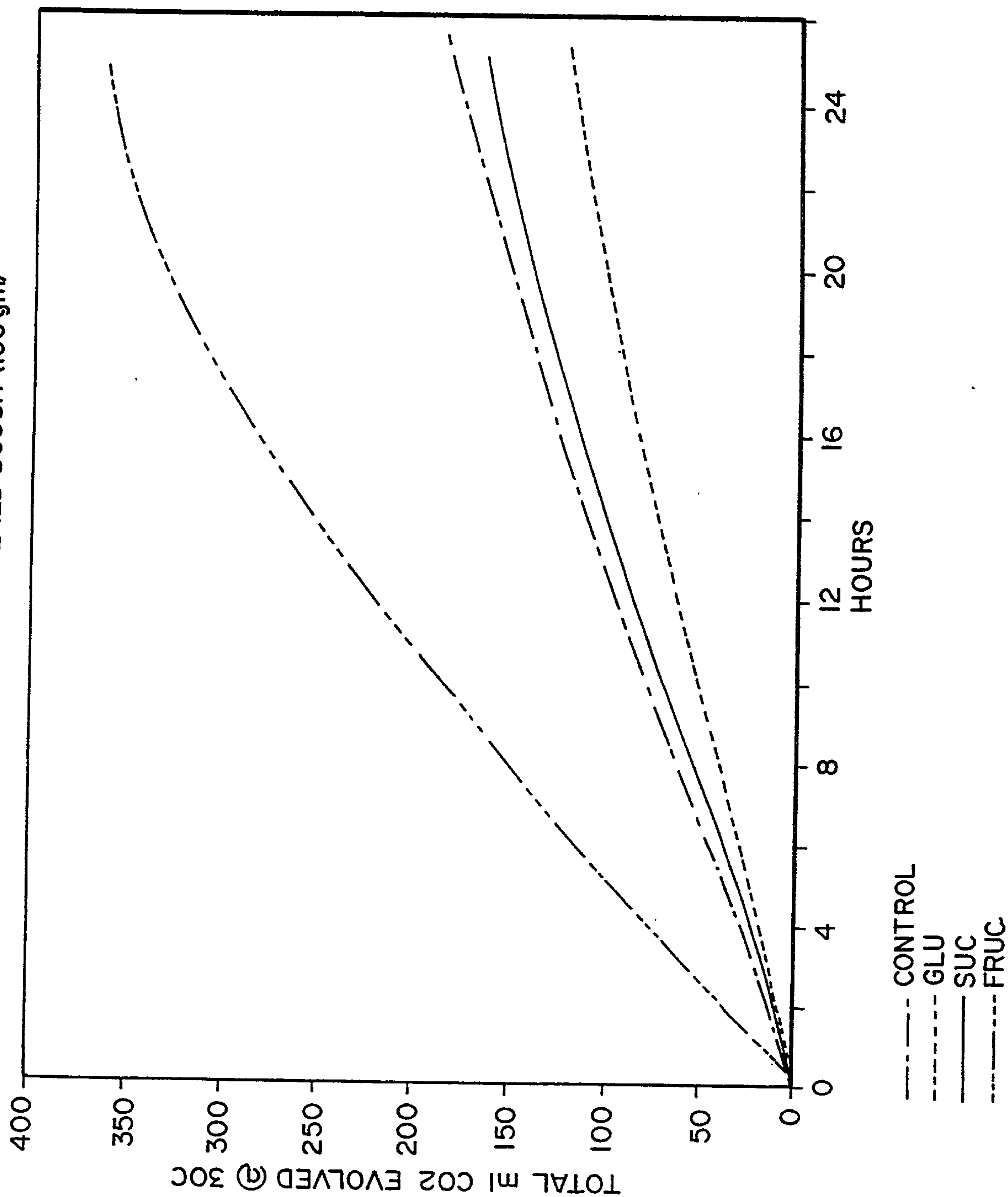
2155675

Fig. -9B



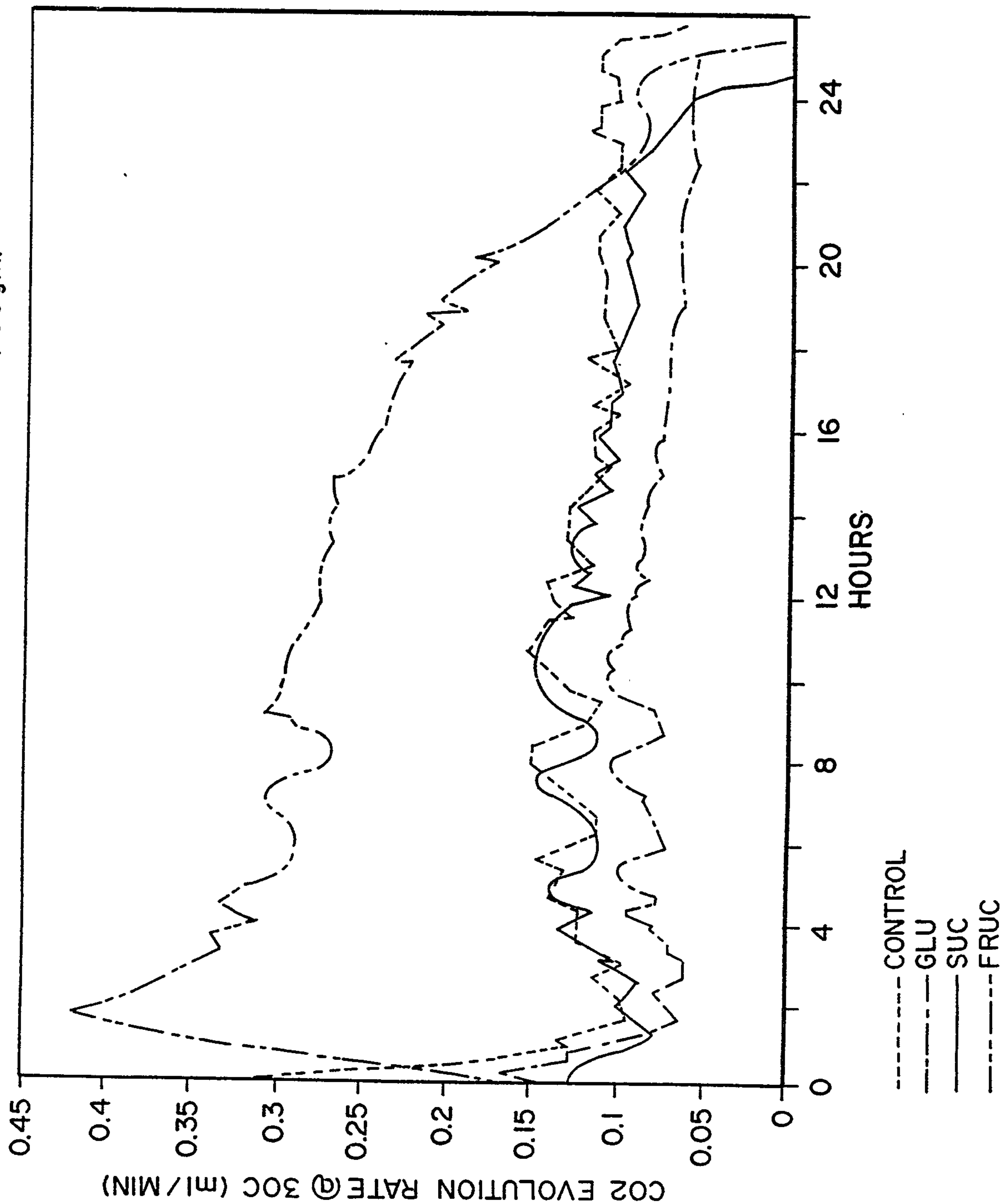
12/18

Fig. -10 EFFECT OF SUGAR ON TOTAL ml CO₂ EVOLVED
(pgi-/zwf- YEAST LEAVENED DOUGH (100 gm))

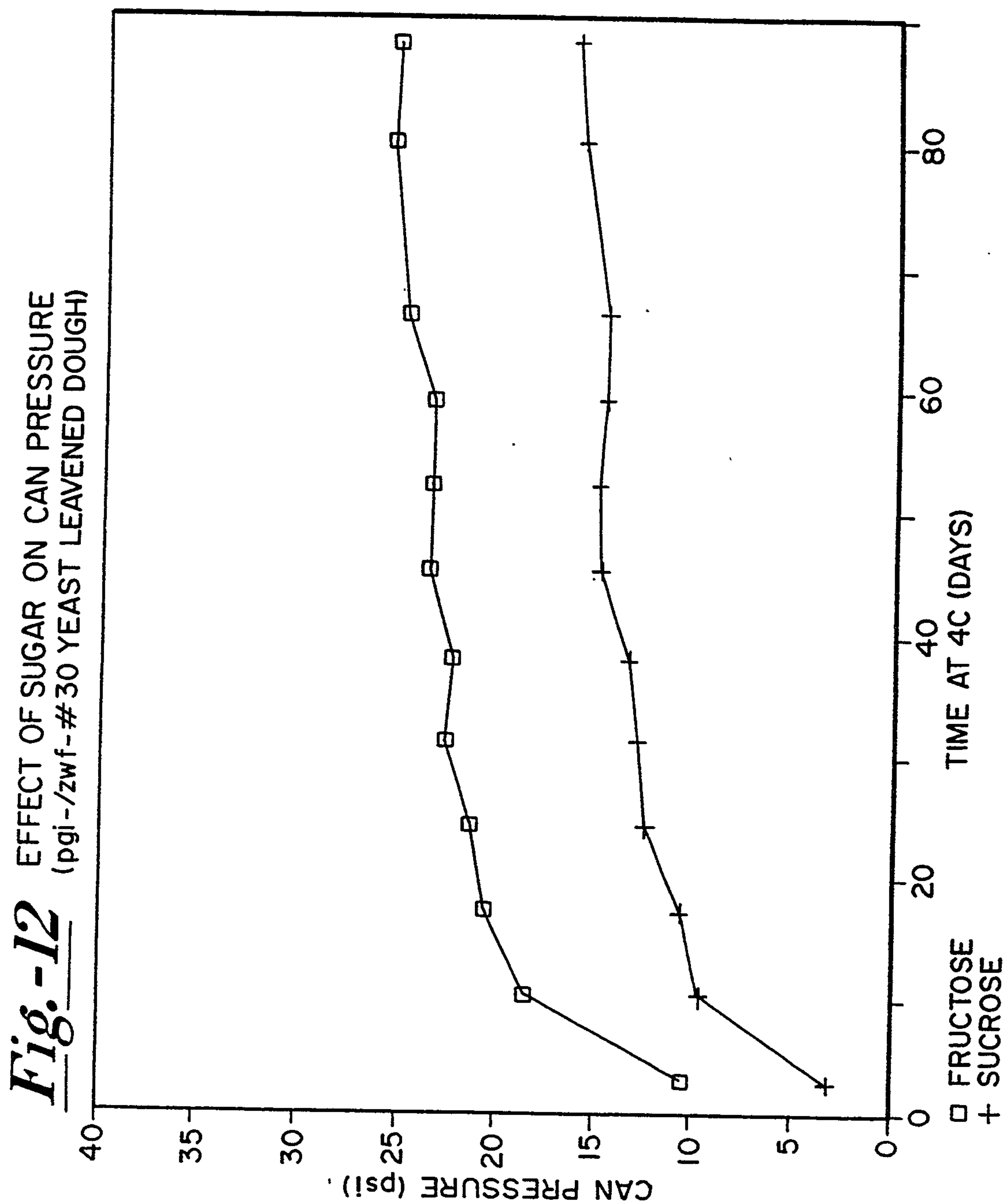


13/18

Fig. -11 EFFECT OF SUGAR ON CO₂ EVOLUTION RATE
pgi-/zwf-YEAST LEAVENED DOUGH (100gm)

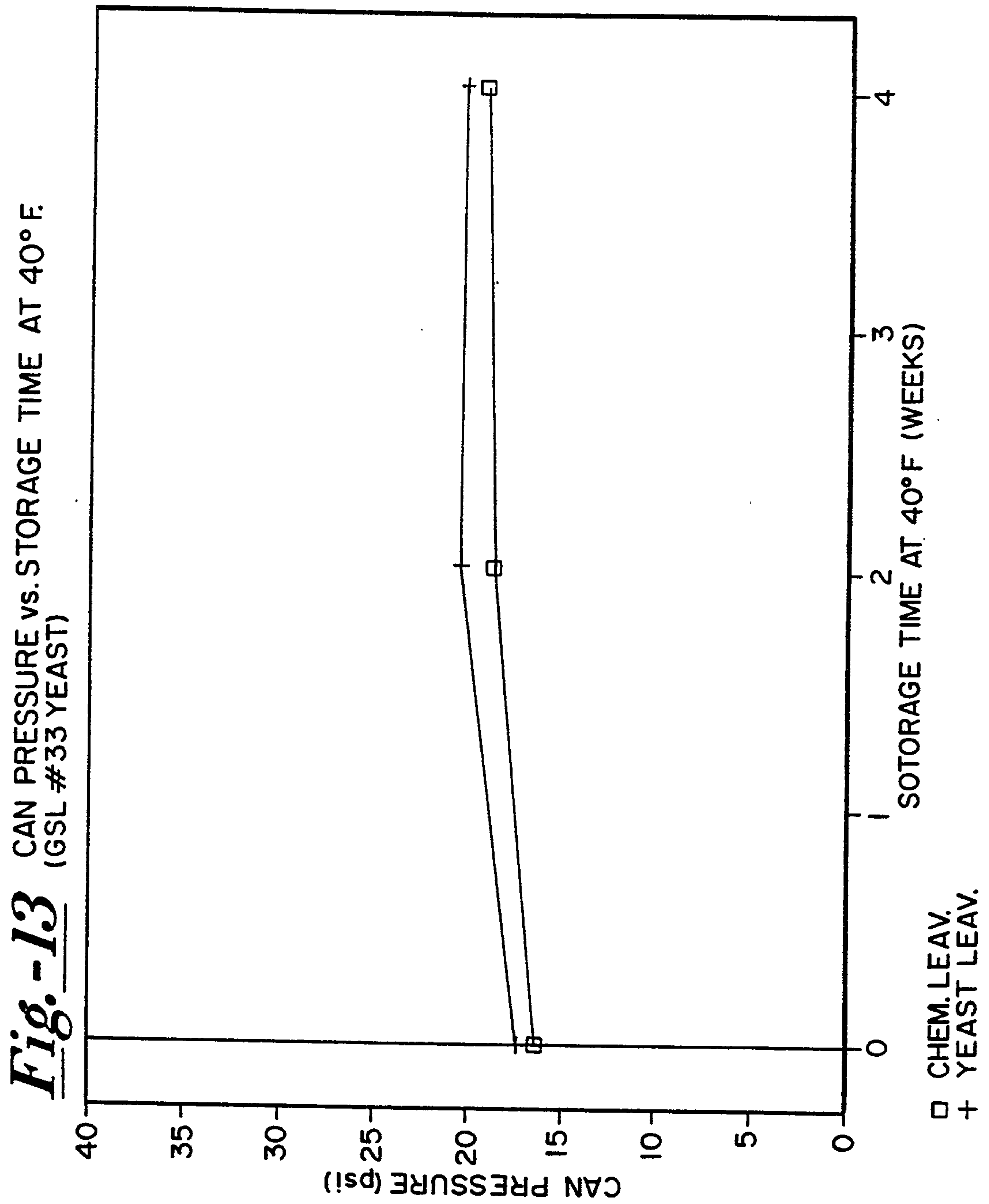


14/18



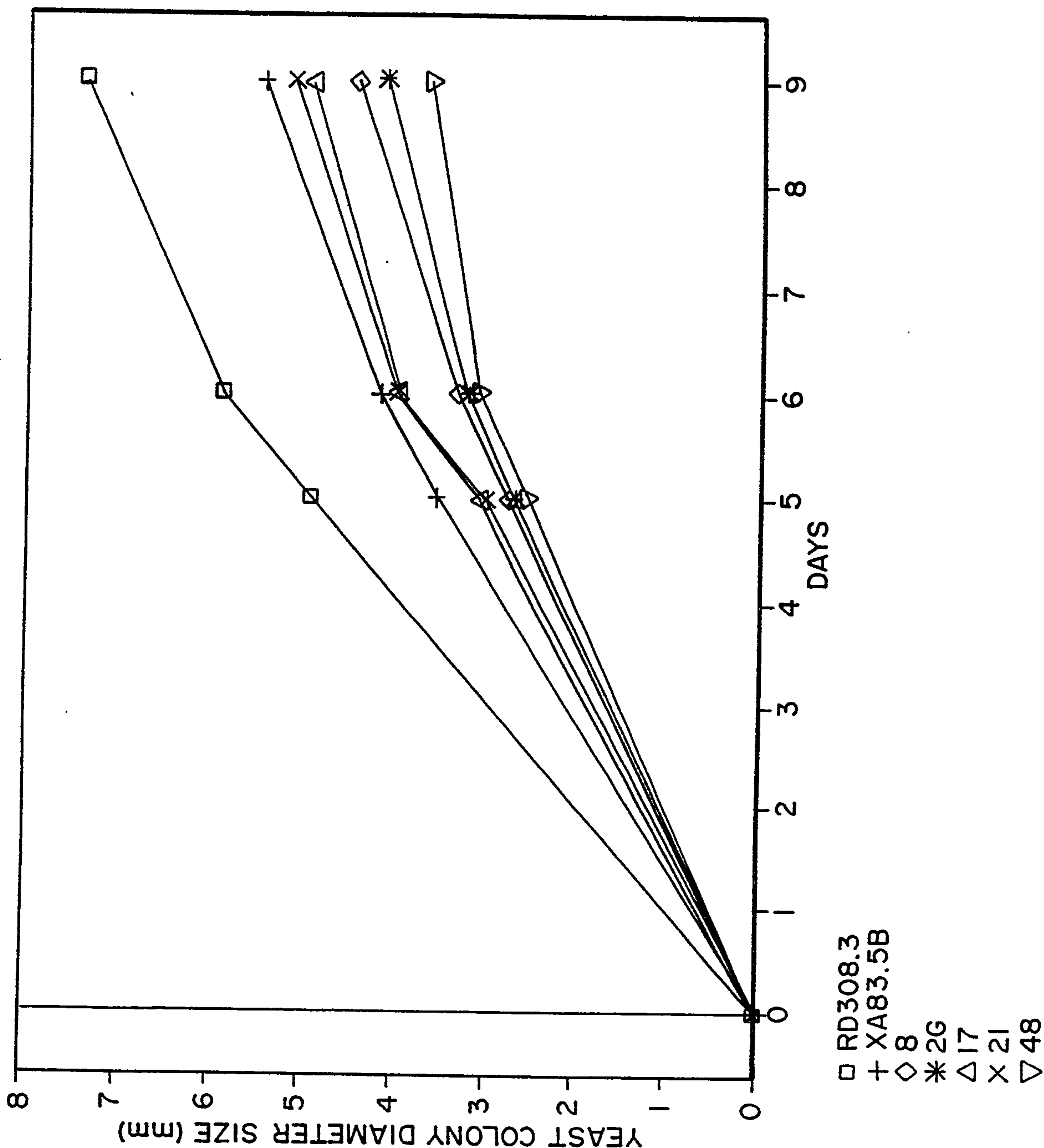
2155675

15/18

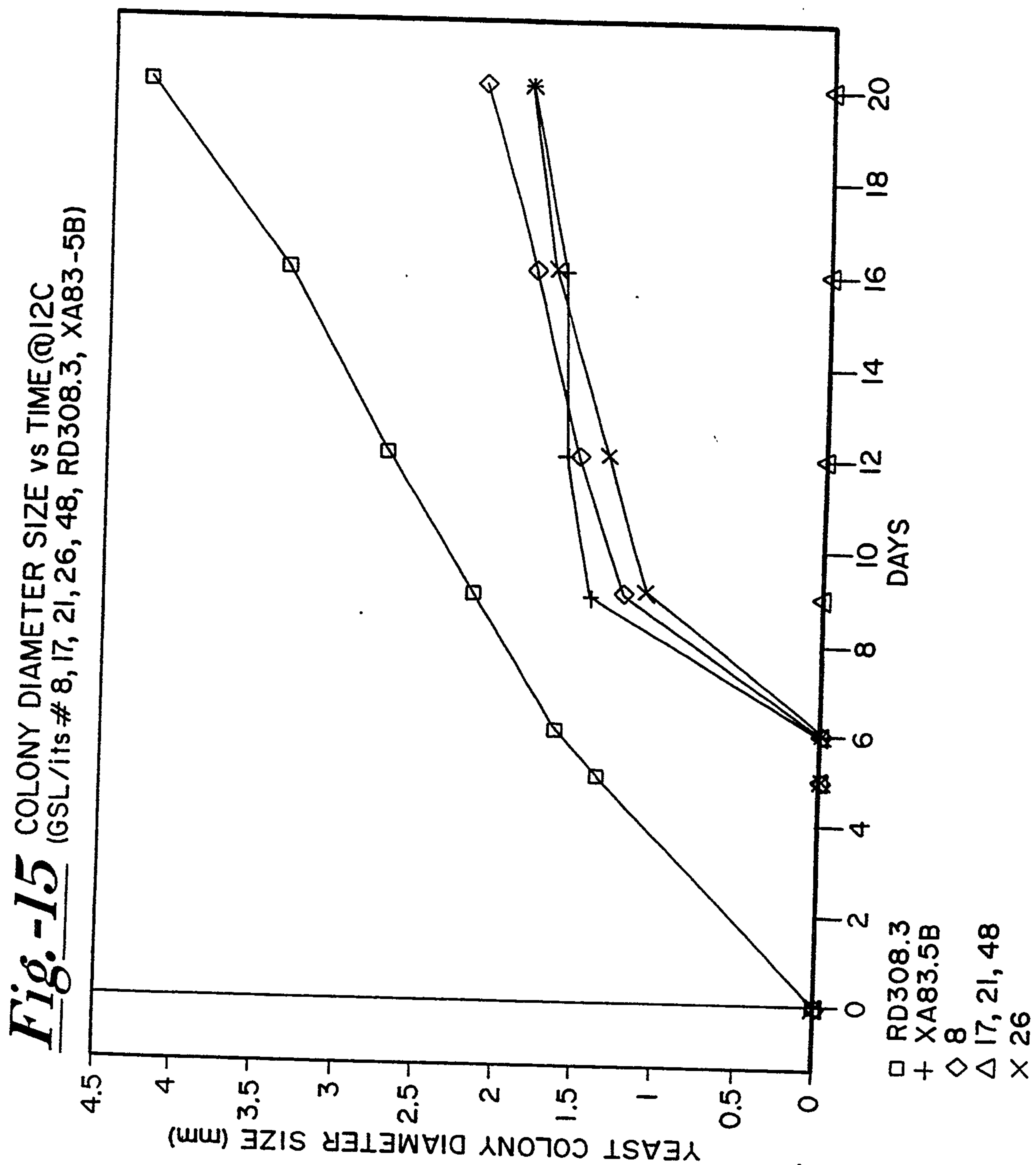


16/18

Fig. 14 COLONY DIAMETER SIZE VS TIME @ 30C
(GSL/its # 8, 17, 21, 26, 48, RD308.3, XA83-5B)



17/18



18/18

Fig. -16 ANAEROBIC cdc 19 ABS. vs TIME @ 25C
(0-4 HR INCUBATION 40C PRIOR TO STORAGE)

