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ALCOHOLIC FERMENTATION WITHOUT YEAST CELLS*

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Until now it has not been possible to separate fermenting activity from living yeast cells; the following describes a procedure that solves this problem.

One thousand grams of brewer's yeast¹ that had been cleaned as a prerequisite for the preparation of compressed yeast, but to which no potato starch had been added, is carefully mixed with the same weight of quartz sand² and 250 g Kieselguhr. It is then trituated until the mass has become moist and pliable. Now 100 g of water are added to the paste, it is wrapped in filter cloth and gradually subjected to a pressure of 400–500 atmospheres: 350 cc press juice are obtained. The residual cake is again trituated, sieved, and 100 g water are added. A further 150 cc of press juice result when the cake is again subjected to the same pressure in the hydraulic press. One kg of yeast hence yields 500 cc press juice, containing about 300 cc cell substances. Traces of turbidity are now removed by shaking the press juice with 4 g of Kieselguhr and filtering through paper with repeated refiltration of the first portions.

The resulting press juice is a clear, slightly opalescent yellow liquid with a pleasant yeast odour. A single determination of the specific gravity gave a value of 1.0416 (17°C). A large amount of coagulum separates upon boiling, so that the liquid almost completely

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¹ Any water adhering to the yeast has been removed to the point that no more water comes off at a pressure of 25 atm.

² Powdered glass is less suitable since it is weakly alkaline.

BUCHNER

solidifies. Insoluble flakes begin to form at only 35–40°; gas bubbles, shown to be carbon dioxide, are seen to rise even below this temperature. This gas hence saturates the liquid³. The press juice contains over 10 percent dry weight. A press juice prepared by an earlier, inferior procedure contained 6.7 percent dry weight, 1.15 percent ash and, judging from nitrogen content, 3.7 percent protein.

The most interesting property of the press juice is its capacity to institute fermentation of carbohydrates. Upon admixture of the same volume of a concentrated solution of cane sugar, a regular evolution of carbon dioxide starts after only 1/4 to 1 hour, and continues for days. The same behaviour is elicited by glucose, fructose, and maltose. No fermentation occurs, however, in mixtures of press juice with saturated solutions of lactose or with mannitol, consistent with the fact that these substances are not fermented by living brewer's yeast cells. After fermenting for several days, mixtures of press juice and sugar solution gradually turn turbid upon storage in the icebox, although no organisms are found with the microscope. On the other hand, 700-fold magnification shows plenty of protein coagula, probably precipitated by the acids formed during the fermentation. Fermentation is not prevented when the mixture of press juice and saccharose solution is saturated with chloroform, but this results in early slight protein separation. Similarly the fermenting activity is not abolished by filtration of the press juice through a sterilized *Berkefeldt-Kieselguhr* filter, a process that undoubtedly retains all yeast cells. Even at icebox temperature a mixture of the absolutely clear filtrate and sterilized cane sugar solution begins to ferment, albeit after about a day's delay. It is found that when a parchment paper tubing filled with press juice is hung in a 37 percent cane sugar solution, its surface after a few hours becomes covered with innumerable tiny gas bubbles. Naturally a vigorous formation of gas was also be observed inside the tubing because of diffusion of sugar solution. Further experiments must decide whether the carrier of the fermenting activity can in fact dialyse through the parchment paper as appears to be the case*. In time press juice gradually loses its fermenting activity. Thus upon storage for five days in a half-filled bottle, the activity of the juice

³ The plant physiologists may decide whether this carbon dioxide is possibly derived from oxidative processes connected with respiration.

*This observation was soon retracted in Buchner and Rapp [*Ber. Dt. Chem. Ges.* **31**, 209–217 (1898), p. 213], submitted exactly a year (31st January 1898) after the present paper [*Translators' note*].

ALCOHOLIC FERMENTATION WITHOUT YEAST CELLS

toward saccharose had been lost. It is remarkable, however, that press juice containing added cane sugar, i.e. press juice with fermenting activity, retains its activity in the icebox for at least two weeks. In this connection one should probably first consider that the carbon dioxide produced during the fermentation has a favourable effect, exerted by keeping atmospheric oxygen away; possibly, however, the readily assimilated sugar could also contribute towards the preservation of the agent.

Only a few experiments have been performed previously to learn something about the *nature of the active substance* in the press juice. When the press juice is heated to 40–50° one first of all observes development of carbon dioxide, and then a gradual separation of coagulated protein. The material obtained here was filtered off after an hour, and repeatedly refiltered. In one experiment, but not in another, the clear filtrate still possessed weak fermenting power. The active substance therefore either loses activity, even at this remarkably low temperature, or it coagulates and precipitates. In another experiment, 20 cc of the expressed juice were added to three volumes of absolute alcohol, and the precipitate was filtered by suction and dried *in vacuo* over sulphuric acid; the resulting 2 g of dry substance was only very slightly soluble upon stirring with 10 cc water. The filtrate obtained from this had no fermenting activity toward cane sugar. These experiments will have to be repeated; in particular an attempt will be made to isolate the active principle by means of ammonium sulphate.

As to the *theory of fermentation*, the following conclusions can be drawn. First, it is established that an apparatus as complicated as the yeast cell is not required to institute the fermenting process. Rather, the carrier of the fermenting activity of the press juice must be regarded to be a dissolved substance, undoubtedly a protein. This will be called *zymase*.

As early as 1858, *M. Traube* expressed the view that fermentation is brought about by a special protein derived from yeast cells. Later this *enzyme theory* or *ferment theory* was defended in particular by *F. Hoppe-Seyler*. At that time, however, the separation of such an enzyme from yeast cells had not been accomplished.

Even now the question remains open whether zymase can be added directly to the established list of enzymes. *C. von Nägeli*⁴ emphasized earlier that there are important differences between fermenting activity and the activity of the usual enzymes. The latter merely

⁴ *Theorie der Gährung* (Theory of Fermentation), Munich, 1879, p. 15.

bring about hydrolyses that can be imitated by the simplest chemical means. Although *A. v. Baeyer*⁵, by using analogies to relatively simple principles, has brought us nearer to an understanding of alcoholic fermentation as a chemical process, the decomposition of sugar into alcohol and carbon dioxide remains as one of the more complicated reactions. In this process carbon linkages are broken with a completeness that has not until now been attained by other means. In addition, a significant difference exists in the heat effect of the reaction.⁶

Invertin can be extracted from yeast cells that had been killed by dry heat (1 hour to 150°) and isolated upon precipitation with alcohol as a powder that is readily soluble in water. In this manner it is not possible to obtain the substance that brings about fermentation. Yeast cells that had been heated to such high temperatures probably contain no more of it; alcohol precipitation, if the preceding experiment permits a conclusion, converts it into a water-insoluble modification. Hence it will scarcely be wrong to assume that zymase should be viewed as a genuine protein, and that it is much closer than invertin to the living protoplasm of yeast cells.

The French bacteriologist *Miquel* has expressed similar views about urase*, the enzyme excreted by the bacteria that carry out so-called urea fermentation. He designates this enzyme directly as protoplasm that has done away with the protection of the cell wall, that in addition functions outside the cell wall, and that by and large differs from the protoplasm of the cell contents only in this manner⁷. In addition, one has to include here the experiments of *E. Fischer* and *P. Lindner*⁸ concerning the effect on cane sugar of the yeast fungus *Monilia candida*. This fungus ferments saccharose, but neither *Ch. E. Hansen* nor the above authors could obtain an aqueous extract from the fresh or from the dried fungus that contained an invertin-like enzyme able to carry out the preceding cleavage into glucose and fructose. The experiment proceeded quite differently when *Fischer*

⁵ These *Berichte* **3**, 73.

⁶ *A. Bouffard* has recently again determined the heat developed by budding yeast during alcoholic fermentation. *Compt. rend.* **121**, 357.

* This term, rather than urease, is used here [*Translator's note*].

⁷ In this connection one has to note, however, that the so-called urea fermentation, the decomposition of urea to ammonia and carbon dioxide, differs chemically in an extraordinary fashion from the actual fermentation processes. Therefore many do not consider it as a fermentation. It is a simple hydrolysis that can be brought about by water alone at 120°.

⁸ These *Berichte* **28**, 3037.

ALCOHOLIC FERMENTATION WITHOUT YEAST CELLS

and *Lindner* used fresh *Monilia* in which a part of the cells had been opened up by means of careful grinding with glass powder. Now one could observe an unmistakable inverting activity. "In this case, however, the inverting agent seems not to be a stable water-soluble enzyme but a constituent of the living protoplasm."

It is true that the fermentation of sugar by zymase can occur inside the yeast cells⁹; it is more likely, however, that the yeast cell has secreted this protein into the sugar solution¹⁰. The events that occur during alcoholic fermentation may perhaps therefore be regarded as physiological only because the zymase is excreted by the living cells. *Nägeli*¹¹ and *O. Löw* have shown that already after 15 hours at 30° considerable amounts of proteins, coagulable by boiling, diffuse out of yeast cells in a nutrient solution that, initially weakly alkaline (by means of K_3PO_4), later becomes neutral. In fact, as shown in the above experiment, zymase can apparently go through parchment paper.

FERMENTATION EXPERIMENTS

| No. | Press juice (cc) | Carbohydrate Solution (cc) | Sugar content (%) | Experimental Temperature | Remarks |
|-----|------------------|----------------------------|-------------------|--------------------------|---|
| 1 | 30 | Saccharose 30 | 37 | Ice-box | After 1 hour distinct gas development, not yet complete after 14 days. The froth layer eventually is 1 cm high. |
| 2 | 50 | " 50 | 37 | " | Strong gas development and froth layer. After 3 days without a precipitate the initially clear solution becomes opaque. |

⁹ The diosmotic conditions make this appear possible. Compare *v. Nägeli*, l.c., p. 39.

¹⁰ In this way one can probably also explain the experiments of *J. de Rey-Pailhade* (*Compt. rend.* **118**: 201) who prepared a weakly alcoholic (22 percent) extract from fresh baker's yeast to which some glucose had been added. After removal of microorganisms via filtration through a sterile *Arsonval* candle, this sugar-containing extract spontaneously developed carbon dioxide in the absence of oxygen.

¹¹ l.c., p. 94. The experiments were repeated successfully; however, it was found that they occur not only in saccharose but also in lactose solutions. Therefore, contrary to these authors' assumption, the diffusion processes are not linked to the fermenting activity.

BUCHNER

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|----|-----|----------|-----|-------|------------|---|
| 3 | 150 | " | 150 | 37 | " | After 3 days the froth layer is 3/4 cm high. |
| 4 | 20 | " | 20 | 37 | " | Gas development becomes visible after 2 hours and is not complete after 14 days; the initially clear solution at the end shows only minimal turbidity; froth layer 1 1/2 cm high. |
| 5 | 30 | " | 30 | 37 | " | Gas development begins after 1 day and the initially clear solution shows only minimal turbidity at the end; during this time solution is still completely clear. |
| 6 | 20 | " | 20 | 37 | Room temp. | After 1 hour vigorous gas development; even after 2 weeks there is still slight gas bubble formation with only minimal turbidity. |
| 7 | 20 | " | 20 | 37 | 40° | After 2 hours already 10 cm high froth layer; after 1 day strong coagulum separation; gas formation is complete |
| 8 | 30 | " | 30 | 12 | Ice-box | After 6 days still strong gas formation; in addition, turbidity consisting of very fine coagulum. |
| 9 | 5 | Maltose | 5 | 33 | " | After 1 hour start of gas development that still continues after 12 days |
| 10 | 10 | " | 5 | 26 | " | Gas development is extraordinarily strong already after 3 hours. |
| 11 | 10 | Glucose | 10 | 33 | " | Strong gas development only after 20 hours, but persists after 12 days; froth layer 3/4 cm high. |
| 12 | 10 | " | 5 | 26 | " | Already after 1/2 hour rather strong gas development lasting 12 days the solution becomes turbid and deposits some precipitate (see below). |
| 13 | 10 | Fructose | 10 | 37 | " | Very strong gas development after only 1/4 hour and still vigorous after 3 days; the solution remains clear. |
| 14 | 10 | " | 5 | 25 | " | Considerable frothy layer after only 15 minutes, measures 1 cm after 3 days. |
| 15 | 10 | Lactose | 10 | Sat.. | Room temp. | No gas development, not even after 6 days. |
| 16 | 10 | Mannitol | 10 | " | " | As for lactose. |

ALCOHOLIC FERMENTATION WITHOUT YEAST CELLS

NOTES

In experiment 1, the escaping gas, 4 hours after it began to develop, was passed into lime water and identified as *carbon dioxide*. In experiments 2 and 3, the *alcohol* formed after 3 days of fermentation was identified: in experiment 2, 1.5 g, and in 3, 3.3 g ethyl alcohol were present. In this calculation, the amounts of alcohol that still adhered to the yeast from beer manufacture were deduced. In experiment 2 the yeast was washed 4 times with 5-litre portions of water before preparation of the press juice; then the alcohol was determined in 2/3 of the whole, and the rest was worked up for press juice. The results showed the yeast to contain at the most 0.3 g alcohol. In experiment 3 commercial brewer's yeast that had been purified for compressed yeast manufacture but containing no added starch was worked up directly; yeast required for preparing 150 cc of press juice was shown by analysis to contain 1.2 g of alcohol. Hence 1.2 g of alcohol were formed by fermentation in experiment 2, and 2.1 g in experiment 3. In all cases the alcohol was identified by the iodoform reaction and it was finally salted out of the aqueous solution by means of potash. The precipitate obtained in experiment 3 distilled completely between 79–81° (734 mm); the distillate was colorless, combustible, and possessed the odour of ethyl alcohol.

Microscopic investigation was performed on experiments 2 and 3 after they had proceeded for 3 days, while the slight sediment in experiment 8 was examined after 6 days of fermentation, and in experiment 12 after 12 days; in all cases, the more or less strong turbidity was due not to organisms but merely to coagulated protein. Furthermore, in experiment 3, interrupted after 3 days, 6 *plate cultures* were started. Portions of 1 cc of the liquid were inoculated in each of 3 tubes containing liquefied beer spice gelatin, and 1 cc portions in 3 tubes of liquefied meat water peptone gelatin. After 6 days one of the former plates showed 11 colonies while the two others had remained sterile; each of the three peptone gelatin plates showed uniformly 50-100 colonies and had become liquefied. In view of the large volumes inoculated in these experiments, the results prove that the fermenting activity was not due to microorganisms; this conclusion, besides, already follows from the rapid appearance of the fermenting process.

Finally, in experiments 4 and 5 *the press juice was filtered by suction through sterilized Berkefeldt-Kieselguhr filters*. Furthermore, in experiment 5 the cane sugar solution had been sterilized in the autoclave, and the two liquids were mixed under completely aseptic conditions.

It has been found that the method described above for press juice preparation can also be used for obtaining the contents of bacterial cells. The corresponding experiments are proceeding with pathogenic bacteria at the Hygiene Institute in Munich.

Tübingen, 9th January 1897