

Factors affecting lecithinase activity and production in *Clostridium welchii**

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INTRODUCTION

Interest in *Clostridium welchii* food poisoning was generated by the work of Hobbs *et al.* (1953). In order to explain the pathogenicity of *Cl. welchii* in food poisoning, Nygren (1962) proposed that phosphorylcholine, the end product of the action of lecithinase upon lecithin, was responsible for the symptoms of food poisoning. On the other hand, Dische & Elek (1957) and Dack (1947) have suggested that large numbers of living organisms were responsible for clinical disease.

Although the role of lecithinase produced by *Cl. welchii* in food poisoning is unclear, Weiss & Strong (1967) and Nakamura & Cross (1968) studied the lecithinase production by strains of *Cl. welchii* isolated from food-borne outbreaks.

Collee, Knowlden & Hobbs (1961) reported that optimum growth of *Cl. welchii* occurred at temperatures between 43° and 47° C. Boyd, Logan & Tytell (1948) obtained maximum growth of this organism after 16–20 hr. of incubation. The optimum temperature for growth of *Cl. perfringens* may not be identical with the optimum temperature for lecithinase production.

The lecithinase of *Cl. welchii* is resistant to inactivation by heat. Macfarlane & Knight (1941) reported that lecithinase preparations retained 45% of their activity when exposed to 100° C. Smith & Gardner (1949) found that lecithinase was greatly inactivated at 65° C. but could be partially reactivated by further heating to 100° C. They suggested that enzymically inactive complexes linked by calcium ions were formed at 65° C. However, at 100° C. these complexes dissociated with the liberation of active lecithinase. Purified lecithinase, but not culture filtrates, were heat-sensitive (Kushner, 1957). Weiss & Strong (1967) reported that lecithinase retained more activity after heating at 100° C. than at 75° C. They noted that the degree of heat resistance of lecithinase was somewhat dependent upon the strain of *Cl. welchii* studied.

Optimum lecithinase activity was observed at pH 7.0–7.6 (Macfarlane & Knight, 1941). However, Smith & Gardner (1949) demonstrated that lecithinase activity occurred at a pH range of 5.0–9.0.

Very little is known about the nutritional factors that stimulate lecithinase production by *Cl. welchii*.

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In this paper we describe studies on the effect of time and temperature of incubation on lecithinase production by *Cl. welchii*. We also describe experiments on the effects of heat and pH on lecithinase activity. Studies on the stimulation of lecithinase production by lecithin, in a chemically defined medium, are also described.

GENERAL METHODS

Assay of lecithinase activity

The lecithinase activity was assayed using the lecithovitellin reaction method of van Heyningen (1941) as modified by Nakamura & Cross (1968). In the modified method the lecithinase activity is reported as $\mu\text{g./ml.}$ of lecithinase in culture filtrates as determined from a standard assay curve using commercial purified lecithinase (Nutritional Biochemicals Corp., Cleveland, Ohio).

Some of the data on the lecithinase activity are recorded in terms of changes in optical density produced as a result of the effect of lecithinase upon the egg-yolk-saline substrate. The readings were made with a Coleman Junior, Model 6C, spectrophotometer with the wavelength set at 650 $m\mu$.

Culture media

Complex medium for lecithinase production by *Cl. welchii* consisted of trypticase, 20 g.; yeast extract, 5 g.; soluble starch, 2.5 g.; sucrose, 1 g.; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.; $\text{Fe}_2(\text{SO}_4)_3 \cdot n\text{H}_2\text{O}$, 0.1 g.; distilled water, 1000 ml. The pH was adjusted to 6.8.

Lecithinase production was studied in a chemically defined medium. The medium was commercially available tissue-culture medium NCTC 109 (Grand Island Biological Company, Grand Island, N.Y.) to which synthetic glycylglycine (Nutritional Biochemicals Corp., Cleveland, Ohio) was added in varying concentrations. The pH of the medium after pressure filtration was 7.2.

EXPERIMENTAL STUDIES

THE EFFECT OF TEMPERATURE AND LENGTH OF INCUBATION ON LECITHINASE ACTIVITY OF *CLOSTRIDIUM WELCHII*

Weiss & Strong (1967) incubated cultures of *Cl. welchii* at 37° C. and found that lecithinase activity reached a peak between 2–4 hr. and then decreased rapidly to a minimum in 6–10 hr.

In this study we have extended the time of incubation and varied the temperature of incubation in order to determine the lecithinase activity under various conditions.

Methods

Three strains of type A *Cl. welchii* were used in these studies. Strain UMJS-12 was isolated from human faeces. Strain UMJS-39 was isolated from soil. Strain UMKK-29 was isolated from cold cut meat. The strains were identified on the basis of morphological and biochemical characteristics.

Screw-capped test-tubes containing 10 ml. of complex medium for lecithinase production were inoculated with 1 ml. of an 18–24 hr. thioglycollate broth culture of each strain. The tubes were incubated at 10°, 30°, 46°, and 52° C. The culture filtrates were assayed for lecithinase activity after 2, 5, 10, 30 and 60 hr. of incubation.

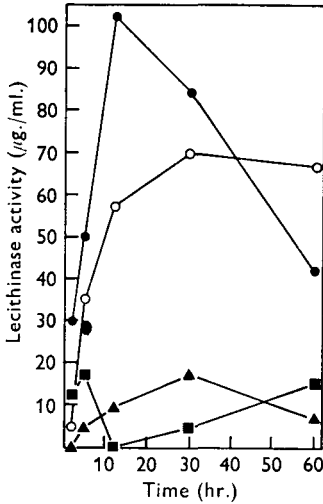


Fig. 1

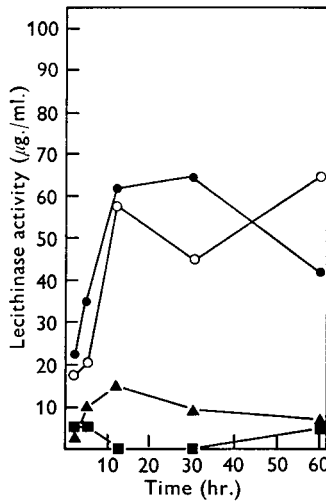


Fig. 2

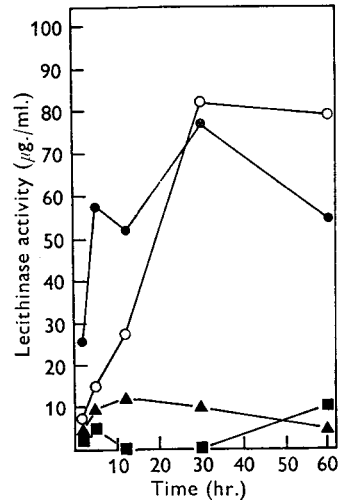


Fig. 3

Fig. 1. The effect of time and temperature of incubation on the lecithinase activity of *Cl. welchii* strain UMJS-12. ●—●, 46° C.; ○—○, 30° C.; ■—■, 52° C.; ▲—▲, 10° C.

Fig. 2. The effect of time and temperature of incubation on the lecithinase activity of *Cl. welchii* strain UMKK-29. ●—●, 46° C.; ○—○, 30° C.; ▲—▲, 10° C.; ■—■, 52° C.

Fig. 3. The effect of time and temperature of incubation on the lecithinase activity of *Cl. welchii* strain UMJS-39. ●—●, 46° C.; ○—○, 30° C.; ▲—▲, 10° C.; ■—■, 52° C.

Results

The lecithinase activity of *Cl. welchii* was time- and temperature-dependent. The results from the three strains studied are presented in Figs. 1–3. The optimum time and temperature of incubation for lecithinase production varied slightly with each strain. Strain UMJS-12 produced maximum lecithinase activity after 12 hr. of incubation at 46° C., whereas strain UMKK-29 produced maximum lecithinase activity after 12–30 hr. of incubation at this temperature. Strain UMJS-39 produced maximum lecithinase activity after 30–60 hr. of incubation at a temperature of 30° C. Lecithinase activity was negligible at 10° and at 52° C., the lower and upper limits for growth of *Cl. welchii*. There were no viable cells present in cultures incubated at 52° C. However, small amounts of lecithinase were detected. It is possible that the cells used in the inoculum possessed lecithinase which was released into the medium after cell lysis.

The lecithinase activity in cultures incubated at 30° C. appeared to be relatively

stable. On the other hand, the lecithinase activity in cultures incubated at 46° C. reached a maximum and then declined upon continued incubation.

EFFECT OF HEAT ON LECITHINASE ACTIVITY

In this study we have examined the susceptibility of commercial lecithinase and lecithinase in culture filtrates to heat.

Methods

Strain PB 6H of *Cl. welchii* was grown for 5 hr. at 46° C. in the complex medium. Cell-free culture filtrates were obtained by centrifuging each culture for 30 min. at 4000 rev./min. The supernatant fluid was passed through a sterilizing filter pad. Each filtrate preparation was adjusted to pH 7.2 by the addition of 1N-NaOH. These filtrate preparations served as the source of crude lecithinase. Two stock

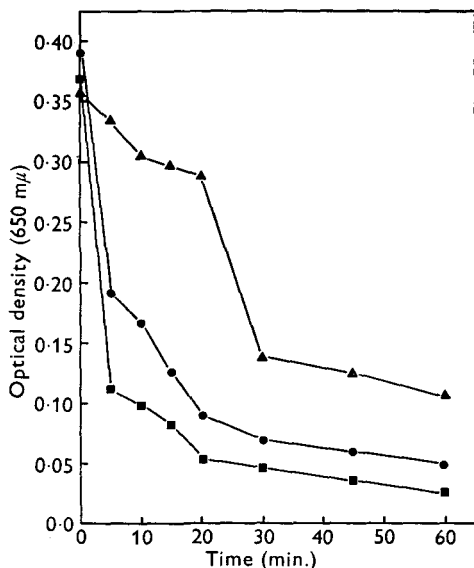


Fig. 4

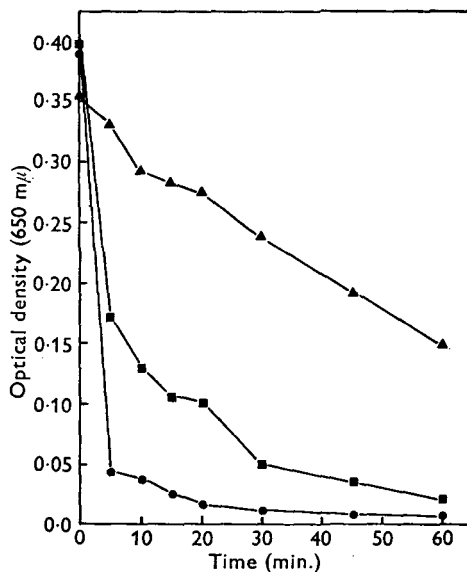


Fig. 5

Fig. 4. The inactivation of lecithinase by heat (60° C.). ●—●, Lecithinase dissolved in 0.05 M tris buffer. ▲—▲, Culture filtrate of strain PB6H. ■—■, Lecithinase dissolved in medium for α -toxin production.

Fig. 5. The inactivation of lecithinase by heat (90° C.). ●—●, Lecithinase dissolved in 0.05 M tris buffer, ▲—▲, Culture filtrate of strain PB6H. ■—■, Lecithinase dissolved in medium for α -toxin production.

solutions of the commercial lecithinase were prepared. One stock solution was prepared by dissolving 100 μ g./ml. of enzyme in 0.05 M Tris (Tris(hydroxymethyl)aminomethane) buffer, pH 7.2. The second solution was prepared by dissolving 100 μ g./ml. of the enzyme in the complex medium, pH 7.2.

Five ml. of each lecithinase preparation were pipetted into 16 \times 125 mm. screw-capped test-tubes. The tubes were placed in a water bath preheated to the desired temperature. After heating for the desired length of time, the tubes were cooled in an ice-bath. One ml. of each preparation was assayed for lecithinase activity.

Results

The lecithinase present in culture filtrates of *Cl. welchii* was relatively heat-resistant. The preparation lost only 20 % of its original activity when heated for 20 min. at 60° C. Figure 4 shows the decline in enzymic activity as a function of heating time. A marked decrease in activity resulted upon heating beyond 20 min. Commercial lecithinase, dissolved in complex medium and buffer, was more rapidly inactivated than the enzyme in the culture filtrate.

Figure 5 shows the effect of a higher temperature, 90° C., upon lecithinase. The enzyme inactivation pattern was similar to that at 60° C. Commercial lecithinase was inactivated much more rapidly than the lecithinase in the culture filtrate.

EFFECT OF pH ON LECITHINASE AND ITS ACTIVITY

Gale & van Heyningen (1942) reported that in the absence of glucose lecithinase is produced when the pH of the medium lies between 5.5 and 7.0, with a maximum production at pH 6.0. In the presence of glucose the maximum production of lecithinase took place at a pH of 7.0–7.5.

In this study we examined the effects of pH on commercial lecithinase and the effects of pH on the hydrolysis of lecithovitellin by this enzyme. We studied the effects of pH on the growth and lecithinase activity of *Cl. welchii*, strain BP 6K, a classical gas-gangrene strain, as well as on strain Hobbs 3, isolated from boiled salt beef (originally National Collection of Type Cultures 8239).

Methods

Commercial lecithinase was dissolved in distilled water to yield a final concentration of 100 µg/ml. The pH of the solvent was adjusted to the desired pH by the addition of 0.1 N-HCl or 0.1 N-NaOH. After exposing the enzyme preparations to the various pH values for 1 hr., the pH of each sample was readjusted to pH 7.0. One ml. of each solution was assayed for lecithinase activity. Lecithinase exposed to pH 7.0 was used as the control (100 % activity).

The effects of pH on the hydrolysis of lecithovitellin (the substrate) by lecithinase were determined by adjusting the pH of the reaction medium from pH values 1–9. In place of the Tris buffer, HCl or NaOH were added to adjust the pH. The control consisted of the optical density produced at pH 7.0, which was arbitrarily defined as 100 % activity.

The enzymic activity remaining after exposure to various pH values was calculated as a percentage by dividing the optical density produced in the experimental tubes by the optical density of the control and multiplying these values by 100.

The effect of pH on growth and lecithinase production in the growth medium was studied. Standard plate count methods were used to enumerate the bacterial population.

Results

Table 1 summarizes the effect of pH on commercial lecithinase. Exposure to pH values of 1–3 for 1 hr. completely inactivated the enzyme. At pH 5 half of the activity was lost. Maximum enzyme activity remained after exposure to pH values of 6–9. After exposure to pH 10, 63.2 % of the activity was lost.

Table 1. *Effect of pH on the activity of commercial purified lecithinase*

pH	Optical density (absorbance at 650 m μ)	Activity remaining (%)
1.0	0.000	0
2.0	0.000	0
3.0	0.000	0
4.0	0.010	3.0
5.0	0.170	50.0
6.0	0.320	97.0
7.0*	0.340	100.0
8.0	0.333	98.0
9.0	0.333	98.0
10.0	0.125	36.8

* Defined as 100% activity.

Table 2. *Effect of pH on the hydrolysis of lecithovitellin
(egg yolk saline) by lecithinase*

pH	Optical density (absorbance at 650 m μ)	Activity compared to control (%)
1.0	0.000	0
2.0	0.000	0
3.0	0.000	0
4.0	0.000	0
5.0	0.000	0
6.0	0.072	27.6
7.0*	0.260	100.0
8.0	0.210	80.7
9.0	0.030	11.5
10.0	0.008	3.0

* Defined as 100% activity.

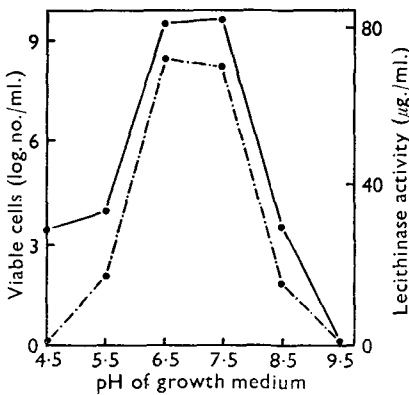


Fig. 6

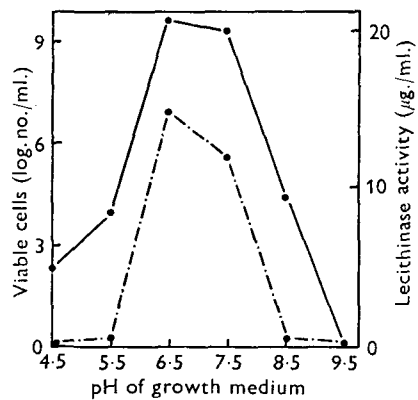


Fig. 7

Fig. 6. The effects of pH on the growth and lecithinase production in *Cl. welchii* strain BP 6K. ●—●, Log. no. of cells/ml. ●- - -●, Lecithinase activity (µg./ml.).

Fig. 7. The effects of pH on the growth and lecithinase production in *Cl. welchii* strain Hobbs 3. ●—●, Log. no. of cells/ml. ●- - -●, Lecithinase activity (µg./ml.).

The effect of pH on the enzymic reaction is shown in Table 2. The hydrolysis of the lecithovitellin was completely inhibited at pH values of 1–5. Although some hydrolysis occurred at pH 6.0 and 9.0, the optimum pH for the reaction to occur appears to be around pH 7–8.

The effects of pH on growth and lecithinase production during growth are shown in Figs. 6 and 7. Although growth occurred over a wider pH range, lecithinase production was more restricted by pH changes. However, the optimum pH for growth paralleled that for lecithinase production. Strain BP 6K produced measurable lecithinase at pH values 5.5–8.5, whereas strain Hobbs 3 produced lecithinase only at pH values 6.5–7.5.

EFFECT OF SYNTHETIC LECITHIN ON LECITHINASE PRODUCTION

The production of large amounts of lecithinase by *Cl. welchii* in a chemically defined medium was recently reported (Nakamura, Cook & Cross 1968). A number of synthetic dipeptides stimulated lecithinase production in the defined medium. A study of the possible precursors of lecithinase synthesis may yield vital information regarding the nutritional conditions necessary for optimum lecithinase production.

Gordon, Turner & Dmochowski (1954) reported that lecithin inhibited the haemolytic activity of lecithinase. Although Adams, Hendee & Pappenheimer (1947) found that lecithin stimulated lecithinase production, their experiments were performed in a complex non-synthetic medium.

In this study we describe the stimulatory activity of lecithin upon lecithinase production by *Cl. welchii* in a chemically defined medium.

Table 3. *The effects of lecithin on lecithinase production by Clostridium welchii, BP 6K, grown in a chemically defined medium supplemented with glycylglycine*

Concentration of glycylglycine (mg./ml.)	Lecithinase activity ($\mu\text{g./ml.}$)	
	Concentration of lecithin	
	0.0	0.1 mg./ml.
0.0	0.0	0.0
0.01	67.0	90.0
0.05	64.0	82.0
0.10	30.0	60.0
0.20	10.0	20.0

Methods

Synthetic lecithin (L- α -lecithin) obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, was added at a concentration of 0.1 mg./ml. to the chemically defined medium fortified with glycylglycine. Experiments were also performed in media devoid of glycylglycine in order to determine the importance of this dipeptide in lecithinase production.

Strain BP 6K of *Cl. welchii* was maintained for over twenty subcultures in the defined medium in order to eliminate the possibilities that carry-over material from the complex medium might stimulate lecithinase production. Twenty-four hr. cultures were assayed for lecithinase activity.

Results

Lecithin stimulated the production of lecithinase. However, the stimulatory activity was dependent upon the concentration of glycyglycine present. The data are presented in Table 3. In the absence of glycyglycine lecithin did not stimulate lecithinase production. There was an optimum concentration for both glycyglycine and lecithin in the production of lecithinase.

DISCUSSION

Quantitatively, lecithinase production by *Cl. welchii* is dependent upon the strain, the medium, the culture conditions, pH, temperature of incubation, and the length of incubation. One of the strains, UMJS-12, in our study produced more lecithinase at 46° than at 30° C. The other two strains produced comparable amounts of lecithinase at both of these temperatures. This reflects the variation in the ability of different strains to produce lecithinase at different temperatures.

Roberts (1957) reported that there was less lecithinase produced when the cultures were incubated at 43° C. compared to 37° C. However, he measured lecithinase activity by haemolysis, whereas in our work we have used the lecithovitellin reaction to measure lecithinase activity. In other words, the activity assayed may indicate different kinds of lecithinase levels. In fact, several investigators have suggested the possibility that the haemolytic activity due to alpha toxin may be due to a component separate from the lecithinase which produces the lecithovitellin reaction (Miles & Miles, 1950; Matsumoto, 1961).

It was reported by Weiss & Strong (1967) that the lecithinase activity of some strains decreased rapidly after reaching a maximum after 4–6 hr. of incubation. In our work the lecithinase activity remained relatively stable or decreased only slightly after maximum activity was observed. Our data indicated that the lecithinase activity in older cultures (30–60 hr.) was greater than the lecithinase activity in young actively growing cultures (5 hr.). Gale & van Heyningen (1942) also reported that the production of lecithinase varied with the age of the culture. The amount of lecithinase produced was small in young cultures and increased to a maximum at the time when active cell division ceased. It is possible that the composition of the medium used for the growth and production of lecithinase plays an important role in the stability of the lecithinase after it is produced. In culture media deficient in nutrients, it is possible that the lecithinase, which is a protein, is broken down and metabolized.

A portion of the lecithinase activity in older cultures may have been due to the lysis of the cells which may release the lecithinase into the culture medium. This is a possibility because it was reported (M. Nakamura & W. R. Cross, unpublished data) that some strains of *Cl. welchii* retain over 50 % of the lecithinase within the cell.

Lecithinase in culture filtrates was more resistant to inactivation by heat than purified commercial lecithinase. A possible explanation is that the crude lecithinase in culture filtrates may differ from that of the purified preparation. Purified lecithinase heated in the sterile complex medium retained its heat sensitivity, suggesting that the complex medium itself did not protect the enzyme against

heat. Therefore, another possibility is that metabolites produced by the growing *Cl. welchii* may have protected the enzyme against heat. Conflicting data were presented by Kushner (1957) who reported that the lecithinase activity, using purified material, was almost completely destroyed after 5 min. at 100° C. On the other hand, he found that lecithinase in the culture filtrate was resistant to decomposition under similar conditions.

Although our data indicate that less inactivation of the enzyme occurred at 90° C. compared to 60° C. the differences were not particularly significant. Smith & Gardner (1949) suggested that the lower temperatures increased inactivation possibly because of the formation of enzymically inactive complexes. They suggested that calcium ions added during the heating procedure mediated the formation of inactive complexes. In our study only trace amounts of calcium ions were present. This might account for the similarity in the heat inactivation patterns we observed at the two temperatures.

It was reported that lecithinase was more resistant to heat at alkaline pH values than at acid pH values (Macfarlane & Knight, 1941; Smith & Gardner, 1949). The decrease in heat-resistance in an acid environment may have been due to partial inactivation of the enzyme by the acid.

Lecithinase was readily inactivated by acid pH, but was more resistant to alkaline pH values. Others reported that the lecithinase was most stable at pH 6, losing activity rapidly at pH 7, and very rapidly above pH 8 (Adams & Hendee, 1945). The pH for optimal hydrolytic reaction of the lecithovitellin was between pH values of 7 and 8. The effect of pH may be upon the substrate as well as on the enzyme system. This possibility is supported by the observation that lecithinase retained 50% of its activity when exposed to pH 5.0 for 1 hr., but no detectable hydrolysis occurred when the enzyme reaction was measured at this pH.

Our data do not permit us to speculate much regarding the reasons why lecithin stimulated lecithinase production in the presence of glycylglycine. However, one possibility is that additional substrate, when present in the culture medium, stimulated enzyme formation because lecithinase is a partially inducible enzyme. The addition of lecithin increased lecithinase production by 50–100%. However, this was possible only if the concentration of glycylglycine was optimal.

The data presented in this study suggest that conflicts in the published literature and variations in the results reported may be due to differences in methodology and strain of *Cl. welchii* used by various investigators.

SUMMARY

A variety of factors that affect lecithinase activity and lecithinase production by *Cl. welchii* were studied. The lecithinase activity was time and temperature dependent. The optimum temperature varied from 30° to 46° C. according to the strain of *Cl. welchii* employed. Maximum lecithinase activity was produced after 12–60 hr. There was considerable strain variation. This could easily account for the differences in the published data.

Commercial purified lecithinase was more readily destroyed by heat than the lecithinase produced by *Cl. welchii* in culture media. The enzyme inactivation

pattern at 60° C. was similar to that at 90° C. This is not in agreement with the reports of others who found that less enzyme was inactivated at the higher temperatures.

Acid pH values completely inactivated the enzyme. However, alkaline pH values did not significantly destroy the enzyme. The lecithovitellin reaction was completely inhibited at pH values of 1–5. The optimum pH for the reaction was around pH 7–8.

The production of lecithinase was dependent upon the pH of the culture medium. One strain produced measurable lecithinase at pH values of 5.5–8.5, whereas another strain produced lecithinase only at pH values 6.5–7.5.

Lecithin stimulated the production of lecithinase in a chemically defined medium.

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