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Growth of *Candida utilis* on a Mixture of Monosaccharides, Acetic Acid and Ethanol as a Model of Waste Sulphite Liquor

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ABSTRACT. Candida utilis cultivated under batch conditions in a synthetic medium with a mixture of different carbon sources utilized first D-glucose and then D-galactose, D-mannose, D-xylose, L-arabinose, ethanol and acetic acid. The effect of acetic acid was primarily a function of pH and the physiological state of the inoculum. At pH 4.5, acetic acid at a concentration of 1 g/l increased the specific growth rate, reduced time of cultivation and increased yield of the yeast dry weight. The yield from acetic acid was 61 %. In the presence of a higher content of acetic acid (3–6 g/l) the yield was only 18–26 %. The yield calculated only from monosaccharides increased but the yield with respect to total carbon sources was lower. The specific growth rate decreased as well. The addition of ethanol also resulted in an increase of the production and yield of the yeast dry weight but the cultivation time was prolonged. The simultaneous utilization of carbon sources of the studied mixture modelling a sulphite fermentation medium with ethanol is advantageous. However, due to physiology of the yeast, it is most suitable to cultivate a strain adapted to utilizable carbon sources in a continuous way, in the presence of their limiting concentrations in the cultivation medium.

One way of intensification of the production of single-cell protein (SCP) is the production of fodder yeasts on a mixture of different waste sulphite liquors with synthetic ethanol (Mostecký *et al.* 1976; Rychtera *et al.* 1977). In such a fermentation medium the yeasts can, under specific conditions, assimilate aerobically not only hexoses, pentoses, acetic acid, formic acid and other acids contained in waste sulphite liquors but also ethanol and, occasionally, acetic acid originating from it (Vernerová *et al.* 1974; Adámek *et al.* 1976).

The specific growth rate of the yeasts differs when the microorganism grows in a medium with only a single carbon and energy source or in the presence of more carbon and energy sources. During cultivation in media containing a mixture of monosaccharides a gradual utilization of individual carbon sources was observed. The rate of their utilization differed from that in media individual monosaccharides under otherwise identical conditions (Andreev 1961; Fencl and Burger 1968; Andreev *et al.* 1970; Pineault *et al.* 1977).

Yeasts assimilate together with sugars different organic acids, with a different specific growth rate and yield of biomass (Málek *et al.* 1957; Semushina *et al.* 1974). In the presence of 0.5 % D-xylose organic acids are assimilated more rapidly and with a higher yield of biomass; in a medium with acetic acid the yield of the yeast dry weight reached 53.1, 44.3, 39.4 and 60.0 % in *Candida tropicalis* SD-3, *Candida*

utilis K-2, *Candida utilis* Kr-9 and *Trichosporon* sp. T-3, respectively (Kryuchkova and Vorob'eva 1964). As compared with other volatile acids, acetic acid is least toxic; the inhibitory effect decreases in a homologous series: caproic acid > butyric acid > formic acid > propionic acid > acetic acid (Shvets *et al.* 1976). Certain yeast genera (*Hansenula, Pichia, Candida etc.*) can grow even on 10 % acetate and very good yields of the yeast dry weight can be obtained in media containing 3—5 % acetate even at low pH (Ogata *et al.* 1969). Huňková (1972) studying toxicity of lower fatty acids found that even 0.7 M acetic acid (3.2 %) in the medium with glucose did not inhibit aerobic oxidation of glucose in *Saccharomyces cerevisiae, Candida utilis* and *C. lipolytica*, however, it could partially inhibit CO_2 fixation, uptake of inorganic phosphate by the cell, and its division.

C. utilis cultivated on ethanol always accumulates acetic acid, which is not desirable from the point of view of the growth rate and yeast mass yield (Adámek *et al.* 1976; Průchová *et al.* 1977; Šestáková 1976*b*). Thus, when introducing the production of fodder yeast from waste sulphite liquors with an addition of ethanol is should be kept in mind that the content of acetic acid in the medium during the cultivation may increase, not only due to its accumulation from waste sulphite liquors, but also due to its metabolic formation from ethanol.

In the present work growth characteristics of *C. utilis* 49 during cultivation in synthetic media modelling a mixture of monosaccharides, acetic acid and ethanol in sulphite waste liquors, at two pH values and using inocula prepared in different ways, were investigated.

MATERIALS AND METHODS

Microorganism. The yeast *Candida utilis* 49 from the collection of the Research Institute of the Fodder Industry and Service in Prague was used throughout.

Preparation of inoculum. In experiments 1—5 (Table I) the inoculum was prepared by a twice-repeated 12-h cultivation of *C. utilis* in flasks on a rotary shaker at 30 °C. After the cultivation the yeast was centrifuged, washed and resuspended in a physiological saline in such a way that the amount of yeast added equaled 1—2 % of the total volume of the medium (the initial content in the medium was 1—2 g yeast dry weight in one litre) — inoculum I. In experiments 6—9 the yeasts were adapted by an 8-h batch cultivation to monosaccharides and acetic acid in a 2—1 fermentor — inoculum 2.

Cultivation medium. 6 g $(NH_4)_2SO_4$, 0.45 g $MgSO_4.7H_2O$, 0.45 ml (85 %) H_3PO_4, 1.05 g K_2SO_4, 1 ml of microelement solution, 10 g of a mixture of monosaccharides and tap water ad 1000 ml. The solution of microelements contained $FeSO_4$, $ZnSO_4$ and $MnSO_4$. The mixture of monosaccharides (%): D-xylose 40, D-mannose 40, D-glucose 10, D-galactose 5, L-arabinose 5. pH of the medium was 4.5 (pH adjusted with sulphuric acid or sodium hydroxide), sterilization was done for 2 h at 0.12 MPa.

Cultivation was performed at 30 °C, either in cultivation flasks containing 100 ml of the medium or in a 2 litre fermentor with a mixer, automatic temperature regulation, defoaming device and regulatory pH-meter. Transfer of oxygen was 300 mmol l^{-1} h^{-1} (determined by the sulphite method). Acetic acid was added to the cultivation medium in the form of sodium acetate prior to pH adjustment. Ethanol was added at the beginning of the cultivation (Table I).

Analytical methods. Determination of yeast dry weight, pH of the medium, GLC-determination of ethanol, determination of acetic acid and ethyl acetate, as well as purity and origin of chemicals have already been described (Šestáková 1976*a*). Monosaccharides were separated by descending paper chromatography on

Whatman 1 in ethyl acetate—pyridine—water (2 : 1 : 2). Chromatograms were detected with acidified anilinium phthalate followed by heating (105 °C, 5 min). The spots were quantitatively evaluated in a photometric densitometer PH-1-5 (Vernon) with a built-in integrator.

RESULTS AND DISCUSSION

It follows from the course of curves representing the utilization of individual carbon source (Fig. 1) and from the detected changes of the specific growth rate (Fig. 2) that the carbon sources are utilized at different rates. Glucose was utilized first and most rapidly. Galactose was also always utilized from the beginning of the cultivation but at a substantially lower rate. Xylose and mannose were assimilated in most experiments only 1—2 h after the beginning of the cultivation but the rate of their utilization was then higher than that of galactose. Arabinose was utilized most slowly. As the content of individual sugars in the medium at the beginning of the cultivation varied, the rate of exhaustion of a monosaccharide from the medium need not be identical with the rate of its assimilation. The highest specific growth rate of the cells was detected during the utilization of glucose (*i.e.* after a 1—3 h cultivation). In most experiments the same specific growth rate (0.36—0.40/h) was detected in the exponential part of the growth curve. Some important characteristics of all experiments are summarized in Table 1.

It follows from the literature data (Semushina *et al.* 1963; Pineault *et al.* 1977) that out of a mixture of sugars hexoses are utilized more rapidly than pentoses (hexose effect), however, when the yeast is adapted to these compounds this phenomenon cannot be observed as during the cultivation the specific growth rate can change only slowly. Similar phenomena were often observed during cultivation of yeasts in a mixture of glucose (hexoses) and oligosaccharides, ethanol and acetic acid. The hexose, or glucose effect is sometimes explained by repression of synthesis of enzymes of the pentose cycle or by catabolite repression and inhibition of a series of further functional enzymes (Zabrodskii 1972). Other authors explain the mechanism of the glucose effect by enzymic degradation of the corresponding enzymes by proteinases (lyases) in vacuoles of the cells with participation of a catabolite of glucose (Shaltiel 1976). Burger *et al.* (1959) demonstrated that a step-rise assimilation of monosaccharides was also associated with their different affinity to the transport system of the cell membrane. For the production of fodder yeasts from waste sulphite liquors Fencl (1962) recommends a two-step continuous cultivation; hexoses are utilized during the first step and pentoses and other more slowly utilizable compounds are used up during the second step (at a higher dilution rate). However, it is apparent (Rosa *et al.* 1977) that at low dilution rates *C. utilis* can utilize all monosaccharides in waste sulphite liquors also in a one-step continuous cultivation.

Effect of acetic acid at pH 4.5

The addition of acetic acid (1 g/ml) at pH 4.5 stimulates utilization of monosaccharides and improves all important characteristics of the cultivation (*e.g.* increases specific growth rate, reduces time of cultivation, increases yield of the yeast dry weight, *etc.*; Table I). The abnormally high yield of dry weight from acetic acid (61 %) indicates that this compound (or monosaccharides) is better utilized for production of the yeast dry weight. Thus, addition of acetic acid (1 g/l) can be recommended for the cultivation of *C. utilis* on a mixture of monosaccharides at pH 4.5. However, with an increasing content of acetic acid specific growth rate and

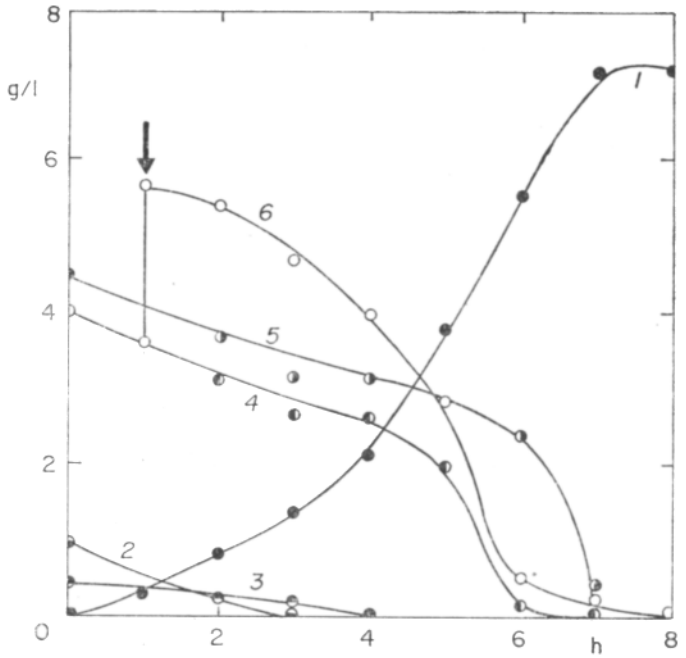

FIG. 1. Growth curve of *C. utilis* (*1*) and utilizaiton of individual carbon and energy sources on a mixture of D-glucose (*2*), D-galactose (*3*), D-xylose (*4*), D-mannose and L-arabinose (*5*), acetic acid (*6*, × 2.5), and ethanol (*7*, × 2.5); experiment 3.

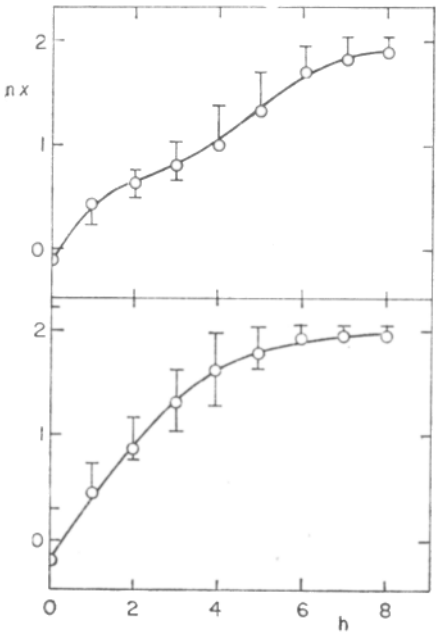

FIG. 2. Growth curves of *C. utilis* from experiments 1—3 with inoculum I (*upper part*) and from experiments 6—9 with inoculum II (*lower part*) for calculation of kinetic changes of specific growth rate; x concentration of yeast dry weight in the medium.

TABLE I. Characteristics of cultivation experiments 1—9 with *C. utilis* 49 grown in a 2-l fermentor at pH 4.5 and 30 °C

Characteristic	Inoculum I (from shakers)					Inoculum II (from fermentor)			
Experiment number	1	2	3[a]	4	5[b]	6	7	8	9
cultivation time, h	8	7	8	4	8	7	6	—	8
initial concentration of acetic acid, g/l	0	0.1	0.1	0.5	0.5	0	0.3	0.4	0.6
maximal production, g l^{-1} h^{-1}	1.6	1.6	1.8	0	1.5	1.6	2.2	—	1.8
specific growth rate[c], h^{-1}									
μ_1	0.54	0.65	0.59	0	0.54	0.60	0.40	0.35	0.30
μ_2	0.36	0.36	0.36	0	0.36	0.40	0.40	—	0.30
yield of yeast dry weight, %, calculated									
per available monosaccharides	53	59	76	0	62	56	64	—	72
per total carbon sources	53	54	55	0	41	56	49	—	45
per acetic acid	0	61	61	0	18	0	25	—	26
Residual acetic acid in the medium, %	0	0	0.017[d]	0.496	0.168	0	0	0.36	0.008

[a] Initial concentration of ethanol 3 g/l; yield of yeast dry weight from ethanol 58 %. [b] pH of the medium 6.5. [c] μ_1 was determined after a 1-h cultivation (utilization of glucose), μ_2 from the middle part of the exponential phase of the growth curve. [d] Metabolic acetic acid from ethanol.

yield of yeast dry weight calculated with respect to total carbon sources decrease. After the addition of 3—6 g/l of acetic acid the compound is predominantly oxidized and a smaller portion is assimilated. The yield of yeast biomass from acetic acid varied within 18—26 %. Hence the content of acetic acid commonly observed in calcium and sodium-bisulphite waste liquors (lower than 6 g/l) can be utilized for increasing the yield. However, in the presence of acetic acid at a concentration higher than 3 g/l lower dilution rates should be applied.

Similar data concerning the favorable effect of low concentrations of acetic acid on the utilization of carbon sources were presented by Arima *et al.* (1969), Kinsel and Leathen (1973), Nakamura *et al.* (1975), and Sustina *et al.* (1973). The optimal concentrations of acetic acid were primarily a function of pH of the medium, species or mutant of the yeast, and varied within 1—30 g/l. It is added mostly in the form of different industrial wastes, *e.g.* exhaust water (formed during production of 2-furaldehyde) or as a 20 % potassium acetate or powder calcium acetate (formed also during the production of 2-furaldehyde), sugar-beet molasses, *etc.*

The addition of substrates containing acetic acid increased its content in the medium up to 1.7—4.8 g/l. *Candida tropicalis* utilizes acetic acid to 82—92 % and its residual content in the medium is 0.3—0.4 g/l. The addition of volatile acids (0.7—1 g/l) increases the yeast yield by 6—13 % (Rosa *et al.* 1977).

Inhibitory effect of acetic acid at different pH values

Experiments 4 and 5 showed that the inhibitory effect of acetic acid was a function of the actual concentration of its non-dissociated form. Whereas the physiologically weak inoculum did not grow in the medium with 5 g/l of acetic acid at pH 4.5, mostly better characteristics of cultivation were observed at pH 6.5 as compared with cultivation in a 1 % mixture of pure sugars. In comparison with cultivations at pH 4.5 (see Table I) the cultivation at pH 6.5 produced very low yields of yeast mass as calculated with respect to acetic acid. Only in this experiment acetic acid was not fully metabolized during the cultivation (its residue in the medium was 34 %). The increase of pH of the medium to 6.5 decreased the toxic effect of acetic acid by increasing its dissociation. Due to a higher risk of a bacterial contamination, under practical conditions of the cultivation a further adjustment of pH to even higher values would not be suitable. Effect of pH of the medium on the degree of dissociation of molecules of acetic acid and its inhibitory effect on yeasts were studied by numerous authors (Neal *et al.* 1954; Ogata *et al.* 1969; Ikeda *et al.* 1973; Barinova *et al.* 1975; Shvets *et al.* 1976). Most authors found that optimal pH for *C. utilis* was close to neutral values (5—6). The mechanism of the inhibitory effect of acetic acid is explained by penetration of undissociated molecules of the acid into the yeast cell that dissociate in the cytoplasm, decrease the intracellular pH and limit respiration of the yeasts, decrease production of certain enzymes, *e.g.* proteases, *etc.* However, a relatively easy penetration of non-dissociated molecules of acids into the cell suppresses transport of other components of the medium (inorganic phosphate in particular) impairing the normal metabolism. Ions of non-utilizable acids, added hydroxides and also CO_2 influence the concentration of acetate ions in the cultivation medium (Ikeda *et al.* 1973; Ko and Edwards 1975). Neutralization of different plant hydrolyzates removes a considerable portion of nondesirable ingredients and, thus, accelerates growth of the yeasts in these media. In addition to dissociation of volatile acids, release of monosaccharides from saccharide-bisulphite bonds is associated with pH of waste sulphite liquors and other plant hydrolyzates (Sapotnitskii 1960).

Cultivation on a mixture of sugars, acetic acid and ethanol

It follows from cultivations 1—3 (Table I, Fig. 1) that the presence of 1 g/l of acetic acid and 3 g/l of ethanol in mixture with 10 g monosaccharides per litre cultivation medium did not inhibit the growth of the yeasts. In experiment 3 acetic acid was utilized more rapidly than in experiment 2, indicating that ethanol stimulates the rate of utilization of acetic acid. After a 3—5 h cultivation also a small amount of ethyl acetate (less than 80 mg/l) was detected in the cultivation medium. Utilization of ethanol at the beginning of the cultivation was very slow. Only after exhaustion of a substantial portion of saccharides from the medium was ethanol utilized rapidly. The course of utilization of other carbon sources differed from that during cultivations without ethanol. Thus for instance, 1 g of glucose but also approximately 1 g of mannose (with arabinose) and 1 g of xylose but only 0.49 g ethanol, 0.38 g acetic acid and 0.18 g galactose were utilized after 3 h. We assume that arabinose was still intact at this time. After 5 h, when all acetic acid was utilized (*i.e.* 1 g/l), 1.1 g/l ethanol (*i.e.* about one-third of the initial content) were utilized as well. It follows from the experiment 3 that the maximal production of the yeast dry weight was higher than that on a pure mixture of monosaccharides but that the cultivation time was prolonged. Glucose, galactose and acetic acid were not detected after a 5-h cultivation but later (due probably to a rapid oxidation of ethanol in the presence of only negligible content of xylose and arabinose) metabolic acetic acid accumulated in the medium (Fig. 1). As the specific growth rate in experiment 3 was lower than that in experiment 2 it appears that ethanol stimulates oxidation of acetic acid and the utilized sugars stimulate assimilation of acetic acid preventing its undesirable accumulation in the cultivation medium.

Effect of the physiological state of the inocilum on growth of cells and utilization of monosaccharides, acetic acid and ethanol

In experiment 1, inoculum I exhibited a longer cultivation time, slower utilization of individual sugars and lower yield of the yeast dry weight than inoculum II in experiment 6 (Table I). Also the experiments with higher amounts of added acetic acid showed that the physiological state of the inoculum is of utmost importance for a successful course of the cultivation. For instance, inoculum I did not grow with acetic acid at a concentration of 5 g/l (pH 4.5) but inoculum II grew well even in the presence of 6 g/l (or 5.6 g/l at the same pH; Fig. 3). It is likely that this substantial importance of preparation of the inoculum for the course of the batch cultivation and yield of the yeast dry weight can be suppressed during a long-term continuous cultivation, particularly under conditions of limitation by carbon and energy sources (Rosa *et al.* 1977). Under such conditions the yeast can produce a pool of inducible enzymes sufficient for a rapid utilization of non-sugar carbon sources and is not influenced by inhibition and catabolite repression caused *e.g.* by monosaccharides.

Gonzáles (1977) found that the carbon and energy source in the medium influenced to a varying degree the oxidative and assimilatory activity of the yeast cells during cultivation on non-sugar substrates. The author found that glucose used for preparation of the inoculum of *Saccharomyces cerevisiae* could stimulate catabolic reactions of ethanol or acetic acid in a subsequent cultivation with these non-sugar substrates but that it simultaneously slowed down anabolic reactions in the yeast cell. The inoculum grown on glucose as a carbon and energy source exhibited a low assimilation activity during utilization of ethanol (or acetic acid). This activity increased proportionally with increasing activity of isocitrate lyase. Also the ex-

periments with the inoculum of *C. utilis* prepared in the medium with monosaccharides could demonstrate a slower utilization of ethanol and a lower yield of the yeast dry mass as compared with the experiments with the inoculum prepared in the medium with ethanol (Šestáková 1976*b*).

It can be concluded that the presence of acetic acid (1 g/l) and ethanol (up to 3 g/l) in a mixture with monosaccharides representing dominant sugars in sulphite waste liquors stimulate metabolism of the substrates in *C. utilis* with a simultaneous

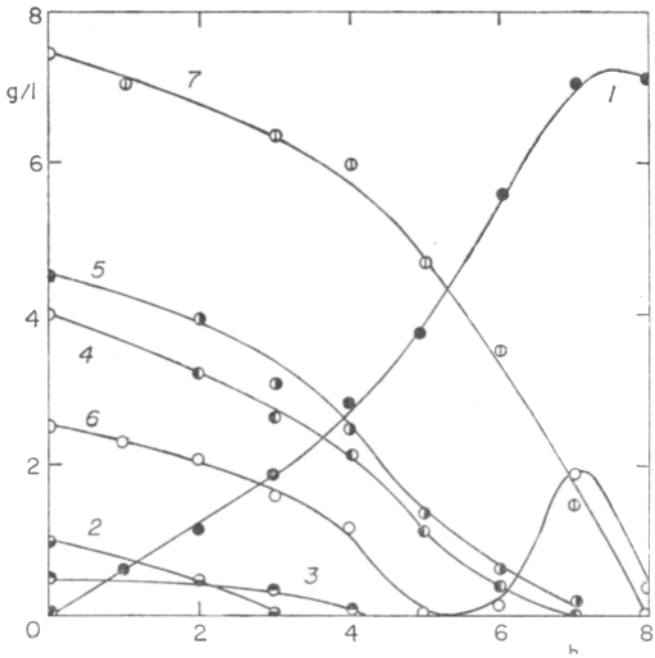

FIG. 3. Growth curve of *C. utilis* (*1*) and utilization of individual carbon sources on a mixture of D-glucose (*2*), D-galactose (*3*), D-xylose (*4*), D-mannose and L-arabinose (*5*) and acetic acid (*6*); arrow indicates addition of further 2 g acetic acid per litre; experiments 8 and 9.

increase of the specific growth rate and yield of the yeast dry mass as calculated with respect to total carbon sources. In spite of the fact that *C. utilis* can grow at pH 4.5 even in the presence of acetic acid at a concentration of 6 g/l and 10 g of monosaccharides, the highest yield of the yeast dry weight was reached at a concentration of 1 g/l. As compared with the yield in the presence of acetic acid as the only carbon source or with a pure mixture of monosaccharides, the yield of the yeast dry weight from acetic acid increased by up to 100 % during cultivation on a mixture of this acid with a mixture of monosaccharides, *i.e.* from 30 to 60 % (Šestáková 1976*b*). The addition of ethanol stimulated utilization of acetic acid but ethanol alone was utilized more slowly at the beginning of the cultivation than most sugars present (due to the fact that the inoculum was not adapted to this substrate). The increased rate of utilization of acetic acid can be explained by stimulation of its transport inside the yeast cell and by an increased activity of isocitrate lyase caused by the addition of ethanol, which is a well-known inducer of this enzyme (Gonzáles 1977). The importance of preparation of the inoculum for a successful course of the

continuous cultivation is especially pronounced at the beginning of the experiment, when *C. utilis* need not yet be adapted to all carbon and energy sources in the cultivation medium due to insufficiency or excess of one of the substrates present. It is apparent that the continuous cultivation of *C. utilis* on a mixture of sulphite waste liquors with synthetic ethanol facilitates the maximal adaptation of the yeast to the substrates present. When the cultivation proceeds in the presence of carbon and energy sources at concentrations close to limiting values, all carbon sources present are utilized even if at different rates. At the same time yield of the yeast dry weight is increased (Rosa *et al.* 1977). The addition of non-sugar substrates may thus improve technology of the production of yeasts from sulphite waste liquors and other raw materials.

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