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# On the Formation of Reducing Sugars in Thermophilic Cellulose Fermentation

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The behavior of cellulose fermenting bacteria towards cellobiose and glucose is rather interesting. By checking the bacterial growth in crude cultures with chemicals or by suddenly changing the incubation temperature from 55 to 20° C Pringsheim [1] as early as 1912 was able to obtain these sugars as intermediate products in thermophilic cellulose fermentation. When the fermentation is carried out with crude cultures under normal conditions, however, these sugars seldom appear in the fermented medium, owing to consumption by the subsidiary flora. Not until recently when pure cultures of thermophilic cellulose bacteria became available was it possible to show that these bacteria produce glucose (Imsenecki [2], McBee [3]) or glucose and cellobiose (Enebo [4]) as regular fermentation products which are accumulated in the medium.

The present report deals with the formation of sugars in cellulose fermentation performed without special precautions by a pure culture of a cellulose fermenting thermophile and also with the action of this organism on sugars, chiefly glucose and cellobiose.

## A. FORMATION OF REDUCING SUBSTANCES IN THERMOPHILIC CELLULOSE FERMENTATION

### Methods

Fermentations were carried out anaerobically at 55° C under reduced $CO_2$-pressure. The medium consisted of an inorganic nutrient salt solution according to Simola [5] which also contained 2 % Bacto tryptone, 2 % autolyzed baker's yeast, 2 % precipitated chalk and 0.03 % thioglycollic acid. Cellulose wadding was used as cellulose source. Inoculation was always made with 5 % of a pure culture of a thermophilic cellulose bacterium, grown on the same medium (Enebo [6]). For further details concerning the fermentation method see Enebo [7].

*Table 1. Reduction values obtained when fermenting a 1 % cellulose suspension. (Incubation for 14 days at 55° C.)*

| ml 0.1 *N* sodium thiosulfate consumed per 5 ml medium in determination of reduction according to Schoorl | | | | | |
|---|---|---|---|---|---|
| I | II | III | After fermentation with baker's yeast | | |
| | | | IV | V | VI |
| Before hydrolysis | After hydrolysis | Per cent increase | Before hydrolysis | After hydrolysis | Per cent increase |
| 7.22 | 8.65 | 19.8 | 2.44 | 4.03 | 65.4 |

Determinations of reducing substance in the medium were performed according to Schoorl after purification of the solution with lead acetate. Cellobiose and higher saccharides were hydrolyzed by adding conc. HCl until a concentration of 10 % HCl was reached and then keeping the sample at 100° C for 60 minutes. Glucose was removed from mixtures of other sugars by fermentation with baker's yeast.

The increase in reduction after HCl-hydrolysis is, under the conditions mentioned above, ca. 46 % for a pure solution of cellobiose at a concentration corresponding to 2.44 ml 0.1 *N* thiosulfate per 5 ml sample. The low value under III in table 1 thus makes it probable that the fermented medium contains glucose together with higher saccharides. After fermentation with baker's yeast the reduction before hydrolysis has fallen to only one third of the initial value. The increase in reduction after hydrolysis is considerable (VI), indicating that besides the expected cellobiose the solution must contain higher oligosaccharides.

The presence of glucose and cellobiose was shown by the following procedure, based on the different solubility of these sugars in abs. alcohol:

250 ml of fermented medium were distilled *in vacuo* to a volume of 50 ml and then purified by means of lead acetate. The distillation was then continued to a volume of ca. 8 ml. After dilution with water to 10 ml, 90 ml abs. alcohol were added and the precipitate obtained removed by filtering. 100 ml alcohol were added to the filtrate and the mixture concentrated *in vacuo* to a volume of ca. 10 ml. After cooling a crystalline layer was found on the walls of the flask. The crystals were removed, washed with abs. alcohol and dissolved in a few ml of water (fraction I). To the remaining liquor 150 ml alcohol were added and the mixture again distilled to a volume of ca. 10 ml and again a precipitate was obtained. The remaining liquor after addition of water and removal of alcohol formed fraction II.

The method followed produced an enrichment of cellobiose in fraction I and of glucose in fraction II. A Schoorl-test showed that fraction I possessed a reductive power

*Table 2. Influence of different fermentation times on the formation of reducing sugars in a 1 % cellulose-suspension.*

| | ml 0.1 *N* sodium thiosulfate consumed per 5 ml medium in determination of reduction according to Schoorl | | | | | |
|---|---|---|---|---|---|---|
| I | II | III | IV | After fermentation with baker's yeast | | |
| | | | | V | VI | VII |
| Fermentation time (days) | Before hydrolysis | After hydrolysis | Per cent increase | Before hydrolysis | After hydrolysis | Per cent increase |
| 4 | 1.34 | 2.11 | 57.5 | 0.82 | 1.54 | 87.8 |
| 8 | 2.13 | 2.82 | 32.4 | 1.18 | 1.98 | 67.8 |
| 14 | 2.54 | 3.02 | 18.9 | 1.12 | 1.81 | 61.6 |

which after hydrolysis increased with ca. 50 per cent. Thus this fraction seemed to contain only cellobiose as reducing sugar.

Phenylosazones of the fractions I and II were prepared according to the direction given by van der Haar [8] and purified by repeated recrystallizations from alcohol-water and acetone-water solutions. The osazone of fraction II was washed with hot water in order to remove any traces of cellobiose-osazone.

The osazone of fraction I had the m. p. 197—198° C (corr.), corresponding to the generally accepted value for cellobiose-osazone [9]. For the osazone of fraction II the m. p. 204—205° C was obtained which is somewhat to low for glucose-osazone (m. p. 210° C [8]). Unfortunately the amount of the osazones were too small to permit a determination of their optical rotation.

Table 2 contains reduction values obtained for different fermentation times. As is shown in column II the total amount of reducing sugars increases during the fermentation. According to the values in the columns III and IV a further increase in reduction is obtained after hydrolysis, indicating the presence of higher sugars in the fermented medium. The percentage increase after hydrolysis becomes lower when the fermentation proceeds, showing that the relative amount of glucose gradually increases. Values corresponding to the original amounts of glucose before hydrolysis are obtained by subtracting the values of column II from those of column V. After 4 days the reduction by glucose thus was $0.52/1.34 = 39$ % of the total reduction. After 14 days the reduction by glucose had increased to $1.42/2.54 = 56$ % of the total reduction. Column VII shows that the higher sugars are slowly converted to lower sugars.

*Table 3. Results of fermentation of various sugars by a pure culture of a cellulose decomposing thermophile. Initial carbohydrate concentration 1 %. (Incubation for 10 days at 55° C, mean values of at least 3 experiments.)*

| Sugar | % of added sugar fermented |
|---|---|
| Cellobiose * | 95 |
| *l*-Arabinose | 46 |
| *d*-Xylose | 45 |
| *d*-Glucose | 33 |
| *d*-Fructose | 31 |
| Maltose | 28 |
| *d*-Mannose | 17 |
| Sucrose | 0 |

Even after 14 days the solution contains higher sugars other than cellobiose, as the increase in reduction after hydrolysis is much greater than is calculated for the latter. (For an amount of pure cellobiose corresponding to the value 1.12 ml 0.1 *N* thiosulfate in the Schoorl determination, the increase in thiosulfate consumption after hydrolysis should be ca. 48 % instead of 61.6 % as the table indicates.)

The enrichment of cellobiose and glucose in a cellulose medium on fermentation with a pure culture of a cellulose thermophile indicates that the hydrolytic activity of the bacteria is greater than the fermentative activity. Contrary to the report of McBee [3] these sugars are indeed formed during the fermentation and not only after fermentation has ceased.

## B. FERMENTATION OF CELLOBIOSE AND GLUCOSE WITH A CELLULOSE THERMOPHILE

The literature gives contradictory reports concerning the action of cellulose thermophiles on various sugars. Thus the pure culture of Imsenecki [2] was able to ferment glucose and to a slight extent maltose and sucrose. A culture of Rotmistrov [10], also described as pure, easily fermented several sugars. More recently McBee [3], working with provably pure cultures, has obtained fermentation of cellobiose but not of glucose.

* Remaining reduction is mainly caused by glucose, formed by the cellobiase activity of the bacteria.

*Table 4. Fermentation of cellobiose and glucose with a pure culture of a cellulose decomposing thermophile. Initial sugar concentration 1 %.*

| Fermentation time (days) | I. Fermentation of cellobiose % of added sugar consumed | II. Fermentation of glucose % of added sugar consumed |
|---|---|---|
| 2 | 43 | 10 |
| 3 | 78 | 16 |
| 4 | 90 | 21 |
| 6 | 94 * | 29 |
| 8 | 95 * | 34 |
| 10 | 97 * | 38 |

## Experimental

The present author has made sugar fermentations with the same pure culture of a cellulose decomposing thermophile that was used for the cellulose fermentations. The method was the same as described under A only with the cellulose changed for sugars (always sterile filtered, not autoclaved). The results of this test are in Table 3.

The table shows that the cellulose decomposing thermophile is not directed to cellulose only as carbohydrate source, as was once proposed. Contrary to the behavior of certain mesophilic cellulose decomposing bacteria their growth is not inhibited by comparatively high concentrations of fermentable sugars.

As is shown in Table 4 cellobiose is fermented at a rate which is several times as high as that for glucose.

The preference for cellobiose shown by the bacteria makes it probable that they are especially adapted to this sugar as a final product of the hydrolysis of cellulose. It is possible that this effect has a connection with the chemical structure of cellulose, in which cellobiose as well as glucose may be considered to form the ultimate unit. For some reason the bacteria may prefer to break up every second of the glucosidic linkages and then directly ferment the cellobiose formed. On the other hand the accumulation of glucose in the fermented medium indicates a certain cellobiase activity of the bacteria. As is shown in Table 5 part of the cellobiose, during fermentation of this sugar, is hydrolyzed to glucose.

* See also note to Table 3.

*Table 5. Cellobiase activity on fermentation of cellobiose with a pure culture of a cellulose decomposing thermophile. (Incubation for 4.5 days at 55° C.)*

| Expt. no. | Initial concentration of sugar % | ml 0.1 *N* sodium thiosulfate consumed per 5 ml medium in determination of reduction according to Schoorl | | | | | |
|---|---|---|---|---|---|---|---|
| | | Before hydrolysis | After hydrolysis | Per cent increase | After fermentation with baker's yeast | | |
| | | | | | Before hydrolysis | After hydrolysis | Per cent increase |
| 1 | 1.25 | 8.27 | 10.70 | 29.4 | 4.27 | 6.07 | 42.2 |
| 2 | 1.75 | 17.00 | 22.03 | 29.6 | 12.63 | 18.17 | 43.8 |

After the glucose is removed the increase in reduction after hydrolysis corresponds approximately to the value calculated for cellobiose. Synthesis of higher saccharides does not therefore appear to take place.

As an uninoculated blank with cellobiose kept at 55° C for 10 days showed no hydrolysis the glucose formation must be considered to be an entirely enzymatic effect.

## SUMMARY

This paper deals with the formation of lower sugars in thermophilic fermentation of cellulose using a pure culture of a cellulose decomposing bacterium and with fermentation of sugars, especially glucose and cellobiose by the same organism.

By repeated addition of alcohol to the medium from cellulose fermentation and evaporation *in vacuo* to a minute volume, cellobiose and glucose fractions were obtained. The formation of small amounts of saccharides higher than cellobiose could also be shown.

Experiments with fermentations of different sugars with the cellulose bacterium showed that the rate of fermentation was highest for cellobiose, followed by arabinose and xylose (equal), glucose, fructose.

Cellobiose was fermented at a rate, several times as high as that for glucose. During cellobiose-fermentation a certain cellobiase activity was observed.

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