†Lateral axis and dorsi-ventral axis about equal.

2. *Picea rubra* (Lamb.) Link. Strengthening cells very thick walled, occurring in a single row, sometimes doubled or tripled at the angles. Some strengthening cells occur within the sheath.

**Ducts *none* to *two*.

3. *Picea pungens* Engelmann. Strengthening cells thick walled, some thick-walled cells occurring singly or in groups between the stomata, with tendency to double row at the angles.

4. *Picea Engelmanni* Engelmann. Strengthening cells in a single row, sometimes doubled at the angles; thick walled. A single cell sometimes occurs within the bundle sheath.

***Ducts *none* to *one*.

5. *Picea Canadensis* (Will.) B. S. P. (=Picea alba Link.). Strengthening cells in a single row sometimes doubled at the angles; very thick walled: some occur within bundle sheath.

Although the above synoptical arrangements appear to be conclusive within themselves they are valuable only in conjunction with the external features.

A Proteolytic Enzyme of Yeast.

By Katherine E. Golden.

INTRODUCTION.

The enzymes are auxiliary substances which are formed where solid bodies are to be liquefied. They are peculiar in that they decompose complex substances without being affected themselves in any way by the action, and also that even in minute quantities they can produce very marked results. They are important in animal and plant metabolism and occur both in the cells and in solution in secretions of the cells. In the case of unicellular organisms, the metabolic processes are carried on throughout their entire substance, the food substance being absorbed into the cell, where the enzyme is formed and does its work. This, however, is not always the case, the enzyme sometimes being excreted, the work of absorption following its action. This latter process is peculiar to multicellular organisms, having certain parts differentiated for special work,

the enzymes being formed in one set of cells, and excreted into the parts where the absorption of food takes place.

The composition of the enzymes is not known, some inclining to the view that they are of a proteid nature, while others think that they are nucleo-proteid, but as yet it is not definitely settled, as the enzymes have not been obtained in a pure condition, their reactions being connected with those of the substances with which they are associated. On account of this lack of knowledge as to their composition the enzymes have been classified according to the substances which they decompose.

The proteolytic enzymes are those which decompose proteids into less complex substances. There are two classes of these, the peptic and tryptic enzymes. The peptic enzymes decompose proteids to peptones, while the tryptic enzymes go farther, effecting the decomposition of the peptones to amides. In plants the amides are formed as antecedents to proteids, helping in the reconstruction of proteids as well as aiding in their osmosis by decomposition. When the carbohydrate, which was united with the amide, forming the proteid, is used up, the amide unites with a fresh carbohydrate, again forming a proteid. The enzyme in the plant, which causes this decomposition, is often in such minute quantity that it is almost impossible to determine its presence.*

Only a very few of the vegetable proteolytic enzymes have been investigated, and nearly all of these are from the higher plants. Those which have been investigated minutely have been found to be of a tryptic nature. A few of the fungi have also been found to secrete a proteolytic enzyme among these yeast.[1] It is claimed that if yeast be deprived of both oxygen and food material that it breaks up its own reserve proteid, and also, that if yeast be pressed, and the extract collected and heated to 45° C., a bulky coagulum is formed, which disappears in a few days, the extract in the meantime being kept under antiseptic conditions. The digestion of the reserve proteid and the disappearance of the coagulum indicate the presence of a proteolytic enzyme.

Proteolysis by yeasts has been noted but indirectly, except in the case of the pressing from the yeast of an extract by Büchner.[2] Jörgensen,[3] in describing S. Jörgensenii and S. membranaefaciens states that the

* Kerner and Oliver. Natural History of Plants. Vol. I, part 2.

[1] Green, J. R. The Soluble Ferments and Fermentation, 1899, pp. 215–217.

[2] Büchner, E. Ber. d. deut. chem. Gesell., 1897.

[3] Jörgensen, A. Micro organisms and Fermentation, 1893.

yeasts cause a slow liquefaction of wort gelatine; and Frankland,[4] in his description of S. liquefaciens, states that it liquefies gelatine fairly rapidly. This action of the three yeasts indicates the excretion of proteolytic enzymes.

Though the statement relative to the extraction of a proteolytic enzyme from yeast is made of pressed yeast, no particular species being named, and there are various pressed yeasts, yet only in the three cases cited has the direct liquefaction of gelatine been noted.

EXPERIMENTS.

Among some wort gelatine yeast cultures I found one which had liquefied the gelatine. On examining the culture it proved to be contaminated by another yeast from the air. The yeasts were separated, and when grown apart, the "wild" yeast was found to be the one which caused the liquefaction. Cultures were made into both the ordinary beef broth gelatine and wort gelatine[5] to determine the constancy of this characteristic of the yeast. Tube and plate cultures of both kinds of gelatine showed liquefaction, the wort gelatine, however, being liquefied sooner than the beef gelatine. From thirty to forty days were required to liquefy a tube containing 6 cc. wort gelatine. The liquefaction was not uniform, even when conditions of media and temperature were alike. Wort gelatine plate cultures became liquefied in about two weeks. These results show undoubtedly the excretion of a proteolytic enzyme by the yeast.

Investigations conducted by Fermi[6] have shown that antiseptics in small amounts are not injurious to enzymes. This property is taken advantage of in the testing for enzymes, and also in determining, relatively, their strength. Water is saturated with thymol to which 5 per cent. of gelatine is added, then placed on a water bath until the gelatine is dissolved, after which 10 cc. are placed in tubes. These are ready for use as soon as the gelatine sets.[7]

To test the strength of the enzyme produced by the yeast, I obtained extracts by filtering the liquefied gelatine from tube cultures. In the first experiment 3 cc. of the extract were used in each tube of thymol gelatine

[4] Saccardo, P. A. Sylloge Fungorum, Vol. VIII, pp. 916-922.
[5] Wort to which is added 7% gelatine. The wort had .23% acid, estimated as lactic.
[6] Lafar, F. Technical Mycology, Vol. I, 1898, p. 300.
[7] Same as ref. 6.

and a small amount of thymol added to the extract to prevent any further development of the yeast. The liquefaction of the thymol gelatine did not run quite uniformly: In twenty-five days one tube had 8 cc. gelatine liquefied, another had 7½ cc., while a third had 7¼ cc. A second set having extract from cultures six weeks old, in ten days, one liquefied 2 cc. gelatine, a second one 2.2 cc., and a third one 2.5 cc.

Wort in which yeast had been grown for ten days was filtered and 3 cc. of the filtrate used in thymol gelatine tubes, but this was very weak in enzyme. In ten days a cup-shaped depression was formed in the top of the gelatine, but no further action could be discerned. Both the wort gelatine and wort extracts were turbid when placed in the thymol gelatine. It required eight days for the wort extract to become clear and ten days for the wort gelatine extract.

As has been said already, the proteolytic enzymes are of two kinds, the peptic and the tryptic, the pepsin of the gastric juice and the trypsin of the pancreatic juice being taken as types. Besides differing in their decomposition products, they differ in other respects. Pepsin can act only in the presence of dilute acid and is injured by the presence of even a small quantity of the alkaline salt, Na_2CO_3, which is most favorable to the action of trypsin. Trypsin can also act in neutral or slightly acid solutions. A neutral salt in solution is deleterious to both enzymes, but especially so to pepsin, though according to Edkins[5] trypsin is aided by the presence of from 1 to 2 per cent. NaCl, though greatly retarded by 8 per cent. The vegetable trypsins which have been investigated are most active in faintly acid solutions.

In determining the kind of ferment, whether of a peptic or tryptic nature, the thymol gelatine was used for control, and the thymol gelatine with 1 per cent. NaCl, and 1 per cent. Na_2CO_3 added. Tubes of egg albumen were also used.

The following table shows the result of the experiment:

[5] Green, J. R. Fermentation, 1899, p. 193.

S. LIQUEFACIENS EXTRACT.

Experiment.	Age of Culture.	No. cc. Extract.	Time of Clearing.	Time of Liquefaction.	No. cc. Gelatine Lique.
Thymol gelatine	52 days.	2.5	6 days.	17 days.	5.50
Thymol gelatine + 1% NaCl	52 days.	2.5	3 days.	17 days.	6.00
Thymol gelatine + 1% Na_2CO_3	52 days.	2.5	4 days.	17 days.	2.70
Thymol gelatine	50 days.	1.5	1½ days.	17 days.	4.50
Thymol gelatine + 1% NaCl	50 days.	1.5	5 days.	17 days.	4.75
Thymol gelatine + 1% Na_2CO_3	50 days.	1.5	3 days.	17 days.	1.50
Thymol gelatine	40 days.	1.5	4 days.	17 days.	5 00
Thymol gelatine + 1% NaCl	40 days.	1.5	3 days.	17 days.	7.00
Thymol gelatine + 1% Na_2CO_3	40 days.	1.5	2 days.	17 days.	1.75
Egg albumen	52 days.	2.5	6 days.	17 days.	1.00
Egg albumen	50 days.	1.5	8 days.	17 days.	Cup-shaped depression.

Average—T. g., 5.00; T. g. + 1% NaCl, 5.92; T. g. + 1% Na_2CO_3, 1.98.

As indicated in the table, the presence of Na_2CO_3 seems to aid in the enzymic action, as the liquefaction was greater in each case when this was present than in the tubes without any salt. The extract in all the tubes, with the exception of that containing Na_2CO_3 gave a slightly acid reaction. The Na_2CO_3 seemed to hinder the action somewhat, as that was so much lower than either of the others.

In the work of Hahn,[9] and of Hahn and Geret,[10] it would seem as if they draw conclusions in regard to the presence of proteolytic enzymes in pressed yeast from somewhat indefinite causes. In the one case Hahn used pressed yeast, mixing it with *kieselguhr* and squeezing from it a liquid in the same manner as Büchner extracted his zymase. This liquid was treated with chloroform, to which was added gelatine and a trace of phenol. The extract liquefied the gelatine. Then Hahn and Geret used extract obtained in the same way with chloroform alone, keeping the solution at 37° C. for several weeks. The chloroform served to precipitate the proteids and keep the solution free from living organisms. A bulky precipitate was formed, which gradually disappeared. The liquid again became turbid, the second turbidity being due to the formation of amido compounds (tyrosin and leucin). From these experiments they conclude that they have extracted a proteolytic enzyme from the yeast. If the pressed yeast consisted of yeast only, there would be no question in regard to the results, but pressed yeast always contains a relatively large number of bacteria and a few moulds. Among the bacteria one is pretty sure to find some liquefiers.

To test for the presence of liquefiers I made some gelatine plate cultures from pressed yeast; and a description of one which contained only one colony of a liquefying bacterium will serve to indicate the power of the enzyme which was excreted. When the liquefying colony was first noted it was ½ mm. in diameter but at the end of twelve hours it had liquefied a spot 19 mm. in diameter; in twenty-three hours the spot had increased to forty-seven mm. in diameter and in forty-eight hours the gelatine of the entire plate was liquefied. Cultures were made into beef gelatine and the gelatine (6 cc.) was liquefied in forty-eight hours. The liquefied gelatine from a tube was filtered, and the filtrate used to determine enzymic action of the bacteria as in the former experiments for the yeast. At the same time 60 grams of pressed yeast were mixed with

[9] Hahn, M. Ber. d. deut. chem. Gesell., 1898, No. 2, pp. 200-201.
[10] Hahn, M., and Geret, L., l. c., pp. 202-205.

emery and ground in a mortar for an hour. Then a mash of the mixture was made with 20 cc. distilled water. The mash was put in a press and squeezed, the extract being filtered. The extract was used as in the former experiments, in addition to which 10 cc. were heated to 45° C. to precipitate the proteid matter.

The same quantity of pressed yeast was made into a mash with 20 cc. dis. water, saturated with thymol, but without any previous grinding. The extract from this was used in the same manner as that obtained from the ground yeast. This was allowed to stand for one hour, then pressed and filtered, the filtrate used as in the former experiment.

The purpose of this experiment was to determine if it be necessary to crush the yeast cells in order to obtain the enzyme. The following table shows the results obtained in the various experiments.

COMPRESSED YEAST EXTRACT, GROUND.

Experiment.	Age of Culture.	No. cc. Extract.	Time of Clearing.	Time of Liquefaction.	No. cc. Gelatine Liq.
Thymol gelatine	.....	1.5	.....	13 days.	2.0
Thymol gelatine + 1% NaCl	.....	1.5	.....	13 days.	3.0
Thymol gelatine + 1% Na_2CO_3	.....	1.5	.....	13 days.	3.0
Thymol gelatine	.....	5.0	11 days.	17 days.	2.0
Thymol gelatine + 1% NaCl	.....	5.0	Not clear.	17 days.	2.5
Thymol gelatine + 1% Na_2CO_3	.....	5.0	Not clear.	17 days.	3.0
Egg albumen	.....	1.5	.....	13 days.	Shallow depression.
Egg albumen	.....	5.0	Not clear.	17 days.	1.0
Yeast solution, heated to 45° C.	.....	10.0	Not clear.	17 days.	No apparent action.

Average—T. g., 2.0; T. g. + NaCl, 2.75; T. g. + Na_2CO_3, 3.00.

COMPRESSED YEAST, WITHOUT GRINDING.

Thymol gelatine	.....	5.0	Clear at start.	8 days.	2.0
Thymol gelatine + 1% NaCl	.....	5.0	Clear at start.	8 days.	1.0
Thymol gelatine + 1% Na_2CO_3	.....	5.0	Clear at start.	8 days.	3.5
Yeast solution	.....	10.0	Clear at start.	8 days.	No apparent action.

BACTERIAL EXTRACT.

Experiment.	Age of Culture.	No. cc. Extract.	Time of Clearing.	Time of Liquefaction.	No. cc. Gelatine Liq.
Thymol gelatine	4 days.	1.5	Clear at start.	13 days.	2.25
Thymol gelatine + 1% NaCl	4 days.	1.5	Clear at start.	13 days.	2.00
Thymol gelatine + 1% Na_2CO_3	4 days.	1.5	Clear at start.	13 days.	2.75
Thymol gelatine	7 days.	3.0	Clear at start.	8 days.	4.00
Thymol gelatine + 1% NaCl	7 days.	3.0	Clear at start.	8 days.	1.75
Thymol gelatine + 1% Na_2CO_3	7 days.	3.0	Clear at start.	8 days.	3.00

Average—T. g., 3.12; T. g. + NaCl, 1.87; T. g. + Na_2CO_3, 2.87.

The results of the experiments show that the pure yeast excretes a proteolytic enzyme that is fairly active, and from the fact that it works in the presence of neutral and alkaline salts, it must be of a tryptic nature. It seems to be of the same nature as the trypsin extracted by Edkins, since it works best in the presence of NaCl.

The experiments on the compressed yeast and the bacteria obtained from the compressed yeast show undoubtedly the presence of an enzyme, but the indications point more strongly to a bacterial than to a yeast origin, since it was not necessary to break the yeast cells before the pressing in obtaining the enzyme, and also, since in experience with pure yeast cultures, only three cases have been noted in which any perceptible enzymic action took place. Then the bacterial extract was very strong, so that though only a comparatively small number of bacteria are present in the compressed yeast as compared with the yeast, the activity of the extract would be accounted for. Then again though the bacterial and compressed yeast extracts did not act uniformly, they showed the same peculiarity in the greater activity of the extract in the presence of Na_2CO_3. Work with a mixture of organisms is always open to the doubt in regard to the action of each organism.

DESCRIPTION OF THE YEAST.

The cells of this species are very variable in shape, being round, elliptic, elongated and irregular, slender at one end and widening out toward the other, or showing projections from the sides (Ill. 1). These irregularities occurring to the greatest extent in wort gelatine cultures (Ill. 2). In wort the cells become much elongated and are in long chains (Ill. 3), while in lactose solution the round cells predominate and occur mostly in pairs. Occasionally giant cells are found in the cultures. The cells which are round in a lactose solution, when placed in wort in a moist chamber, lengthen inside of twenty-four hours. (Ills. 4, 5.)

They vary in size, the round cells averaging 3.3 μ in diameter, while the average of the elongated are 3.3 μ by 10 μ.

This yeast does not ferment sucrose or lactose. It forms a fairly heavy sediment in the sucrose, but only a slight growth in the lactose. In dextrose it required six days for fermentation to start and twenty-four hours to form 3 cc. gas. In wort, which contains maltose, fermentation started in four days, and 25 cc. of gas were formed in three days, when the cul-

ture was kept at room temperature, but at 30° C., gas is given off in twenty-four hours.

In wort gelatine tubes the growth tapers from the surface along the needle track, having fine line of growth radiating from the main growth, then the gelatine gradually breaks down with the liquefaction.

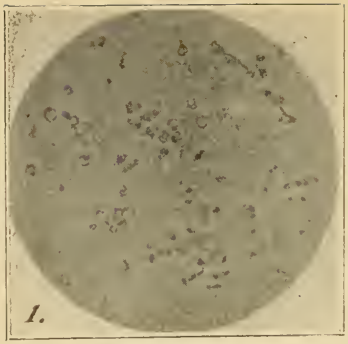
The colonies at first are rather thick in the center with filaments radiating from the central mass. When liquefaction begins, which is inside of three days, the central mass breaks down and spreads as a sort of mycelial mass over the plate, resembling very strongly a mould growth when seen under the microscope; Ills. 6, 7, 8 show successive stages in the growth and liquefaction.

The species is undoubtedly S. liquefaciens, as described in Saccardo,[11] the cells showing the same variations and varying only slightly in size from that description.

EXPLANATION OF ILLUSTRATIONS.

1. Ten days' growth in wort. x320.
2. Two weeks' growth in wort gelatine. x450.
3. Sixteen days' growth in wort gelatine. x320.
4. Twenty-four hours' growth in wort in moist chamber. x334.
5. Round cells from lactose solution in moist charmber. x320.
6. Colony grown in wort gelatine, three days old. x30.
7. Colony grown in wort gelatine, four days old. x35.
8. Colony grown in wort gelatine, four days old. x35.

[11] Saccardo, P. A. Sylloge Fungorum, Vol. VIII, pp. 916-922.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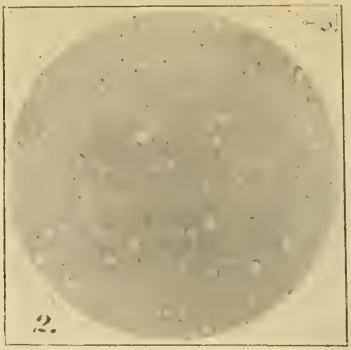
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