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THE PROTEOLYTIC ACTION OF BACILLUS GRANULOBACTER PECTINOVORUM AND ITS EFFECT ON THE HYDROGEN-ION CONCENTRATION[1]

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RECEIVED APRIL 21, 1924 PUBLISHED SEPTEMBER 5, 1924

This paper presents the results of a study of the nitrogen metabolism of *Bacillus granulobacter pectinovorum* when grown in corn mash.

The literature on the nitrogen metabolism of bacteria has been brought up to date by De Bord[2] and by Wagner, Dozier and Meyer[3] and need not be repeated here. In most of these investigations the object has been either to learn what compounds of nitrogen can be utilized by bacteria or to determine what degradation products are formed by the breaking down of the nitrogenous compounds. In some cases the latter information has been sought as a means of classifying bacteria. The hydrolysis products of proteins not only serve as a source of nitrogen for bacteria but because of the buffer action of proteoses, peptones and amino acids play a role in regulating the hydrogen-ion concentration of the medium.

Although vegetable proteins are both abundant and widely distributed in nature, the action of bacteria on these proteins is comparatively unknown. One of the chief reasons for this lack of information is the fact that most bacteria grow but little in a medium where these proteins are the sole source of nitrogen. *B. granulobacter pectinovorum*, however, attacks the proteins of corn, wheat and other cereals with great vigor and brings about very extensive hydrolysis. The products formed from carbohydrates and the general fermentation characteristics of this micro-organism have been extensively studied by Speakman[4] and his associates and to a less extent by Reilly, Hickinbottom, Henley and Thaysen.[5]

Experimental Part

Medium.—The bacteria were grown in 5% corn mash prepared by stirring the meal into cold water, steaming for one hour and then sterilizing for two to three hours. Fermentation is noticeable in 2–4 hours after inoculation, reaches its height in about 20–24 hours, and continues vigorously for 24–36 hours longer. At the end of 72 hours the fermentation is practically complete.

[1] This work was supported in part by a grant from the special research fund of the University of Wisconsin. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] De Bord, *J. Bact.*, **8**, 7 (1923).

[3] Wagner, Dozier and Meyer, *J. Infectious Diseases*, **34**, 63 (1924).

[4] Speakman, *J. Biol. Chem.*, **41**, 319 (1920); **43**, 401 (1920); **58**, 395 (1923). *J. Ind. Eng. Chem.*, **12**, 581 (1920). Robinson, *J. Biol. Chem.*, **53**, 125 (1922).

[5] Reilly, Hickinbottom, Henley and Thaysen, *Biochem. J.*, **14**, 229 (1920).

Forms of Nitrogen and Methods of Analyses.—The total and soluble nitrogen were determined by the Kjeldahl method and the insoluble obtained by difference. The soluble nitrogen was divided into protein and non-protein nitrogen by means of Folin and Wu's tungstic acid method.[6,7]

The filtrate from the tungstic acid precipitation was separated into ammonia nitrogen by the Folin[8] method and into free and combined amino nitrogen by Van Slyke's[9] procedure. The undetermined nitrogen was calculated by difference. The ammonia nitrogen proved to be so small in amount that it was omitted in many of the analyses.

The soluble nitrogen in the corn meal was determined by extracting 500 g. of corn meal with 1000 cc. of distilled water for four hours in a shaking machine. At the end of this time the solids were filtered off and the filtrate was used for the given determinations.

Soluble Nitrogen in a Fermented Culture.—In order to gain some idea of the extent of the proteolysis, the soluble nitrogen was determined in 10 fermented cultures. The results obtained are calculated for 100 g. of corn meal dried at 100° and are as follows: total, 1.65; soluble, minimum 1.04, maximum 1.24, av. 1.13 g. Of the total nitrogen more than 65% was in soluble form. If allowance is made for the soluble nitrogen in the corn meal (13%) it will be seen that more than 50% of the total nitrogen in the corn meal is rendered soluble during the 72–96 hours of fermentation. An especially vigorous fermentation showed at the end of 24 hours 38.8% of soluble nitrogen, of which 15.0% was in the form of amino nitrogen.

Proteolysis, Hydrogen-Ion Concentration and Titratable Acid

Series 1.—Twelve flasks were inoculated at the same time; each contained 350 cc. of 5% corn mash, and was inoculated with 10 cc. of inoculum taken from a single flask. At four different periods three flasks were combined to give a uniform sample for analysis. The first analysis of the mash was made 12 hours after inoculation, while the acidity was still increasing rapidly, the second after 24 hours of fermentation, or immediately after the acidity began to fall, and the third and fourth at the end of 48 and 96 hours, respectively. It was felt that this timing of the analyses would fall at the most important periods in the course of the fermentation.

The data are given in Table I, and disclose a rapid and continuous hydrolysis of the proteins. The soluble nitrogen increases uniformly from 210 mg. per 100 g. of corn at the beginning to about 1000 mg. at the end of 96 hours. The rate of increase is approximately 200 mg. in 24 hours.

The increase in soluble nitrogen is at first mainly in the form of protein or of intermediate products. There is very little amino acid or peptide production during the first 12 hours and not much during the first 24 hours. After this the intermediate products increase more slowly while the amino acid and peptide nitrogen increase rapidly. After 48 hours the quantity of soluble protein and intermediate products decrease, while the other forms continue to increase. At the end of 96 hours the bulk of the ni-

[6] O. Folin and H. Wu, *J. Biol. Chem.*, **38**, 81 (1919).

[7] Hiller and Van Slyke, *ibid.*, **53**, 253 (1922).

[8] Hawk, "Practical Physiological Chemistry," P. Blakiston and Co., **1921**, p. 519.

[9] Van Slyke, *J. Biol. Chem.*, **12**, 275 (1912).

trogen is still in the form of proteoses, peptones and peptides. The ammonia nitrogen rises from nothing at the beginning to 20 mg. at the end of the fermentation.

TABLE I

FORMS OF NITROGEN AT VARIOUS STAGES DURING THE FERMENTATION OF CORN MASH

Calculated for 100 g. of corn dried at 100°

Culture	Time after inoc. Hrs.	Insol. N Mg.	Sol. N Mg.	Sol. N pptd. by tungstic acid Mg.	*Forms of nitrogen in* tungstic filtrates: Free NH_2 nitrogen Mg.	Peptide N Mg.	Rest N Mg.	Reaction: Titr. acid[a] Cc.	Reaction: H-ion value P_H
				SERIES I					
A	0	1435	210	120	38	16	36	0.2	6.0
A	12	1320	325	215	42	21	43	2.8	4.4
A	24	1192	453	273	49	48	76	4.4	4.1
A	48	970	675	378	90	102	94	1.8	4.4
				SERIES II					
A	96	657	988	325	133	229	281	1.5	4.6
	Days								
A	180	393	1252	417	319	385	131	...	...
B	8	381	1401	343	365[b]	...	...	...	...
B	12	290	1488	355	491[b]	492	150	...	...
B	21	272	1494	353	461[b]	416	264	...	...
Percentage of total av.		23.4	76.6	20.8	20.3	22.1	12.4	...	...

[a] 0.1 *N* acid in 10 cc. of mash.

[b] Free NH_2N in the total soluble nitrogen was 445, 568 and 536 mg., respectively.

The longer fermentations of Series II show some increase in proteolysis over Series I but the digestion is completed mainly within four days. While there is considerable variation in the figures for the several forms of nitrogen obtained from different cultures an average shows that almost equal amounts, about 20%, of protein-free amino and peptide nitrogen are formed.

It is somewhat difficult to compare the proteolytic activity of this bacillus with that of other strongly proteolytic organisms owing to the fact that most of these have been grown in a medium containing a much higher percentage of nitrogen than we employed. The recent work of Wagner, Dozier and Meyer with *B. botulinus*, *B. sporogenes* and *B. tetani*, shows an extensive proteolysis of beef heart and peptic digest. These investigators used a medium containing about 1% of nitrogen and found that approximately 50% of this was converted into non-protein nitrogen and 16% into amino nitrogen during 6 days of fermentation. Even these unusually high figures are exceeded by those given in Table I. In eight days the granulobacter organism converted 60% and 20% of the total nitrogen into non-protein and amino nitrogen, respectively.

That the soluble nitrogen is not due to the action of the acetic and

butyric acids produced during the fermentation was proved by adding equivalent amounts of these acids to uninoculated corn mash and incubating at 37° for 5 days. Analysis of this acidified mash failed to show any increase in soluble nitrogen beyond that found in the untreated control.

The rise and fall in titratable acid and the relatively constant Sörensen (P_H) value after the first few hours are shown in the last two columns of the table. The slight buffer action of the corn meal is indicated by the fact that during the first four or five hours the Sörensen value drops quickly, although the titratable acid shows only a slight increase. In the succeeding hours the Sörensen value changes but little while the titratable acidity rises rapidly to a maximum, approximately 5.0 cc. of 0.1 N per 10 cc. of mash and then falls as acetone and butyl alcohol are rapidly produced. The increase in acid without a corresponding decrease in the Sörensen value may be accounted for by the buffer action of the protein hydrolysis products and the low dissociation of acetic and butyric acids.

Distribution of Soluble Nitrogen as Indicated by Various Precipitants

A series of flasks were inoculated with a third culture, C, and were used to determine the different forms of soluble nitrogen precipitated by various protein precipitants. According to Hiller and Van Slyke,[7] tungstic acid precipitates not only the native proteins but also the chief digestion products while trichloro-acetic acid leaves the derived proteins in solution. Phosphotungstic acid has long been used as a precipitant for proteins, proteoses, peptones and diamino acids. In Table II are given the data on both the weight and percentage basis for the different forms of nitrogen at the end of 96 hours' fermentation.

TABLE II

FORMS OF SOLUBLE NITROGEN IN A FERMENTED CORN MASH AS DETERMINED BY VARIOUS PROTEIN PRECIPITANTS

Calculated for 100 g. of corn dried at 100°[a]

Reagent	Sol. N pptd. by reagent		Forms of N not pptd.					
			Free NH_2 nitrogen		Peptide N		Undeter. N	
	Mg.	%[b]	Mg.	%[b]	Mg.	%[b]	Mg.	%[b]
None	0	0	366	22.2	271	16.5	225	13.7
Phosphotungstic acid	357	21.7	346	21.1	4	0.2	155	9.4
Mercuric sulfate	77	4.7	352	21.3	241	14.7	192	11.7
Trichloro-acetic acid	42	2.6	361	21.9	244	14.8	215	13.1

[a] Total N, 1645 mg.; soluble N after fermentation, 862 mg.

[b] Percentage of total nitrogen.

In one case, the analysis was made without precipitating the soluble proteins. The free amino nitrogen was only 20 mg. greater than when phosphotungstic acid was used to remove the proteins, peptides and diamino acids.

Trichloro-acetic acid and mercuric sulfate remove but little of the hy-

drolysis products, while phosphotungstic acid, as might be expected, leaves practically no peptides in solution. The large quantity of free amino nitrogen in the filtrate from the latter precipitation indicates the existence of amino acids in solution. This indication was verified by the isolation of glutamic acid. A more extended examination will doubtless show the presence of other amino acids in the free state.

Summary

In the fermentation of corn mash *B. granulobacter pectinovorum* brings about a rapid hydrolysis of the proteins. From 50 to 75% of the total protein is converted into soluble products during the fermentation which is approximately complete in from 3 to 4 days. One-half of the total soluble products may be formed in 24 hours.

The hydrolysis results chiefly in the formation of simple peptides and amino acids. Due to these buffers and to acids of low dissociation, a high titratable acidity may be produced without causing much change in the hydrogen-ion concentration.

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SOME NEW AROMATIC ORTHOFORMATES

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RECEIVED APRIL 29, 1924 PUBLISHED SEPTEMBER 5, 1924

Although numerous alkyl orthoformates have been described, the phenyl ester is the only simple aromatic compound of this type known.[1] It has been obtained by Tiemann[2] and by Auwers[3] as a by-product of the Reimer-Tiemann reaction. Tiemann stated that attempts were being made to prepare similar compounds from other phenols, but nothing else was published on the subject.

It has recently been shown[4] that phenyl orthoformate can be conveniently prepared by the action of chloroform on dry potassium phenolate at a fairly high temperature. This method has now been employed for the preparation of the *o*-, *m*- and *p*-tolyl esters from the corresponding cresols. The yields of these compounds are poor, and their purification is rendered difficult by the fact that they are accompanied by comparatively large quantities of dark, viscous, alkali-insoluble glues. The

[1] Since the completion of this work, the author finds that Keil [*Ann.*, **352,** 273 (1907)] records, in a footnote, the formation of the *p*-tolyl ester as a by-product of the action of chloroform on *p*-cresol in alkaline solution.

[2] Tiemann, *Ber.*, **15,** 2686 (1882).

[3] Auwers, *Ber.*, **18,** 2657 (1885).

[4] Baines and Driver, *J. Chem. Soc.*, **125,** 907 (1924).