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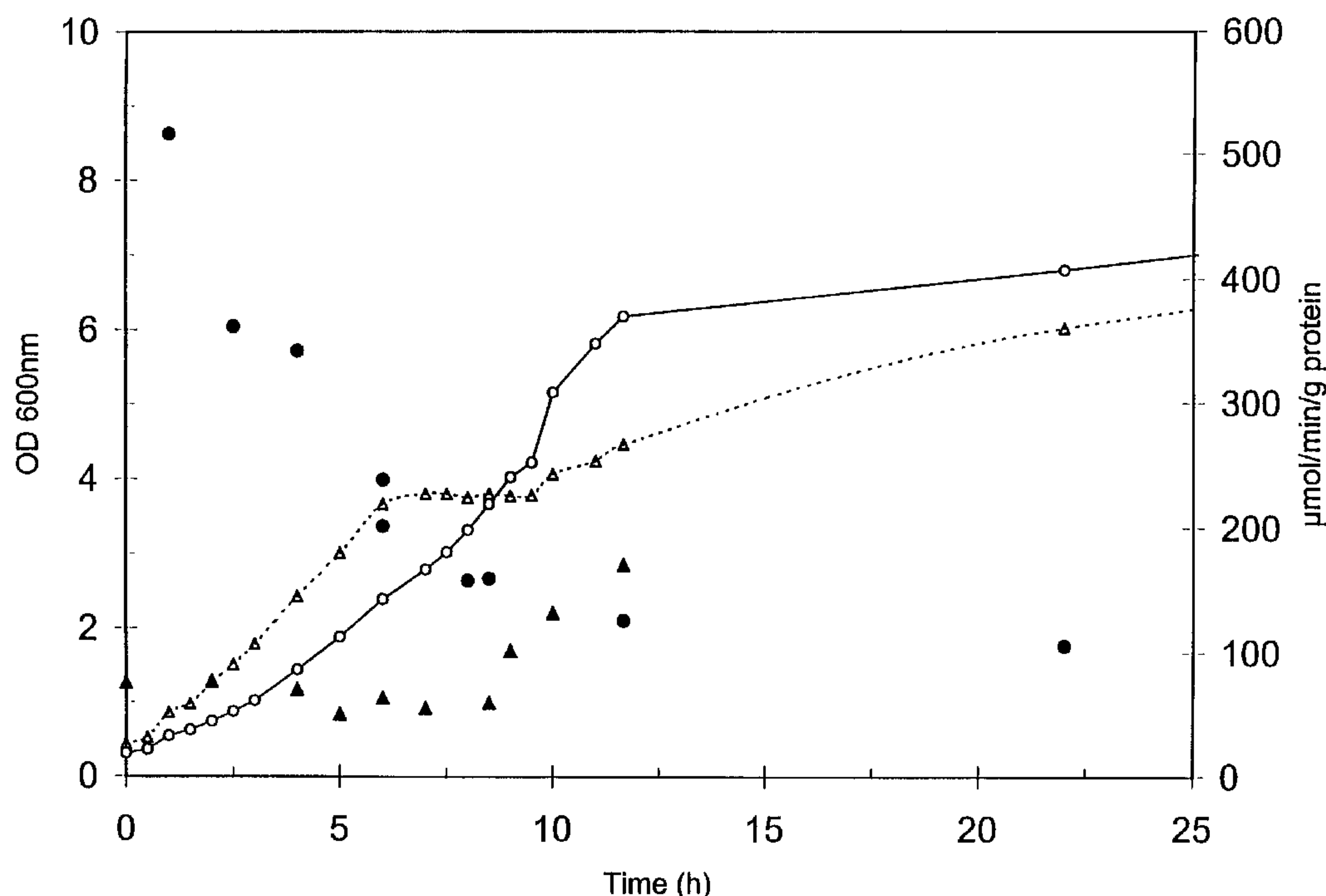
(71) Demandeur/Applicant:  
UNIVERSITEIT VAN AMSTERDAM, NL

(72) Inventeurs/Inventors:  
KRUCKEBERG, ARTHUR LEO, NL;  
DIDERICH, JASPER ANDRIES, NL;  
VAN DAM, KAREL, NL;  
RAAMSDONK, LOURINA MADELEINE, NL;  
BERDEN, JOHANNES ANTONIUS, NL

(74) Agent: OSLER, HOSKIN & HARCOURT LLP

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(54) Title: PROCESS FOR THE PRODUCTION OF YEAST BIOMASS COMPRISING FUNCTIONALLY DELETED  
HXK2 GENES



(57) Abrégé/Abstract:

The invention relates to a process for the production of yeast biomass by cultivation of a yeast culture on a commercial scale wherein the yeast culture comprises one or more functionally deleted HXK2 genes or analogues and to yeast biomass obtainable by said process. The invention also relates to compressed yeast, cream yeast or dried yeast made from said biomass. Furthermore, the invention relates to a process for the production of proteins or secondary metabolites using a yeast culture that comprises one or more functionally deleted HXK2 genes or analogues thereof.

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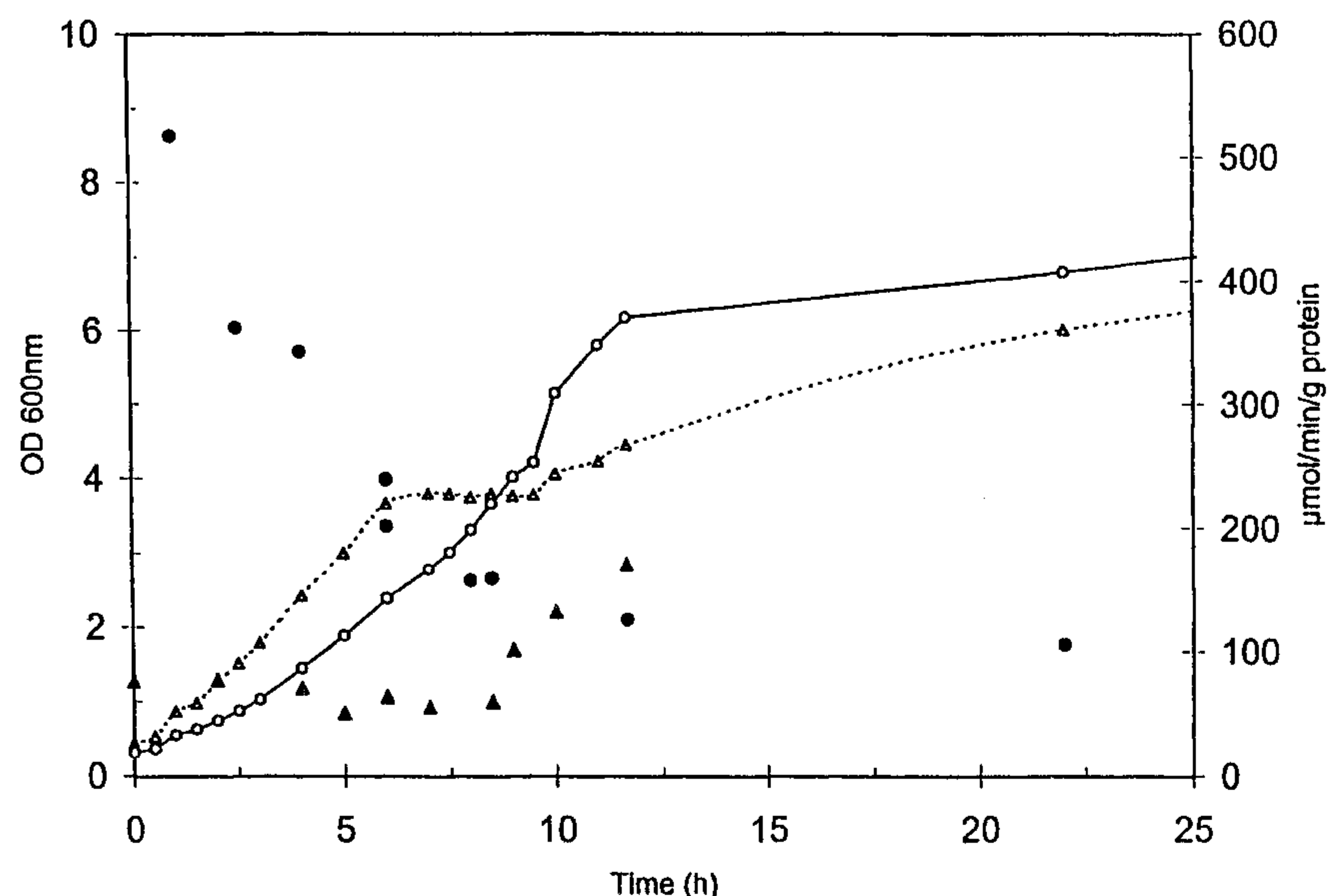
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99201162.7 13 April 1999 (13.04.99) EP(71) Applicant (for all designated States except US): UNIVER-  
SITEIT VAN AMSTERDAM [NL/NL]; Spui 21, NL-1012  
WX Amsterdam (NL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): VAN DAM, Karel  
[NL/NL]; Amsterdamsestraatweg 13, NL-1398 BR  
Muiden (NL). BERDEN, Johannes, Antonius [NL/NL];  
Koningsvaren 119, NL-1391 AH Abcoude (NL). RAAMS-  
DONK, Lourina, Madeleine [NL/NL]; Knobbelzwaansingel  
15, NL-2633 AG Nootdorp (NL). DIDERICH, Jasper,  
Andries [NL/NL]; Churchilllaan 110', NL-1078 EM  
Amsterdam (NL). KRUCKEBERG, Arthur, Leo [NL/NL];  
Oosterpark 50", NL-1092 AP Amsterdam (NL).(74) Agents: VISSER-LUIRINK, Gesina et al.; DSM N.V., DSM  
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(54) Title: PROCESS FOR THE PRODUCTION OF YEAST BIOMASS COMPRISING FUNCTIONALLY DELETED HXK2 GENES

**(57) Abstract**

The invention relates to a process for the production of yeast biomass by cultivation of a yeast culture on a commercial scale wherein the yeast culture comprises one or more functionally deleted *HXK2* genes or analogues and to yeast biomass obtainable by said process. The invention also relates to compressed yeast, cream yeast or dried yeast made from said biomass. Furthermore, the invention relates to a process for the production of proteins or secondary metabolites using a yeast culture that comprises one or more functionally deleted *HXK2* genes or analogues thereof.

## PROCESS FOR THE PRODUCTION OF YEAST BIOMASS COMPRISING FUNCTIONALLY DELETED HXK2 GENES

**FIELD OF THE INVENTION**

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The invention relates to a process for the production of yeast biomass by cultivation of a yeast culture on a commercial scale wherein the yeast culture comprises one or more functionally deleted *HXK2* genes or analogues and to yeast biomass obtainable by said process.

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**BACKGROUND OF THE INVENTION**

Yeast, in particular *Saccharomyces cerevisiae*, is used in many different biotechnological processes. These can be broadly divided into two categories depending on the metabolic state of the yeast cell.

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Production of ethanol for beverages such as wine or beer or for fuel as well as the generation of CO<sub>2</sub> during the leavening of dough for the production of bread, occur under fermentative growth conditions. Combinations of these fermentative processes are known as well. Under respiratory growth conditions, yeast biomass is produced commercially in large scale fermentation processes. The yeast biomass thus obtained can be used as the classical ingredient in the baking industry or be further processed to yeast extracts that are used as savoury flavours in the food industry. Yet another example comprises the cultivation of yeast for production of proteins or other nutrients. More recently, yeast cultures are increasingly used for the production of recombinant (heterologous) proteins, of secondary metabolites, or other modern biotechnological substances. The production of sufficient amounts of yeast biomass stands also central to, and often initiates or is prerequisite for all fermentative yeast processes and also for making enough yeast available to satisfactorily start a fermentative process.

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The two different process categories depend on the presence of a fermentative and a respiratory route for the metabolism of fermentable substrate for energy production. Fermentation is the conversion of fermentable substrate into mainly ethanol and CO<sub>2</sub>. Respiration is the conversion of substrate with the consumption of oxygen into completely oxidised end products (e.g. CO<sub>2</sub>).

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Yeast is able to adapt its cellular composition towards each mode of growth, making it a versatile, but sometimes intractable organism. These modes differ considerably in the yield of biomass on substrate. For the production of yeast biomass aerobically, respiratory growth is the preferred method. For the application of yeast often metabolic characteristics are required, that are normally obtained during fermentative growth conditions. Both modes need to be dealt with satisfactorily to arrive at optimised production. For example, both types of growth are necessary for the baking industry.

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One challenge in baker's yeast production is to optimise the efficiency of conversion of assimilable carbon into biomass during the production phase, while ensuring that the yeast will have good leavening characteristics in the dough. Also important is the maintenance of the leavening characteristics during storage. Other respiratory yeast cultivation processes have similar complicated demands for balance between biomass production and optimal end-use characteristics (flavour, heterologous protein production, etc.).

A fermentative mode of growth is obtained under anaerobic conditions, in which the metabolic balance does not allow the complete oxidation of the substrate without the supply of oxygen. Also, many yeast strains show at aerobic conditions fermentative activity parallel to respiratory metabolism - the so-called respiro-fermentative mode - if fermentable substrates are present in excess. Pure respiratory growth is obtained at aerobic conditions and at non-repressive substrate concentrations. The onset of fermentative activity, in addition to pure respiratory activity, marks the critical growth rate that correlates with a critical concentration of fermentative substrate. This critical growth rate is an expression of the respiratory capacity of yeast.

High concentrations of fermentable substrates, especially glucose, repress a number of metabolic functions, including the respiratory capacity, a process known as glucose repression. Glucose repression results in a shift towards the fermentative mode. For an efficient production of yeast biomass the fermentative growth mode must be avoided, meaning that both anaerobic conditions (under-aeration) and excess of substrate must be avoided.

Glucose repression is seen with a yeast such as *Saccharomyces cerevisiae* which uses glucose preferentially to all other sources of carbon and energy. This preference is exercised by repression of genes necessary for the utilisation of other carbon sources when glucose is available (Johnston and Carlson, 1992.). Genes required for utilisation of sucrose, maltose and galactose are glucose-repressed. Moreover, a number of genes required for synthesis of mitochondrial enzymes involved in respiration, hence for utilisation of gluconeogenic carbon sources, are also repressed by glucose (Gancedo, 1998).

Yeasts comprising a functionally deleted *HXK2* gene or functional analogue thereof exhibit no functional glucose repression, in particular when grown aerobically on glucose (Ma & Botstein, 1994). Said gene (*HXK2*) encodes the enzyme hexokinase 2 (Hxk2). Hexokinase 2 (Hxk2) is one of three enzymes that are able to phosphorylate glucose in *S. cerevisiae* (Lobo and Maitra, 1977). Glucose phosphorylation is the first intracellular step in the glycolytic pathway leading to pyruvate (which is the metabolite that occurs at the branch point between the fermentative and respiratory routes of dissimilation). Two other enzymes, hexokinase 1 and glucokinase, are partially

redundant to Hxk2; they have different kinetic characteristics and are normally expressed at different phases of growth (for a review see Gancedo, 1998.).

5 Mutant strains lacking functional Hxk2 are able to grow on glucose. In a study on a large number of *HXK2* mutations, strains carrying different point mutations revealed growth rates ranging from the wildtype rate to undetectable (these strains carried null mutations in *HXK1* as well). These same authors found that a null mutation in *HXK2* alone (i.e. wildtype *HXK1* was present) led to a decrease in growth rate from 17 to 52%, depending on the growth medium (Ma *et al.*, 1989a). Surprisingly, these authors did not find a clear correlation between the residual hexokinase activity of the mutant enzymes  
10 *in vitro* and the growth rates of strains expressing those enzymes as their sole sugar-phosphorylating activity *in vivo* (Ma *et al.*, 1989b). It has been shown that the function of Hxk2 in hexose phosphorylation is formally distinct from its function in regulating the glucose repression status of the cell (Entian & Frohlich, 1984). The glucose concentration in the environment is converted into a signal that affects the regulatory  
15 status of Hxk2.

The production of microbial biomass by fermentation can be carried out in several ways: batch mode, fed-batch mode, continuous culture, a combination of batch and fed-batch mode, repeated fed-batch mode or any other combination.

20 Batch mode fermentation processes are characterised by the fact that the growth medium containing the substrates is added to the fermentor followed by inoculation of the medium with a preculture of the micro-organism. Oxygen can be supplied by aeration of the growth medium. After a certain time, one or several nutrients are depleted and a maximal cell density is obtained after which the biomass can be  
25 collected. The initial excess of fermentable substrate and possible under-aeration are not favourable for the efficient production of yeast biomass. The culture rapidly changes to fermentative growth and starts producing ethanol and CO<sub>2</sub> which decreases the yield and the productivity of the process.

Fed-batch fermentation processes are characterised by the fact that the  
30 complete medium containing the substrates is not added at the onset of the fermentation process, but part of the medium is added as a continuous or intermittent feed. For instance, glucose and other essential nutrients are fed to the culture in a growth-limiting fashion. The feed rate is adjusted to a rate that does not exceed the oxygen transfer capacity of the fermentor. For example, baker's yeast production usually  
35 uses molasses as a substrate, which has a high saccharose concentration. The saccharose is hydrolysed by the enzyme invertase into glucose and fructose and, in order to maintain efficient conversion of substrate into biomass, the production process is carried out aerobically by feeding at a rate below the maximum oxygen transfer capacity. Irregularities in the mixing may locally cause high concentrations of the sugar

substrate with concomitant production of ethanol and repressive effects on the respiratory capacity. Consumption of the ethanol later on at non repressive conditions, still results in a loss of yield due to the properties of the metabolic pathways.

5 Production processes under continuous culture conditions resemble the fed-batch processes except that biomass-containing effluent is removed from the fermentation continuously or intermittently.

Production processes may also be carried out by a combination of different modes of batch and fed-batch and continuous cultivation.

10 In general, the costs of yeast biomass production depend heavily on the yield, expressed as amount of biomass per amount of substrate and the productivity of the fermentor. Ethanol production by the yeast decreases the yield and therefore increases the costs. The need to keep the growth rate below a critical value in order to avoid ethanol formation reduces the productivity. Therefore, yield and productivity need to be carefully balanced in order to obtain the lowest production costs, whereby the  
15 equilibrium point is dependent on the metabolic properties of yeast. The sensitivity of yeast strains for glucose repression shifts the aforementioned equilibrium point towards lower productivity. Further demands on the application properties of the biomass product aggravate the establishment of the equilibrium point and the process is still far from being optimised, despite years of biotechnological and genetic development. Indeed, the  
20 status of research & development on baker's yeast strains has not changed much since 1971 when Harrison (1971) wrote "as a number of properties besides fermentative activity are required in baker's yeast in different degrees, some being antagonistic, commercial yeasts provide the balance considered to be the best compromise". Little progress has been reported in improvement of baker's yeast production via increase of  
25 the resistance of the strains to glucose repression.

The present invention provides more cost effective, commercial processes for the production of yeast biomass, by using yeast cultures that are less or not sensitive to glucose repression.

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### DESCRIPTION OF THE FIGURES

30 Figure 1 - Influence on growth characteristics of the *HXK2* deletion. The wildtype strain (o) and the Hxk2 deficient mutant strain (squares) were grown on YNB, 2% glucose. Growth was monitored by measuring the optical density at 600 nm.

Figure 2 - Glucose consumption during growth on glucose. The wild type strain (o) and the Hxk2 deficient mutant strain (squares) were grown on YNB, 2% glucose

35 Figure 3 - Ethanol production during growth on glucose. The wild type strain (o) and the Hxk2 deficient mutant strain (squares) were grown on YNB, 2% glucose

Figure 4 - Oxygen consumption capacity during growth on glucose. The wildtype strain ( $\Delta$ , open and closed) and the Hxk2 deficient mutant strain (o, open and closed) were grown on YNB, 2% glucose. During growth (OD 600 nm, open symbols) samples

were taken and analysed for oxygen consumption capacity ( $\mu\text{mole}/\text{min}/\text{g}$  protein, closed symbols).

### **SUMMARY OF THE INVENTION**

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In one aspect, the invention provides a process for the production of yeast biomass characterised by cultivation of a yeast culture on a commercial scale, wherein the yeast culture comprises one or more functionally deleted *HXK2* genes or analogues thereof, and by growing said yeast culture aerobically in a growth medium containing one or more fermentable carbon sources

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In another aspect, the invention provides a process for the production of proteins or secondary metabolites using a yeast culture that comprises one or more functionally deleted *HXK2* genes or analogues thereof by cultivating the yeast culture according to a process as provided by the invention.

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In a further aspect, the invention provides yeast biomass or a substance derived thereof obtainable by a process as provided by the invention.

In another aspect, the invention provides compressed yeast, cream yeast or dried yeast made from yeast biomass as provided by the invention.

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### **DETAILED DESCRIPTION OF THE INVENTION**

Yeast biomass is defined as accumulated yeast or yeast cells, or compounds or substances derived from yeast cells, which can subsequently be used for a great many purposes such as ingredient for dough rising (in the form of compressed yeast, cream yeast or dried yeast), as food component in for example processed foods, as a source of (heterologous) proteins or peptides, as a source of nutrients such as amino acids, vitamins, as a source of secondary metabolites, as source of pharmaceutical ingredients.

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A functionally deleted *HXK2* gene is defined as a complete deletion of the gene or a gene carrying mutations made by substitution, insertion or deletion that result in a functionally inactive Hxk2. Analogues of *HXK2*-genes are genes encoding proteins that exhibit the same function as Hxk2 with respect to sugar utilisation and regulatory properties.

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Cultivation on a commercial scale is defined herein as a fermentation process carried out in a fermentor with a volume of more than 0.5 litre.

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In one aspect, the invention provides a process for the production of yeast biomass characterised by cultivation of a yeast culture on a commercial scale, wherein the yeast culture comprises one or more functionally deleted *HXK2* genes or analogues

thereof, and by growing said yeast culture aerobically in a growth medium containing one or more fermentable carbon sources. Carbon sources are preferably sugars and more preferably belong to the group consisting of glucose, fructose, maltose, saccharose, galactose and raffinose. The yeast preferably belongs to the genus  
5 *Saccharomyces* or *Kluyveromyces*. More preferred species are *Saccharomyces cerevisiae*, *Kluyveromyces lactis* or *Kluyveromyces marxianus*. Yeast cultures in which the glucose repression pathway is functionally deleted, such as those carrying a functionally deleted *HXK2* gene or analogue thereof, have great advantages over classical strains in the production of yeast biomass, specifically under aerobic  
10 conditions, because in commercial scale biomass production processes, they improve the productivity of said processes and maintain or improve the yield. Furthermore, it was found that, in contrast to yeast cultures that are traditionally used for biomass production, yeast cultures comprising one or more functionally deleted *HXK2* genes or analogues maintain their leavening capacity.

15 In particular, said yeast culture showing no or diminished glucose repression when grown aerobically in the presence of glucose, can advantageously be applied in batch production of inoculation material or in the start up phase of a production culture. Said cultures are not hampered by for example the ethanol that is inadvertently produced at those conditions. Also, the higher respiratory capacity of said yeast cultures  
20 at these conditions allows for higher growth rates in the adaptation time after non repressive conditions are introduced. These cultures immediately show respiratory growth when grown aerobically on glucose, not being hampered by glucose induced repression of enzyme synthesis needed for said respiratory growth, as for example characterised by an increased oxygen consumption capacity during initial growth on  
25 glucose and exhibit higher growth rates in the period thereafter. Reduction of the ethanol production results in improved yield and the application of higher growth rates results in a decreased fermentation time and increased productivity, both resulting in cost price reduction. The application of glucose repression resistant strains results also in a reduced ethanol turnover and in apparently higher critical growth rates when  
30 irregularities in the mixing lead to locally cause high concentrations of the sugar substrate with concomitant production of ethanol and repressive effects on the respiratory capacity.

For those applications where the use of recombinant yeast strains is not desired, mutants with defects in the glucose repression pathway can be isolated through  
35 classical mutagenesis and selection. One way of screening for such mutants is by selection for rapid growth on sucrose or ethanol and glycerol of a mutagenised population of cells after preculture in a medium with a high glucose concentration. This procedure is, when so desired, repeated serially in order to optimise the desired phenotype. These thus selected yeast variants can advantageously be used in a culture

as provided by the invention. Another selection strategy is to use 'gratuitous repressors', i.e. glucose analogues that are not themselves metabolisable (e.g. 2-deoxyglucose, 5-thiogluco-  
10 5e, etc.). Mutagenised strains are selected for growth on media containing sucrose or ethanol and glycerol and a gratuitous repressor.

5 In a second aspect of the invention, the production process of yeast biomass by fermentation is carried out in batch mode whereby the growth medium initially contains the fermentable carbon source at a non-growth-limiting concentration. Preferably the concentration of said carbon source is more than 1 mmole per litre, more preferably more than 5 mmole per litre, most preferably > 10 mmole per litre.

10 In a third aspect of the invention, the production process of yeast biomass by fermentation is carried out in a fed-batch mode whereby a component of the growth medium is growth limiting and supplied to the fermentation medium in the feed stream. This growth limiting component can be a nitrogen source such as ammonia, or a carbon source such as the sugars mentioned above.

15 In a fourth aspect, the production process of yeast biomass by fermentation is carried out as a continuous culture and a component of the growth medium is growth limiting. This growth limiting component can be a nitrogen source such as ammonia, or a carbon source such as the sugars mentioned above.

20 In a fifth aspect, the production process of yeast biomass by fermentation comprises the following steps. First a fermentation in batch mode is carried out as described above. The carbon source at initial non-growth limiting concentration is consumed with concomitant accumulation of yeast biomass. After consumption of the carbon source, the fermentation process is changed to fed-batch mode whereby in this case the carbon source is growth limiting and supplied to the fermentation medium in  
25 the feed stream.

In a sixth aspect, the invention provides yeast biomass or a substance derived thereof obtainable by the production processes of the invention. The yeast preferably belongs to the genus *Saccharomyces* or *Kluyveromyces*. More preferred species are *Saccharomyces cerevisiae*, *Kluyveromyces lactis* or *Kluyveromyces marxianus*. Said  
30 biomass in general comprises no or only little ethanol or other byproducts of fermentative growth, despite that it has been produced on a culture medium comprising substantial amounts of glucose.

In a seventh aspect, the invention provides a process for the production of proteins or secondary metabolites using a yeast culture that comprises one or more  
35 functionally deleted *HXK2* genes or analogues thereof by cultivating the yeast by the production processes of the invention. For example, the yeast comprises a nucleic acid that encodes a desired proteinaceous substance (peptide, polypeptide or protein optionally comprising additional non-protein groups) to be harvested or an enzyme providing for additional enzymatic reaction in said yeast. Such an enzyme (a

proteinaceous substance in itself) is particularly useful to provide for additional modifications of a desired product, such as a protein or secondary metabolite to be harvested from said yeast. Or the yeast, in addition to comprising one or more functionally deleted HXK2 genes also comprise a heterologous nucleic acid, is used to produce any product, such as a heterologous protein or a secondary metabolite with a greatly enhanced conversion efficiency as a result of the fact that the sugar substrate is oxidised without wasteful production of ethanol.

In an eight aspect, the invention provides compressed yeast, cream yeast or dried yeast made from said yeast biomass that is obtainable by the production processes of the invention. The yeast preferably belongs to the genus *Saccharomyces* or *Kluyveromyces*. More preferred species are *Saccharomyces cerevisiae*, *Kluyveromyces lactis* or *Kluyveromyces marxianus*.

### **EXAMPLE 1**

#### **Effects of a HXK2-deletion on growth and glucose metabolism of *Saccharomyces cerevisiae* in batch culture**

##### **1.1 Construction of a yeast strain without HXK2**

The *Saccharomyces cerevisiae* wildtype strain CEN.PK 113-7D (MATa, MAL2-8c SUC2) which was kindly provided by Dr. P. Kötter (Frankfurt, Germany) was used for the construction of a mutant strain which lacks the HXK2 gene. Genetic modification of yeast, i.e. deleting or mutating one or more parts of the nucleic acid of a yeast genome, or providing a yeast genome with additional heterologous nucleic acid is in itself a skill known in the art. Here, the HXK2 gene was functionally deleted by PCR-based gene disruption and replaced by a kanMX-marker. Briefly, the PCR primers AK53 (SEQ ID 1) and AK54 (SEQ ID 2) were used in a polymerase chain reaction with plasmid pFA6A-kanMX4 (Wach *et al.*, 1994), deoxynucleotides, and Expand™ DNA polymerase (Boehringer Mannheim) to produce an HXK2-specific kanMX gene replacement module. This DNA module was transformed into the yeast strain, and transformants were selected for resistance to the antibiotic G418. Resistant isolates were verified to have replacements of HXK2 with kanMX by diagnostic PCR.

##### **1.2 Physiological consequences of the HXK2 deletion**

A wildtype yeast strain and a Hxk2 functional deletion strain were grown in batch cultures in a fermentor with a volume of 1 litre on minimal medium containing 2% glucose. The growth of both strains was monitored by measuring the optical density of the cultures at 600nm. During growth, samples were taken for glucose and ethanol

determination. Samples were taken for determination of oxygen consumption rates as an indicator of respiratory activity. The characteristics of glucose transport were determined at certain key points during growth.

### 1.3 Growth characteristics

Functionally deleting the *HXK2* gene has a remarkable effect on the growth characteristics on minimal glucose medium (Figure 1). At low optical densities both strains showed a comparable growth rate. At a higher optical density (approximately 4) the wildtype showed a lag in growth. In contrast the mutant strain did not show a lag at that same optical density. In the *HXK2* mutant strain the phenomenon of diauxic shift is absent or occurs at a higher optical density. In the wild-type yeast strain three phases of growth can be distinguished: during a first phase of exponential growth a substantial amount of glucose is converted into ethanol (and CO<sub>2</sub> - Figures 2 and 3). Thereafter the wildtype yeast consumes the ethanol (and glycerol) that is produced during growth on glucose. In a functionally deleted Hxk2 mutant strain, during a first period of exponential growth no ethanol or glycerol is produced in substantial amounts. The amount of biomass produced (yield) is higher than for the wildtype strain during exponential growth (Figure 1). Glucose is directly converted into biomass instead of first being fermented to ethanol, as is the case for the wildtype strain. At higher optical densities the mutant strain produces ethanol and glycerol, which is, again, only converted after glucose exhaustion; this is likely to be an artefact of some limitation at high biomass densities.

### 1.4 Oxygen consumption capacity during growth on glucose

In the wildtype strain (Figure 4) the oxygen consumption capacity is relatively low when the cells are growing exponentially on glucose. Only after glucose exhaustion does the oxygen consumption capacity increase (release of glucose repression). In the Hxk2 mutant strain the oxygen consumption capacity is markedly higher when the cells are growing exponentially on glucose. At lower glucose concentrations or higher cell densities the oxygen consumption capacity decreases.

## EXAMPLE 2

### Effects of a *HXK2*-deletion in *Saccharomyces cerevisiae* in fed-batch culture

Scaled down production fermentations were run according to the general scheme presented in Chen and Chiyer (1985). Medium and physiological conditions were according to the procedures known to those skilled in the art and in agreement

with the literature mentioned. The process started with a full grown batch culture of the wildtype strain and the isogenic *HXK2* mutant strain in a yeast-peptone (YP) medium. The material of the batch cultures was transferred to a fermentor of 6 litre working volume and 3 litres initial volume. Molasses and  $\text{NH}_3$ -solution were fed into the fermentor according to a pre-designed computer controlled profile. The  $\text{NH}_4^+$  is fed at a rate that is in excess of the demand of the culture, but prevents too high concentrations in the broth. The molasses feed started at a constant rate, that was slightly in excess of the respiratory capacity in the initial hours of the fermentation, resulting in some ethanol formation, the rate of which decreased with the increase in biomass. After the consumption of the produced ethanol started, the feed rate of molasses was exponentially increased but was kept below the critical rate during the remaining part of the fermentation. The aeration rate is high and no ethanol formation is obtained except occasionally a small amount at the end of the fermentation, when the oxygen consumption rate is close to the oxygen transfer capacity. Part of the biomass of this fermentation is used as inoculum for the next production fermentation after washing and concentration and storage in the refrigerator for a limited amount of time.

The main (production) fermentation follows basically the same scheme, except for the application of a higher amount of inoculum material and by consequences higher feed rates of molasses and  $\text{NH}_4^+$ . The feed rate of the molasses was designed in this experiment with a maximum that did not exceed the respiratory capacity of either the parent or the mutant strain. No ethanol formation occurred under these conditions. Due to limitations in the oxygen transfer capacity the feed rate was kept at a maximum value in the last phase of the fermentation, resulting in a decrease of the growth rate with increasing biomass concentration. A maturation step was included at the end of the fermentation before washing and concentration by centrifugation and final concentration in a filter press. The resultant wet yeast product has a dry matter content of about 30 %.

The design of these fermentations intended to allow the growth of both the wildtype and the mutant strain under physiologically equal conditions of growth rate, oxygen uptake and carbon dioxide production rate and absence of ethanol formation. As the feed rate of the molasses was kept below the critical growth rate of both strains, this result was obtained. The quality of the products can be compared therefore for physiologically equal conditions. The quality of the product is measured in a standard test for the gas development in a dough environment. Relative to the amount of flour the dough contains 55 % water, 2 % salt and 0,45 % dry yeast solids. The dough is mixed in a normal way to get a properly developed dough and then put in a gas-production measurement device essentially as described by Burrows and Harrison (1959) at 28°C and incubated for up to 3 hours. The amount of gas produced is recalculated to the amount of gas produced by a quantity of yeast containing 1 mg of nitrogen determined according to Kjeldahl. The amount of gas produced by the parent strain under these conditions is about 7 ml. The test is repeated on samples that have been stored during 1 to 7 days in an incubator at 30 °C, resulting in a figure for the keepability of the gassing power.

In Table 1 the relative gassing power values of both strains are compared (the values for the parent strain are set at 100%). There is a slight difference in the gassing value of the fresh material, but there is a surprising effect on the keepability of the gassing power. This effect shows that there are more effects of the Hxk2-deletion even when grown at respiratory, non-repressive glucose limited conditions.

Table 1.

| days | parent 1 | parent 2 | parents average | mutant 1 | mutant 2 | mutant average |
|------|----------|----------|-----------------|----------|----------|----------------|
| 0    | 100%     | 101%     | 101%            | 88%      | 88%      | 88%            |
| 1    | 103%     | 106%     | 105%            | 84%      | 88%      | 86%            |
| 2    | 97%      | 95%      | 96%             | 82%      | 82%      | 82%            |
| 5    | 68%      | 68%      | 68%             | 72%      | 73%      | 72%            |
| 7    | 34%      | 36%      | 35%             | 69%      | 68%      | 69%            |

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**EXAMPLE 3****Effects of a HXK2-deletion in *Saccharomyces cerevisiae* on the critical growth rate in continuous culture**

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Continuous cultures were carried out as described by Hoek et al (1998) in a fermentor with a volume of 0.7 litre and a minimal medium as described in Example 1. The concentration of glucose in the feed was 7.5 g/kg. The growth rate was increased in subsequent steps allowing for steady state conditions of 4 times  $1/D$  in each step. At dilution rates above  $0.2 \text{ h}^{-1}$  the steps were maximally  $0.025 \text{ h}^{-1}$  and at least 12 hours passed before a next step was made. The dilution rate at which ethanol formation is seen first, is called the critical growth rate.

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The critical growth rate of the parent strain and the Hxk2-mutant were determined according to this method. Values of respectively  $0.3 \text{ h}^{-1}$  and  $0.35 \text{ h}^{-1}$  were found. This indicates an increase of 16% in the respiratory capacity. This allows a significant increase in growth rate in the first phase of the fermentation and allows an optimisation of the process towards a higher productivity.

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## SEQUENCE LISTING

<110> Universiteit van Amsterdam

<120> PROCESS FOR THE PRODUCTION OF YEAST BIOMASS

<130> EP2988

<140>

<141>

<150> EP 99201162.7

<151> 1999-04-13

<160> 2

<170> PatentIn Ver. 2.1

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<223> Description of Artificial Sequence: PCR Primer

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<223> Description of Artificial Sequence: PCR Primer

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ag 60

ctcg



**CLAIMS**

1. A process for the production of yeast biomass characterised by cultivation of a yeast culture on a commercial scale, wherein the yeast culture comprises one or more functionally deleted *HXK2* genes or analogues thereof, and by growing said yeast culture aerobically in a growth medium containing one or more fermentable carbon sources.
2. A process according to claim 1 wherein the fermentable carbon source is a sugar.
3. A process according to claim 2 wherein the sugar belongs to the group consisting of glucose, fructose, maltose, saccharose, galactose and raffinose.
4. A process according to anyone of claims 1-3 wherein the fermentation is carried out in batch mode and the growth medium initially contains the fermentable carbon source at a non-growth-limiting concentration.
5. A process according to claim 3 wherein the concentration of the fermentable carbon source is more than 1 mmole per litre.
6. A process according to anyone of claims 1-3 wherein the fermentation is carried out in fed-batch mode and a component of the growth medium is growth limiting.
7. A process according to anyone of claims 1-3 wherein the fermentation is carried out as a continuous culture and a component of the growth medium is growth limiting.
8. A process according to anyone of claims 6 and 7 wherein the growth limiting component is the fermentable carbon source.
9. A process according to anyone of claims 1-3 wherein the fermentation comprises the following steps:
  - a batch mode fermentation as defined in claims 4 or 5 and the carbon source is consumed with concomitant accumulation of yeast biomass, followed by
  - a fed-batch mode fermentation as defined in claims 6 and 8.
10. A process according to anyone of claims 1-9 wherein said yeast belongs to the genus *Saccharomyces* or *Kluyveromyces*.
11. A process according to claim 10 where the yeast is *Saccharomyces cerevisiae*.

12. A process according to claim 10 where the yeast is *Kluyveromyces lactis*.

13. A process according to claim 10 where the yeast is *Kluyveromyces marxianus*.

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14. A process for the production of proteins or secondary metabolites using a yeast culture that comprises one or more functionally deleted *HXK2* genes or analogues thereof by cultivating the yeast culture according to a process as defined in anyone of claims 1-13

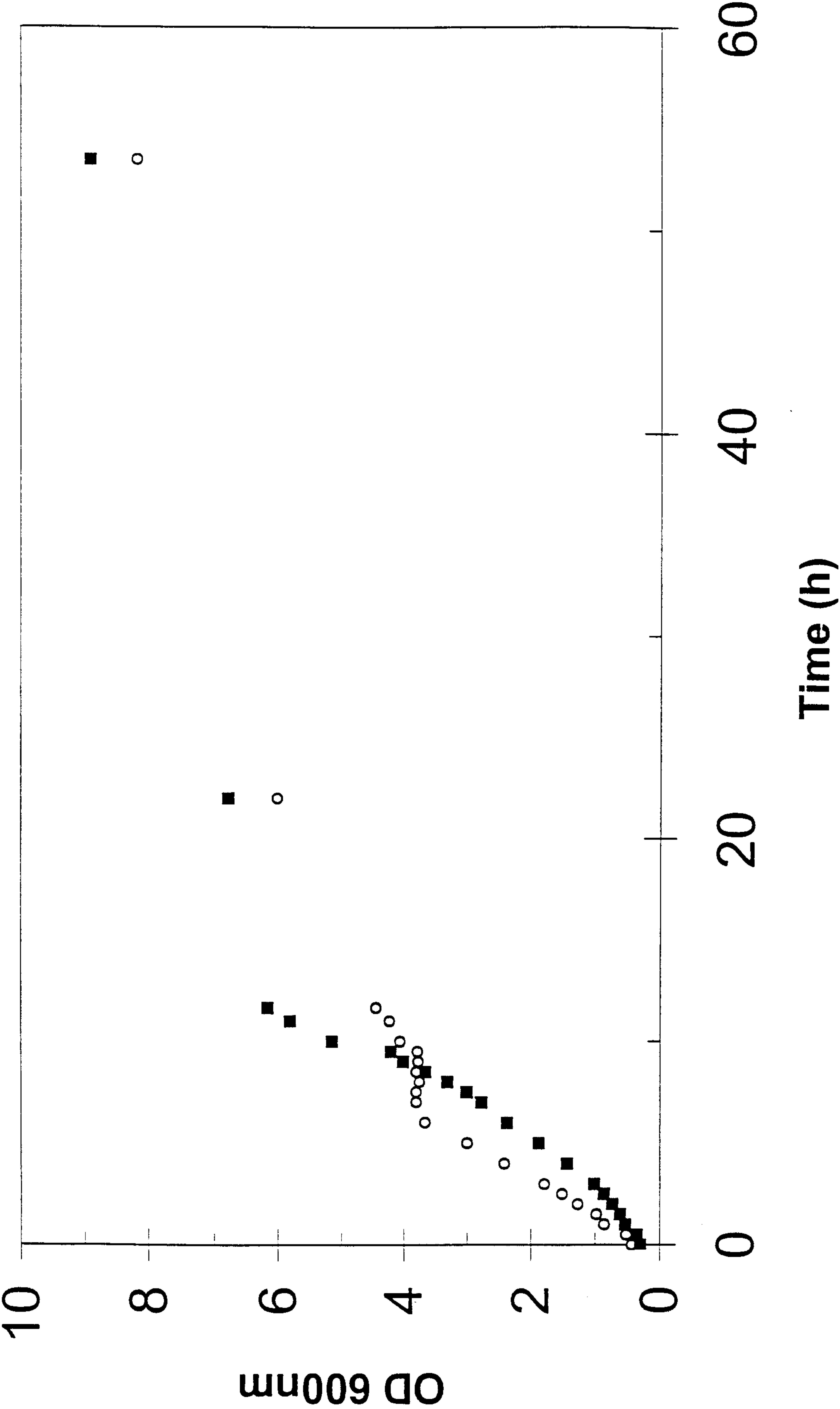
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15. Yeast biomass or a substance derived thereof obtainable by a process as defined in anyone of claims 1-13.

16. Compressed yeast, cream yeast or dried yeast made from yeast biomass as defined in claim 15.

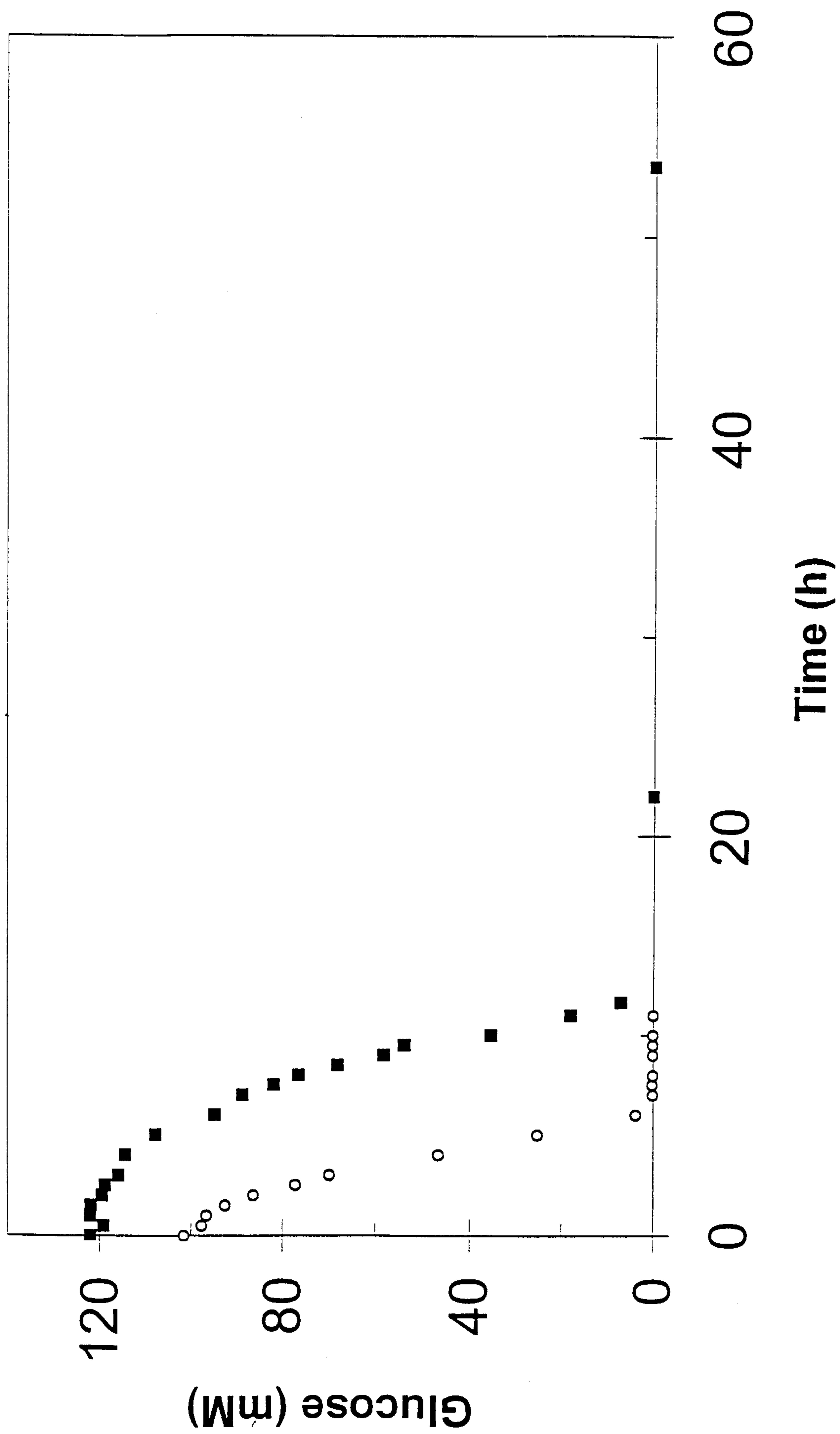
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Figure 1



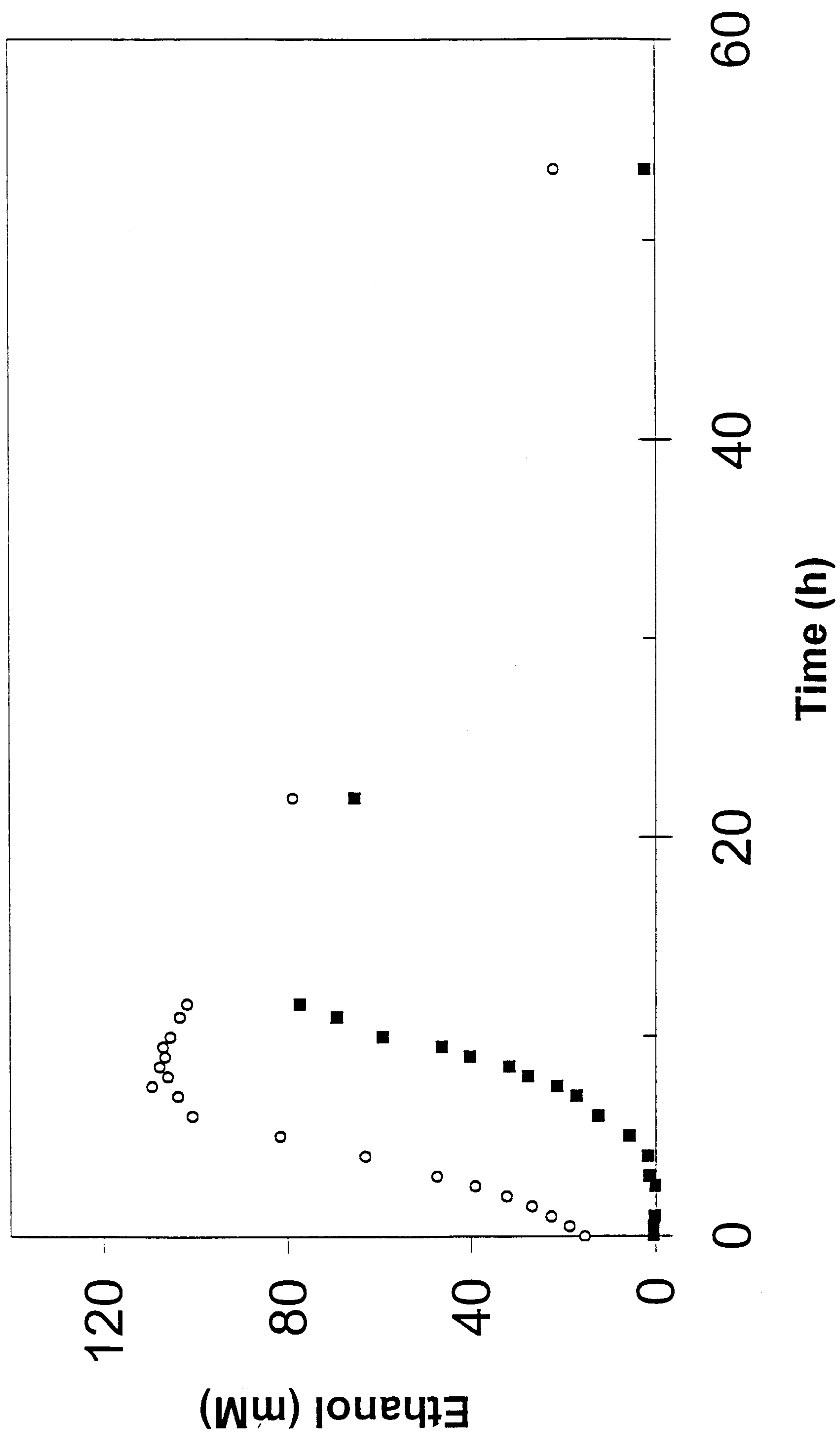
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Figure 2



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Figure 3



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