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From the Department of Microbiology and Immunology, Veterinary College of Norway, Oslo.

THE INTERACTIONS OF PEPTIDE-PRECIPITATING LIGNOSULPHONIC ACIDS AND THE FERMENTATION OF VARIOUS SUGARS BY A STRAIN OF CANDIDA UTILIS

By

Bjørn Næss

NÆSS, BJØRN: *The interactions of peptide-precipitating lignosulphonic acids and the fermentation of various sugars by a strain of Candida utilis.* Acta vet. scand. 1972, *13*, 505—519. — The fermentation of various sugars by Candida utilis in the presence of purified peptide-precipitating lignosulphonic acids has been studied. An inhibitory effect on the fermentation of sucrose, glucose, xylose and an equimolar mixture of glucose and fructose was found, the effect being most pronounced for xylose. Inhibition of the fermentation of xylose was seen when 0.5 % lignosulphonic acids were included, but not when 0.25 % were used. A difference in the inhibitory effect on the fermentation of sucrose and an equimolar mixture of glucose and fructose was observed, and the result is discussed in relation to a possible inhibitory mechanism of the lignosulphonic acids.

The peptide-precipitating ability of the lignosulphonic acids seemed to be little influenced during the fermentation of the sugars by Candida utilis.

lignosulphonic acids; fermentation; sugars; Candida utilis.

Candida utilis has been used in the microbial production of fodder yeast (single cell protein) from industrial waste water for several decades (*Inskeep et al.* 1951; *Pietz* 1967; *van Uden & Buckley* 1970), and production plants are operating in a number of countries (*Inskeep et al.; Peppler* 1968; *Feucl* 1969; *Schönhuth* 1970). The fermentation has been considered bifunctional: 1) removal of sugars and other easily fermentable substances in the waste liquors to prevent microbial spoilage of

lakes, rivers etc. serving as discharge areas and 2) production of single cell protein. One substrate often used for single cell protein production is sulphite spent liquor produced in the sulphite process for the manufacture of chemical wood pulp. This liquor may contain 1.5—2.2 % of sugars of various types (*Tötterman* 1958). The liquor is not considered ideal for fermentation because of the content of various growth inhibiting substances, in particular sulphur dioxide which exists in a number of forms, including total inorganic and organic loosely combined (*Inskeep et al.; Pietz*). The amount of sulphur dioxide can be reduced by the so-called stripping process. In addition, the lignosulphonic acids in sulphite waste liquor inhibit, to some extent, the oxidation of sugars by microorganisms (*Lawrance & Fukui* 1956). The lignosulphonic acids cannot be readily removed, however.

In previous works it was shown that certain lignosulphonic acids from sulphite spent liquor are able to precipitate a number of peptides and proteins (*Næss* 1971a; *Hildrum & Næss* 1972) and that various microbial and animal proteinases are able to dissolve the peptide- and protein-lignosulphonic acid precipitates (*Næss* 1971c). These reactions are considered responsible for an important part of the biochemical activity in the ecosystems into which sulphite spent liquors are released (*Næss* 1971d), and are also assumed to occur to some extent in sulphite spent liquor used in single cell protein production. The aim of the present work was to study the effects of certain purified peptide-precipitating lignosulphonic acids on some biological activities during the fermentation of various sugars with Candida utilis, and to study the effect of the fermentation on the peptide-precipitating ability of the lignosulphonic acids.

MATERIALS AND METHODS

Microorganism. The organism used in this study was Candida utilis (ATCC* 9226).

Fermentor. The experiments were conducted in a 14-l glass fermentor with a circulation stirrer system (type GF0014**). All fermentor parts in contact with the medium are of either glass or stainless steel. The stirring system is regulated by a

* American Type Culture Collection, Rockville, Maryland, USA.
** Chemap AG, Männedorf, Schwitzerland.

continuously variable motor, range 0—3000 r.p.m. Temperature control is assured by water circulating in the hollow elements of the stirring systems, in the built-in electrical heating, and by the use of a contact thermometer. The pH of the medium is recorded automatically during the fermentation by an Ingold glass electrode inside the fermentor, and can be regulated automatically by an incorporated pH control system using 7 M-NaOH in the base reservoir and 5 M-HCl in the acid reservoir. Air is bubbled through the medium after passing through a flowmeter and a ceramic filter.

Medium. The medium used for fermentation was as follows†: NH_4NO_3, 0.5 %; KH_2PO_4, 0.47 %; $Na_2HPO_4 \cdot 2H_2O$, 0.03 %; $MgSO_4 \cdot 7H_2O$, 0.1 %; $CaCl_2 \cdot 2H_2O$, 0.001 %; $FeSO_4 \cdot 7H_2O$, 0.001 %; $MnSO_4 \cdot 4H_2O$, 0.001 %. The sugars used were: Sucrose 1.9 %††, D(+)-glucose 2.0 % (Merck), D(+)-xylose 2.0 % (BDH Chemicals Ltd.) or an equimolar mixture of D(+)-glucose and D(—)-fructose 2.0 % (Merck). The sucrose, glucose and fructose concentrations were corrected for the water of crystallization. Glucose and xylose were used in this study because these sugars comprise a major part of the sugars in sulphite spent liquor (*Pietz* 1967). Sucrose and invert sugar were used to study the effect of lignosulphonic acids on the activity of the invertase produced by Candida utilis for degrading the disaccharide to glucose and fructose (*White et al.* 1968).

Lignosulphonic acids. The sodium salts of the lignosulphonic acids prepared by the Jantzen procedure (*Jantzen* 1964) were used in this study and were kindly supplied by Alwatech A/S, Oslo, Norway. The sulphur content of the lignosulphonic acids was 6 %. The distribution of the molecular weights of the lignosulphonic acids, is given elsewhere (*Næss et al.* in press).

Cultivation. For inoculation, a loopful of cells from a plate of Sabouraud's Maltose Agar (0110—01 Difco*) was cultured in the fermentor with the medium described, using 2 % sucrose as carbon source, for 72 hrs. at 30°C, the fermentor containing 10 l of medium. The cells were centrifuged in a separator (Cepa-Schnell-Zentrifuge**) at 50000 r.p.m. The packed cells were

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† All obtained from Merck AG, Darmstadt, Germany.

†† BDH Chemicals Ltd., Poole, England.

* Difco Laboratories Inc., Detroit, Michigan, USA.

** C. Padberg, Lahr/Baden, Germany.

washed once in distilled water and stored at 4°C. Five g portions of these packed cells were used as inocula in the fermentation experiments. Ten l of medium were used and aeration was maintained at such a rate that oxygen deficiency did not limit growth (flowmeter scale 80). Fifteen-hundred r.p.m. was used for the circulation stirring system, and the temperature was maintained at 30°C. The pH was usually held at 4.5 during the fermentation. Analyses for culture purity were performed by microscopy of broth samples and examination of seeded agar plates (Sabouraud medium) after incubation at 30°C for about 40 hrs. The fermentation experiments were usually started in the morning and samples of the fermentation broth were collected every 2nd hr. during the day and every 5th hr. at night. The samples of broth collected were stored at —20°C.

Growth rates. Five ml of fermentation broth was centrifuged at 2500 × g for 10 min., the cells were washed once with distilled water, recentrifuged, and weighed as packed cells.

Rest sugar analysis. After centrifugation of the samples of broth, the monosaccharides remaining in 1 ml of the supernatant were determined using a Technicon Autoanalyzer* after adding 1 ml 0.5 M-NaOH and diluting the solution to 15 ml with distilled water. The ferricyanide reduction method (Technicon Autoanalyzer Method file N-2b) which utilizes principles developed by *Hoffman* (1937) was used.

The sucrose solutions were hydrolysed at 100°C for 8 min. at pH 1.8 (1 ml 0.5 M-HCl added to 1 ml of the sucrose solution). After hydrolysis, and cooling in an ice bath, 2.0 ml 0.5 M-NaOH was added and the solution diluted to 15 ml before analysis.

* Technicon Instruments Company Ltd., London, England.

Figure 1. a. The variation in pH during the fermentation of sucrose by Candida utilis in the absence (o—o) and the presence (•—•) of 2 % lignosulphonic acids.

b. Growth rate of Candida utilis during the fermentation of sucrose, in the absence (o—o) and the presence (•—•) of 2 % lignosulphonic acids.

c. Removal of sucrose from the fermentation broth in the absence (o—o) and the presence (•—•) of 2 % lignosulphonic acids during fermentation with Candida utilis.

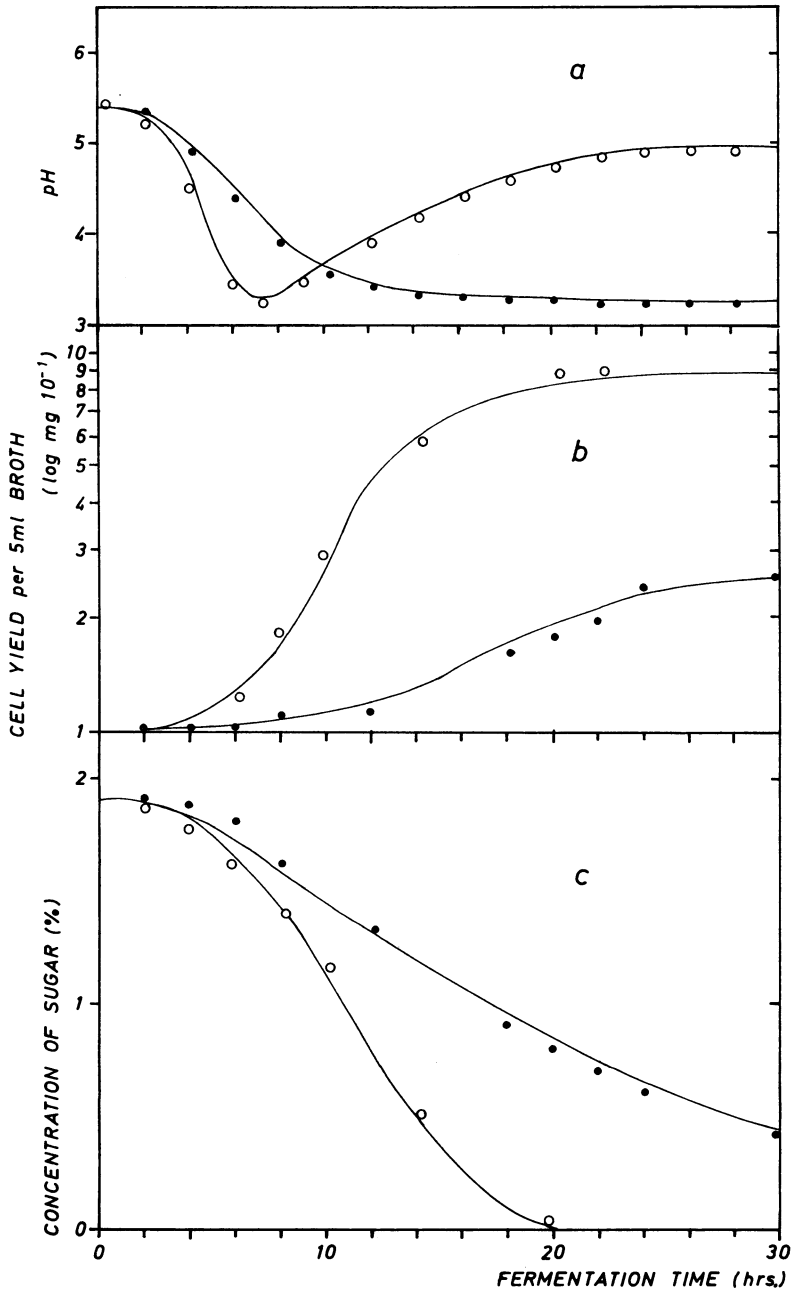
a
b
c
pH
CELL YIELD per 5ml BROTH
(log mg 10⁻¹)
CONCENTRATION OF SUGAR (%)
FERMENTATION TIME (hrs.)
0
10
20
30

Figure 1.

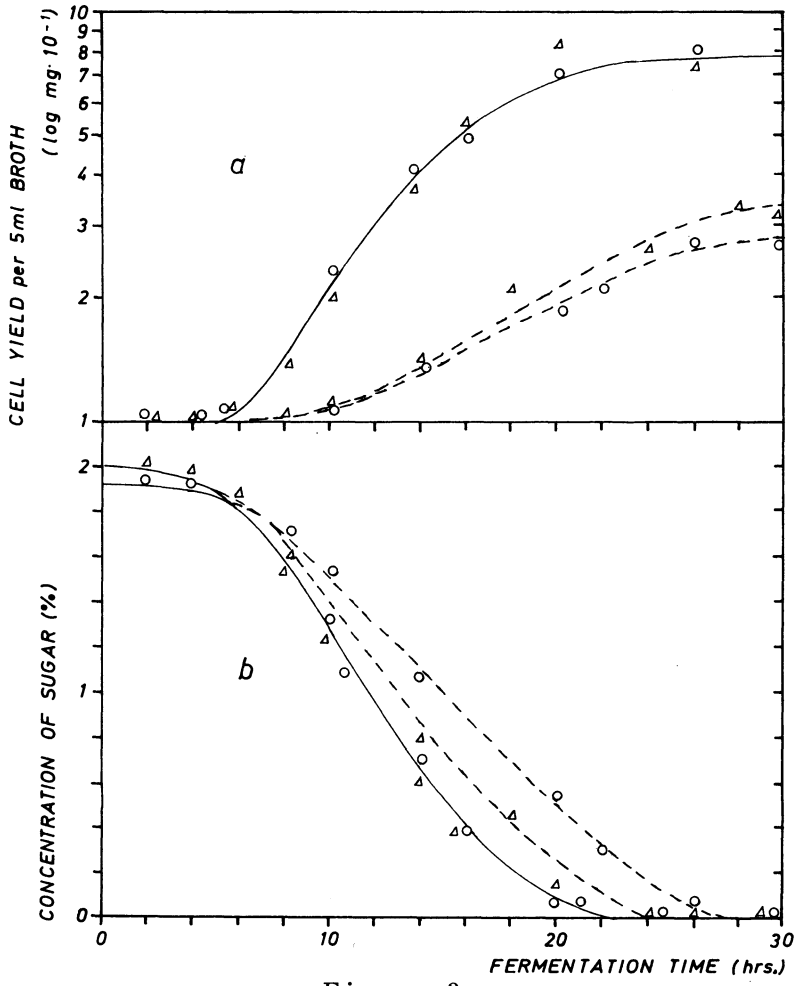

Figure 2.

Figures 2 and 3.

a. Growth rate of Candida utilis in the absence (——) and the presence (- - - -) of 2 % lignosulphonic acids during the fermentation of various sugars.

b. Removal of sugars from the fermentation broth in the absence (——) and the presence (- - - -) of 2 % lignosulphonic acids during fermentation with Candida utilis.

o o sucrose, △ △ invert sugar, • • glucose, ▲ ▲ xylose. pH during the fermentation 4.5.

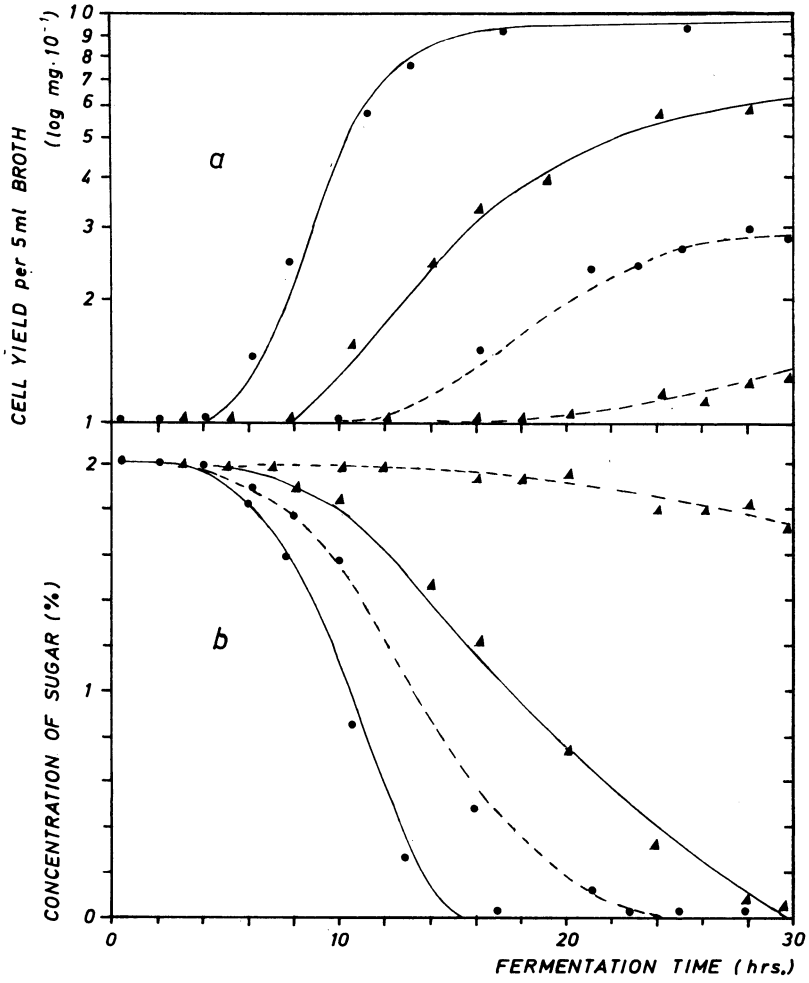

Figure 3.

Analysis of peptide-precipitating activity of the lignosulphonic acids. The neopeptone (Difco) agar system (*Næss* 1971b) for the analysis of the peptide-precipitating activity of the lignosulphonic acids was used with the alteration that pH 3.5 was used in the agar instead of 6.8. Fifty µl of the supernatants, after centrifugation of the samples, were applied into the agar wells. Parallel analyses were also carried out by adding 25 µl of the supernatant to 5 ml of a 5 % neopeptone solution, pH 3.5, and the turbidity measured spectrophotometrically at 610 nm in a Hilger* spectrophotometer. pH 3.5 was used in this study, be-

* Hilger & Watts, Rank Precision Industries Ltd., London, England.

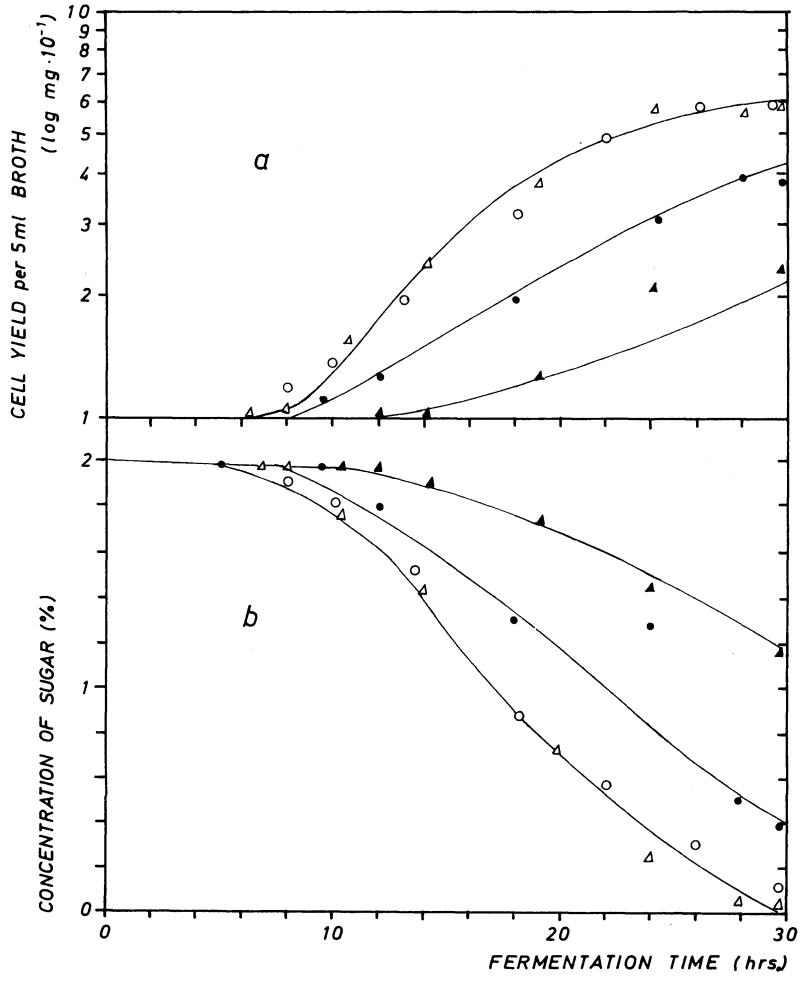

Figure 4. a. Growth rate of Candida utilis in the presence of various concentrations of lignosulphonic acids during the fermentation of xylose.

b. Removal of the xylose from the fermentation broth in the presence of various concentrations of lignosulphonic acids during fermentation with Candida utilis.
o—o 0 %, △–△ 0.25 %, •—• 0.5 % and ▲–▲ 1 % lignosulphonic acids. pH during the fermentation 4.5.

cause it was found that the precipitation of peptides and proteins with the lignosulphonic acid preparation used, was greater at pH 3.5 than at 6.8.

RESULTS

When sucrose is fermented by Candida utilis in the inorganic salt mixture used without pH control, a fall in the pH from 5.4 to about 3.3 is seen when logarithmic growth started, and the pH rises again to about 5.0 when the stationary phase is entered as shown in Fig. 1a and 1b. When 2 % lignosulphonic acids are included in the sugar/salt mixture a similar fall in the pH to about 3.3 can be seen, but no subsequent rise in the pH is observed during the first 30 hrs. of fermentation.

It can be seen (Fig. 1c) that the concentration of sucrose does not reach zero within 30 hrs. of fermentation when 2 % lignosulphonic acids are present, but that no sugar can be detected after 20 hrs. fermentation in the absence of the lignosulphonic acids from the medium. Some of the inhibition of sucrose fermentation by lignosulphonic acids were, in pilot experiments with a constant pH of 4.5, found to be due to an acid effect of the lignosulphonic acids as the sugar level reached zero after about 28 hrs. of fermentation in these experiments. To obtain comparable conditions, a constant pH of 4.5 was therefore used in the remaining experiments.

Figs. 2 and 3 show the effects of including 2 % lignosulphonic acids in media containing various sugars, on the growth rate of the organism and on the removal of the sugars from the broth (hereafter called fermentation of the sugars by the organism, according to *Casida* 1964). It can be seen that under the conditions studied, the lignosulphonic acids have an inhibitory effect on the fermentation of the sugars by Candida utilis. The inhibitory effect is especially pronounced when xylose is used (Fig. 3). A difference can be seen in the fermentation of sucrose and the equimolar mixture of glucose and fructose when 2 % lignosulphonic acids were included in the broth (Fig. 2). The invert sugar is removed more rapidly than sucrose from the broth during the fermentation, and a higher cell yield is found when the invert sugar is used as the carbon source.

In Fig. 4, the inhibitory effects on the xylose fermentation in the presence of various concentrations of lignosulphonic acids are shown. An inhibitory effect was seen, when 0.5 % lignosulphonic acids were present, but not when 0.25 % were used.

Table 1 shows the figures for the peptide-precipitating ability of the lignosulphonic acids at different times during the fermen-

Table 1. Peptide-precipitation caused by lignosulphonic acids during fermentation of various sugar solutions with Candida utilis. The initial concentrations of lignosulphonic acids were 2 %.

Fermentation time (hrs.)	Carbon source: sucrose		Carbon source: invert sugar	
	precipitation zone diameters (mm)	absorbance (610 nm)	precipitation zone diameters (mm)	absorbance (610 nm)
0	12.0	0.90	11.5	0.90
2	11.5	0.88	11.0	0.89
4	11.0	0.88	11.0	0.95
6	12.0	0.91	11.0	0.90
10—12	11.0	0.90	12.0	0.89
15—17	11.5	0.86	11.0	0.86
20—24	11.0	0.92	12.0	0.92

Table 1 (continued).

Fermentation time (hrs.)	Carbon source: glucose		Carbon source: xylose	
	precipitation zone diameters (mm)	absorbance (610 nm)	precipitation zone diameters (mm)	absorbance (610 nm)
0	11.5	0.88	11.0	0.91
2	11.5	0.92	11.5	0.95
4	11.5	0.88	11.0	0.90
6	11.0	0.86	11.0	0.90
10—12	11.0	0.85	11.0	0.90
15—17	11.0	0.86	11.0	0.88
20—24	11.0	0.82	11.5	0.95

tation. No change in the peptide-precipitating ability of the lignosulphonic acids can be seen within 24 hrs. fermentation when neopeptone is used.

DISCUSSION

The observation that lignosulphonic acids inhibit the fermentation of sucrose, glucose and invert sugar with Candida utilis, is of particular interest, because *Lawrance & Fukui* (1956) found that the presence of calcium lignosulphonate did not affect the rates of oxidation of hexoses by the microorganisms which

were collected in a river into which sulphite waste liquor was discharged. A slight depression of the oxidation was first observed by these authors after five days of incubation. When xylose was used, however, a marked retardation in the rate of oxidation was found, which is in accordance with the results of the present study. The difference in the results could be due to the different preparation procedures of the lignosulphonic acids. *Lawrance & Fukui* prepared their lignosulphonic acids by calcium precipitation of sulphite spent liquor and dialysis, and such lignosulphonic acids are probably of relatively high molecular weight (*Hildrum & Næss* 1972). The lignosulphonic acid preparation used in the present work consisted of, in addition to those of high molecular weight, a considerable proportion of medium- and low-molecular weight lignosulphonic acids (*Næss et al.* in press). It is more probable, however, that the difference in the results is due to Candida utilis being more sensitive to the lignosulphonic acids than were the unspecified microorganisms used by *Lawrance & Fukui*. The river where the latter organisms were collected had had sulphite spent liquor discharged into it for 50 years and the microorganisms may thus have become adapted to an ecosystem where the lignosulphonic acids comprised an important part. A genotypic alteration followed by a natural selection of lignosulphonic acid resistant organisms may have occurred. Accordingly, care should be taken in single cell protein production to use strains of microorganisms which have become adapted to, or are selected as resistant to, the inhibiting effect of the lignosulphonic acids and other fermentation inhibitory substances in sulphite spent liquor. When testing the microorganisms, xylose should also be used as carbon source, because the effect of lignosulphonic acids on the xylose fermentation by the cells seems more significant than is the case with the other sugars used in this work. As the inhibitory effect of lignosulphonic acids upon the fermentation of xylose is especially pronounced when using high concentrations of the acids, some dilution of the sulphite spent liquor should be considered in single cell protein production, as a means of obtaining a more effective utilization of the xylose.

In further attempts to find microorganisms for single cell protein production from sulphite spent liquor, it should be possible to find more effective organisms for fermenting both hexoses and pentoses when searching in discharge areas into

which the waste liquor from factories not fermenting the liquor is released. On the other hand it could also be of importance to search for xylose- and other pentose-fermenting strains from discharge areas of alcohol distilleries using sulphite waste liquor (Saccharomyces cerevisiae ferments mainly hexoses) and use these in combination with effective hexose-fermenting strains in single cell protein production.

It has previously been shown that lignosulphonic acids inhibit the proteolytic activity of pepsin in vitro (*Næss* 1971c), and it could be assumed that the inhibition by them of the fermentation of sugars by Candida utilis is due to an effect on certain enzyme systems.

Invertase is an enzyme localized in the cell wall of many yeasts (including several Saccharomyces species) and is released extracellularly to a certain extent when the cells are adapted to sucrose as carbon source (*Frijs & Ottolenghi* 1959). *Frommer & Rauenbusch* (1970) have shown in their work on the invertase production of various yeasts that the extracellular invertase activity of yeasts grown on 6 % sucrose is slightly higher for Candida utilis than for Saccharomyces carlsbergensis and Saccharomyces cerevisiae (8.9 invertase units per 100 ml fermentation broth compared to 6.6 and 7.1, respectively, when corrected to equal cell yield), while the activity of invertase within the yeast cell remains relatively constant (0.7 invertase unit per g packed cells compared to 0.5 and 0.6 respectively). A considerable part of the invertase should thus be in contact with the lignosulphonic acids during the fermentation. The results of the present experiments with sucrose and invert sugar (Fig. 2) seem to indicate that the splitting of sucrose is inhibited only to a small degree by 2 % lignosulphonic acids in the medium. This result may, on the other hand, be due to intracellular hydrolysis of the sucrose catalysed by the invertase released into the yeast cell. It is assumed in this work that the lignosulphonic acids are not absorbed by the cell although sucrose is to some extent admitted into the yeast cell (*Frijs & Ottolenghi*). As the passage of sucrose through the cell membrane is not completely unhindered and because the intracellular invertase activity seems small compared to the extracellular, the difference in the fermentation of sucrose and invert sugar in the presence of lignosulphonic acids may be due to an inhibitory effect on the extracellular invertase activity of the yeast. The invertase activity is

an additional enzyme activity in the broth when using sucrose instead of invert sugar for the fermentation.

It can be seen from Table 1 that the fermentation has little, if any, effect on the peptide-precipitating ability of the lignosulphonic acids used, which indicates that the formation of precipitates with peptides and proteins in the ecosystems into which sulphite spent liquor is released is little influenced by the fermentation.

During the fermentation of sulphite spent liquor it is possible that some of the material of protein nature released by the yeast cells into the broth could form complexes with the lignosulphonic acids. Any peptide- or protein-lignosulphonic acid precipitate, however, will probably be dissolved subsequently because of the excess of lignosulphonic acids (*Næss* 1971a). Another possible reaction is that the lignosulphonic acids react with chemical groups of the cell walls, thus forming aggregates, and a higher sedimentation rate of cells could be expected. However, since the amount of peptide-precipitating lignosulphonic acids present in the supernatant after centrifugation of samples collected during fermentation is relatively constant and at a high level, the amount of lignosulphonic acids forming precipitates, or soluble complexes, with organic substances in the fermentation broth, must be small. On the other hand it has been shown that the content of lignosulphonic acids in peptide-lignosulphonic acid precipitates is relatively small, 10—20 % (*Hildrum & Næss*). Therefore a small amount of lignosulphonic acids may precipitate a greater amount of peptides or proteins.

Further investigations on the effect of lignosulphonic acids on the sedimentation rates of microorganisms would seem to be of interest also with regard to the harvesting of cells in single cell protein production.

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SAMMENDRAG

Vekselvirkninger mellom peptidpresipiterende ligninsulfonsyrer og fermentering av forskjellige sukkerarter med en stamme av Candida utilis.

En har studert fermentering av forskjellige sukkerarter med Candida utilis i blanding med rensede peptidpresipiterende ligninsulfonsyrer. En fant at fermentering av sukrose, glukose, xylose og en ekvimolar blanding av glukose og fruktose ble hemmet. Hemmingseffekten var mest uttalt når xylose ble brukt. Med 0,5 % ligninsulfonsyrer kunne en se hemming av xylosefermenteringen, men ikke når 0,25 % ble brukt.

Det ble funnet forskjell i den hemmende effekt på sukrosefermentering og på fermentering av en ekvimolar blanding av glukose og fruktose, og dette resultat er diskutert i relasjon til en mulig hemmingsmekanisme for ligninsulfonsyrer.

Ligninsulfonsyrenes peptidpresipiterende evne synes ikke å forandres under fermentering av de sukkerarter som er brukt.

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Reprints may be requested from: Bjørn Næss, Department of Microbiology and Immunology, Veterinary College of Norway, Postbox 8146, Oslo Dep., Oslo 1, Norway.