

The Amylase of *Clostridium acetobutylicum*

II. Adsorption¹

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The purification and concentration of enzymes by adsorption on charcoal, tannin, aluminum hydroxide, ion exchange resins, and so forth, has been an established practice in the laboratory. Some years before this work was done in 1949, alkali-cooked lignin was used as an adsorbent for mold and malt amylase (Wallerstein *et al.*, 1945).

In this paper is described our study of the adsorption of the amylase of *Clostridium acetobutylicum* on alkali-cooked lignin and charcoal, elution of the enzyme from the adsorbents, and the underlying relationships governing the adsorption.

General considerations. With treatment time and temperature fixed, the relationship between the amount of adsorbate taken up and the amount of adsorbent used is frequently given by the empirically derived Freundlich equation (Bull, 1943): $X/M = kC^{1/n}$, where X represents the units of enzyme held by the adsorbent, M is the weight of adsorbent used, C is the concentration of enzyme remaining unadsorbed, and k and n are constants determined by the nature of the enzyme and adsorbent and the conditions of adsorption. Thus, the Freundlich equation correlates the adsorptive capacity per unit weight adsorbent (X/M) with the concentration of adsorbate remaining in solution (C). The slope of the Freundlich equation on a log-log plot is $1/n$, which is an index of the ease or difficulty with which the enzyme is adsorbed.

The Langmuir equation has been derived from adsorption in a layer one molecule thick. If a straight line results from plotting C against C/M , the adsorption may be assumed to be proceeding according to the assumptions in the derivation of the Langmuir equation.

EXPERIMENTAL METHODS AND RESULTS

Adsorption on alkali-cooked lignin. *C. acetobutylicum* strain Weizmann, that had been cultured for 48 hr on 5 per cent corn meal, was centrifuged; aliquots of the clear supernatant were adjusted to pH 4.2 and 4.0 with citric acid crystals, and adjusted to pH 5.3 and 6.3 with crystalline sodium citrate. For preliminary ex-

periments, 5 ml of the pH adjusted supernatant were added to different tubes in a series. Graded amounts of alkali-cooked lignin solution were then added to each series of tubes. The lignin solution was a 5 per cent solution, prepared by dissolving the lignin in 1.0 per cent sodium hydroxide and adjusting the pH to 8.4 with sulfuric acid. The lignin, being insoluble in acid solutions, precipitated as a fine, almost colloidal suspension. The mixtures were allowed to remain at room temperature for 20 min and then centrifuged. The clear supernatants were removed and the enzyme was eluted from the lignin by 5 ml 0.04 M phosphate buffer at pH 6.5. After 20 min had been allowed for elution, the tubes were again centrifuged as before and the eluates removed.

The amylase activities of the supernatants and the eluates were determined by the method which was developed previously (Scott, 1950). The results are presented in table 1 and figure 1. The Freundlich equation plots comprise figure 2.

A comparison of the slopes of the Freundlich isotherms in figure 2 shows that adsorption is more efficient at pH 4.0 than at either 3.2 or 5.3. If we assume a symmetrical curve for efficiency versus pH, the peak efficiency comes at about pH 4.5. Furthermore, an examination of the desorption data (see table 1) indicates that maximal desorption is achieved when the adsorption was performed at pH 4.0 to 4.5.

The pH of the fermentation mash after 40 hr is between 4.0 and 4.5. Thus, lignin adsorption seems ideal from the standpoint that no preliminary pH adjustment of the culture filtrate is needed. The yield of enzyme concentrated and purified by adsorption on and elution from lignin is quite good. Consider a typical experiment. Three and one-half L of culture filtrate containing 1610 amylase units were mixed with 400 ml 5.0 per cent lignin. The pH of the precipitation was the same as the end pH of the fermentation, namely, 4.5. The lignin-enzyme complex was centrifuged down, separating as a slurry. The slurry was transferred to a Buchner funnel and the remaining free liquid sucked out through a Whatman no. 3 paper. The entire pasty brown complex was then transferred to a beaker and extracted with three successive 125-ml portions of 0.1 strength MacIlvain's buffer, pH 6.5. The

¹ This work was done in 1949 at the Illinois Institute of Technology, Department of Biology. Another part of this study was published in 1952 (Scott and Hedrick, 1952).

three eluates were combined to give a total volume of 350 ml. The total activity of the eluate was 1370 amylase units, a 10-fold concentration with an 85 per cent recovery of amylase.

Adsorption on infusorial earth. In early work in this research, difficulty was encountered in obtaining active cell-free filtrates. One of the methods used for clarifying the culture filtrate was the addition of infusorial earth. When acid-washed infusorial earth was used, the filtrate was very low in amylase activity. This drop in activity was not due to inactivation because the lost activity could be eluted from the filter

TABLE 1

Adsorption of amylase on varying amounts of alkali-cooked lignin at various pH values

pH	Lignin	Amylase in Supernatant	C	X	X/M	Amylase in Eluate	C'	X'	X'/M
	mg	units				units			
3.2	0	0.650	100	0	—	—	—	—	—
	1	0.320	49	51	51	0.300	46	5	5
	2	0.189	28	72	36	0.450	70	2	1
	3	0.100	15	85	28	0.550	85	0	0
	4	0.060	9	91	23	0.600	92	-1	0
	5	0.025	4	96	19	0.625	96	0	0
	6	0.012	2	98	16	0.635	98	0	0
	7	0.005	0.7	99.3	14	0.638	99	0	0
4.0	0	0.650	100	0	—	—	—	—	—
	1	0.420	65	35	35	0.230	35	0	0
	2	0.270	41	59	30	0.380	59	0	0
	3	0.185	28	72	24	0.465	72	0	0
	4	0.130	20	80	20	0.525	80	0	0
	5	0.090	14	86	17	0.560	86	0	0
	6	0.070	11	89	15	0.580	89	0	0
	7	0.050	8	92	13	0.600	92	0	0
	8	0.030	5	95	12	0.620	95	0	0
	9	0.020	3	97	10.7	0.630	97	0	0
	10	0.010	1.5	98.5	9.8	0.640	98.5	0	0
5.3	0	0.650	100	0	—	—	—	—	—
	1	0.525	81	19	19	0.005	1	18	18
	2	0.435	67	33	17	0.015	3	30	15
	3	0.365	56	44	15	0.035	5	39	13
	4	0.290	45	55	14	0.080	12	43	11
	5	0.230	35	65	13	0.125	19	46	9
	6	0.185	28	72	12	0.190	29	43	7
	7	0.145	22	78	11	0.280	43	35	5
	8	0.095	15	85	10.6	0.360	55	30	3.8
	9	0.055	8	92	10.2	0.440	68	24	2.7
	10	0.001	1	98.5	9.8	0.490	76	23.5	2.4
	11	0	0	100	9.1	0.575	88	12	1.1
6.3	0	0.650	100	0	—	—	—	—	—
	10	0.650	100	0	—	—	—	—	—
	12	0.645	99	1	0.08	—	—	—	—
	14	0.630	97	3	0.21	0.0063	1	2	0.15
	16	0.610	94	6	0.36	—	—	—	—
	20	0.530	81	19	0.95	0.10	15	4	0.20
	25	0.440	68	32	1.3	0.17	26	6	0.24

C = per cent initial activity in supernatant; X = 100 - C.
C' = per cent initial activity in eluate; X' = X - C.

pad. Adsorptions were conducted as previously described for adsorption on lignin at pH 4.5. The results are given in table 2.

Thus, infusorial earth itself could serve as an adsorbent. The Freundlich equation does not apply in this case.

The major importance of this discovery is to serve as a warning against the use of infusorial earth in enzyme work without first determining its adsorbent properties.

Adsorption on activated charcoal. Because of the well known high adsorptive capacity of activated charcoal, this substance was tried as an adsorbent for this amylase. An activated charcoal of high purity (Nu-char C)² was chosen for this work. The results as given in table 3 indicate that activated charcoal can be used for the adsorption of the amylase. This adsorption does not obey the Freundlich equation.

Consider a typical example of adsorption of the amylase on activated charcoal. The pH of 1850 ml culture filtrate containing 850 amylase units was adjusted to 6.5 by the addition of a mixture of 26 g mono-basic potassium phosphate and 10 g dibasic potassium

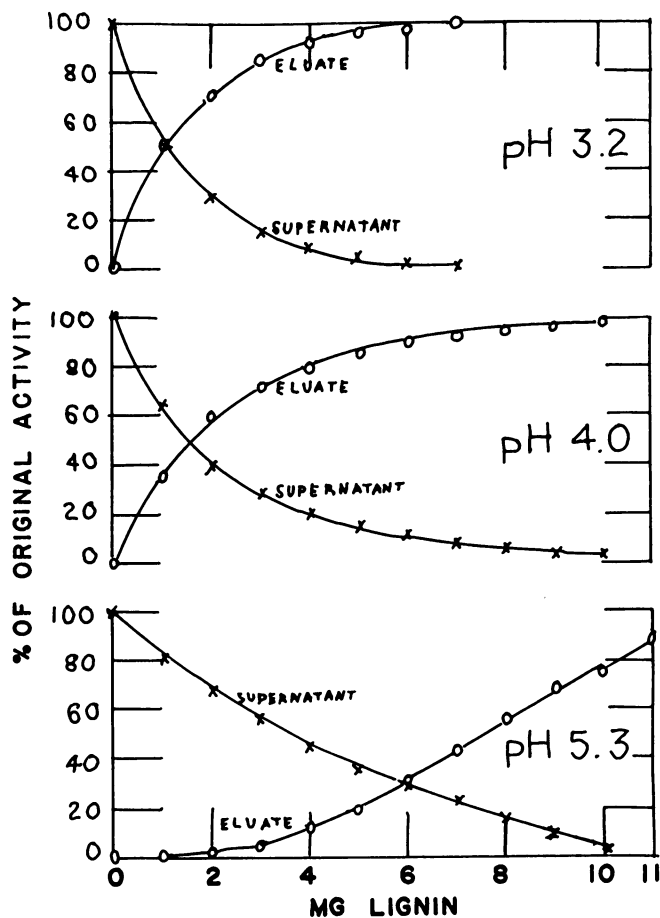


Figure 1. The adsorption of amylase on alkali-cooked lignin; distribution of activity between supernatant and eluate.

² West Virginia Pulp and Paper Company, Chicago, Illinois

phosphate. One g activated charcoal was added. The carbon-enzyme complex was filtered off by a Whatman no. 3 paper with the aid of filter paper pulp. This pulp-paper-carbon-amylase mixture was extracted with four successive portions of 100 ml each of acetate buffer, 0.05 M at pH 4.6. The combined volume of the eluate fractions was 370 ml, equivalent to 20 per cent of the original volume. However, the total activity of the eluate was only 50 units, a yield of only 6 per cent. Since the enzyme could not be eluted under conditions where it could be adsorbed for solution, this represents a case of irreversible adsorption.

DISCUSSION

The adsorption data also indicate the probable isoelectric point of the enzyme. Figure 3 represents a plot of the amount of lignin needed to adsorb 30 per cent of the activity of the preparation (used for table 1) versus pH. The curve approaches pH 6.5 a-

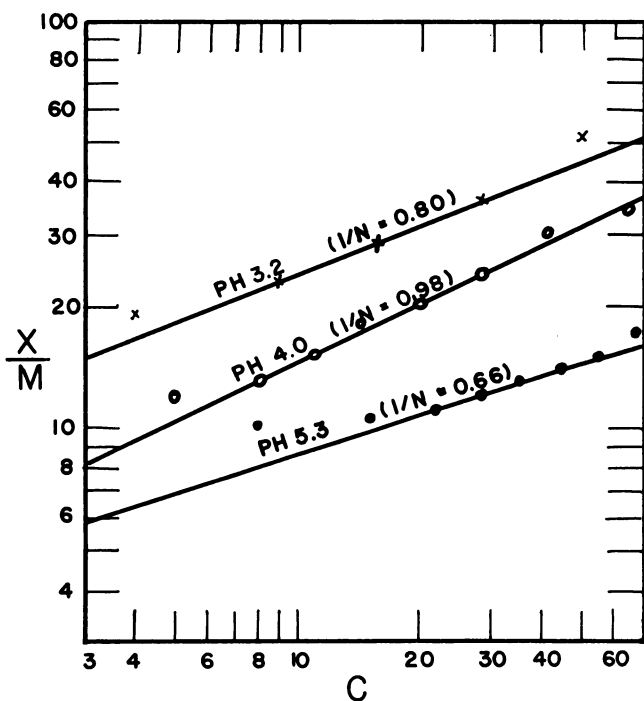


Figure 2. Plot of Freundlich equation for lignin adsorption of amylase at varying pH values.

TABLE 2

Adsorption of amylase on infusorial earth

Infusorial Earth per 5 ml	Amylase in Supernatant	C	X	X/M
mg	units			
0	2.00	100	0	—
2	1.63	82	18	9
8	1.55	77	23	3
14	1.42	71	29	2
20	1.06	53	47	2.4
40	0	0	100	2.5

symptotically. This would indicate that the net charge on the enzyme changes at this pH since the net charge on the lignin does not change at any pH value below 8. Furthermore, the fact that activated carbon adsorbs the enzyme very strongly at pH 6.3, and not at all at pH 4.5, indicates that the isoelectric point of the enzyme is about 6.5. This is in direct contradiction of the belief expressed by Wilson (1939) that the isoelectric point of an enzyme is very close to the pH of its maximal activity.

TABLE 3

Adsorption of amylase on activated charcoal

pH	Nuchar C per 30 ml	Amylase in Supernatant	C	X	X/M	C/M
	mg	units				
4.5	0	6.2	100	—	—	—
	300	6.2	100	—	—	—
6.5	0	6.2	100	—	—	—
	2.5	6.2	100	—	—	40
	5.0	5.8	93	7	1.4	18.6
	7.5	3.95	64	36	4.8	8.7
	10.0	2.3	37	63	6.3	3.7
	12.5	1.55	25	75	6.0	2
	15.0	0.93	15	85	5.7	1
	17.5	0.59	9.5	91.5	5.2	0.5
25.0	0	0	—	—	—	

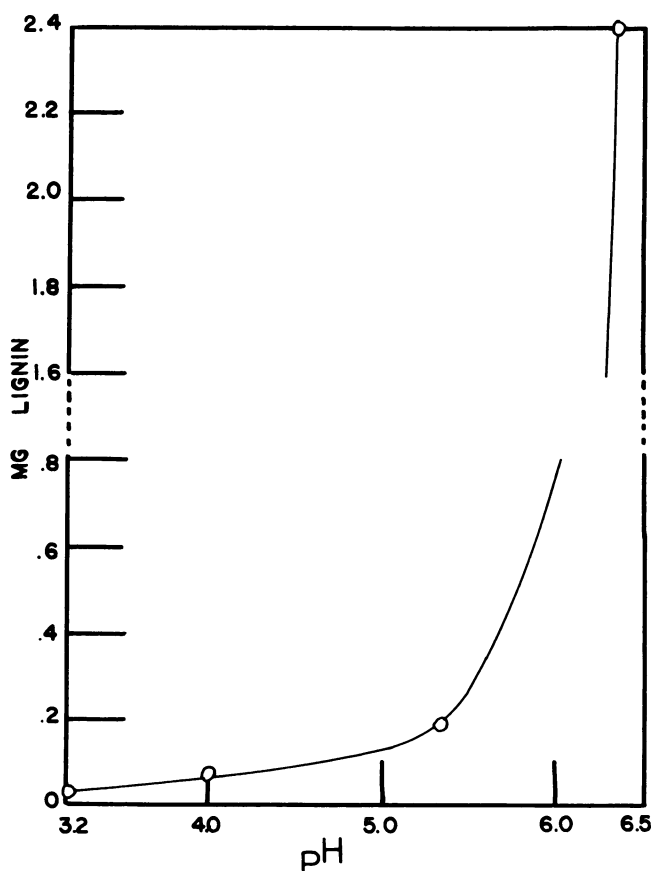


Figure 3. The amount of lignin needed to adsorb 30 per cent of the amylase activity at varying pH values.

The fact that charcoal adsorbs the enzyme strongly at pH 6.5 and not at all at pH 4.5 at first appears surprising inasmuch as lowering the pH enhances the adsorptive action of lignin and infusorial earth. This observation appears somewhat more logical when we consider the fact that activated charcoal adsorbs amphoteric substances (such as enzymes or proteins) best at the isoelectric point of the adsorbate (Hassler, 1941).

SUMMARY

Alkali-cooked lignin may be used for the adsorption of *Clostridium acetobutylicum* amylase, because the enzyme is readily eluted with 0.04 M phosphate buffer at pH 6.5. The adsorption and elution of the enzyme conforms to the Freundlich equation.

The enzyme may also be adsorbed by activated charcoal, however elution is difficult, thus suggesting an example of irreversible adsorption of the enzyme by the activated charcoal. The enzyme is adsorbed by in-

fusorial earth, but the Freundlich equation is not applicable.

Of the adsorbents tested, alkali-cooked lignin proved to be the most practical since no preliminary adjustment of pH is required and good yields may be obtained.

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Microbiology of Meat Curing

II. Characteristics of a *Lactobacillus* Occurring in Ham Curing Brines Which Synthesizes a Polysaccharide from Sucrose¹

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In a previous publication Deibel and Niven (1957) reported that lactobacilli comprised the most common bacterial contaminants found in commercial ham curing brines that were examined. The brine samples were obtained from commercial establishments and represented curing brines that had been in use for 3 to 30 days. These brines were freshly prepared prior to application as a cover pickle for the hams. The containers were the customary large wooden tierces which, along with the meat, provided the initial microbial contamination. Although the total bacterial population attained in any of the batches tested ap-

peared to be too low to be of any significance in the curing and processing of the hams with respect to flavor development or deterioration, it is of interest to record the nature of the salt-tolerant bacteria that are able to grow in the brines provided appropriate times and temperatures are employed.

The most common *Lactobacillus* noted was a motile, homofermentative, salt-tolerant, low-acid-producing variety that had characteristics in common with both *Lactobacillus casei* and *Lactobacillus plantarum*, and yet possessed features which readily distinguished it from either of these two established species (Deibel and Niven, 1958).

Another common *Lactobacillus* found in all of the brines tested was a rather unique group that was capable of synthesizing large amounts of a polysaccharide from sucrose, as manifested by the production of large mucoid colonies on sucrose-gelatin agar. The purpose of this paper is to describe this apparently

¹ Journal paper no. 164, American Meat Institute Foundation. This research was undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number 939 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsements of the Department of Defense.