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RECOVERY OF THE CELLULASE ENZYME SYSTEM OF TRICHODERMA VIRIDE BY ACETONE PRECIPITATION

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Recovery of the Cellulase Enzyme System of *Trichoderma viride* by Acetone Precipitation

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ABSTRACT

The recovery of cellulase from *T. viride* by acetone precipitation was studied to provide basic background information for possible recovery of cellulase in processes for enzymatic hydrolysis of cellulosic wastes. Effects of acetone concentration, temperature, and total soluble protein on the recovery of cellulase and the time required for the precipitate to reach equilibrium were examined. Stability and filtration characteristics of the cellulase precipitate were also studied.

The results showed that more than 85% of the original enzyme activity can be recovered at temperatures as high as 35°C. Most of the cellulase activity is recovered with acetone concentration of 2.0 v/v or greater. Inactivation of the enzyme during precipitation with acetone exhibited first-order kinetics. Solubility of the cellulase protein was independent of temperature and could be described by the salting-out equation. Filtration studies indicated that the enzyme precipitate has a very large specific resistance. Addition of filter aid greatly improved the filtration rates.

INTRODUCTION

Cellulose is the most abundant form of carbohydrate in the world, comprising more than 50% of all carbohydrates. Unlike most resources, the cellulose reserve is being replenished through photosynthesis, at a net rate of between 7.5 and 10×10^{10} tons of cellulose yearly (5,60,75). Large quantities of the cellulose are potentially available in the form of solid wastes.

In America, cellulosic wastes originate primarily from domestic and agricultural sources. More than 250 million tons of domestic wastes are produced each year. Of this, 40 to 60% is cellulosic in nature (1,60). The major portion of the cellulose is in the form of paper, while leaves, grass, and wood also contribute to the total.

The U. S. Department of Agriculture estimates that over 200 million tons of crop residue are produced each year (76). Additionally, 25 million tons of debris from lumbering operations are left in the forests each year. Cellulose is the major component of these wastes. Cellulosic wastes also occur from the food processing and paper industries.

Cellulose is a potential source of food. Unfortunately, few animals can digest it in its natural form and it must first be converted to a more useful form. Cellulose can be converted directly to protein for animal consumption by growing microorganisms such as Cellulomonas on it. Alternatively, it can be degraded enzymatically by cellulase to glucose which can be consumed directly or can be converted to proteins in the form of yeasts.

Cellulose can also be converted into a more convenient form of energy. The first step into the conversion is the hydrolysis of cellulose

to glucose. The glucose can then be converted into a variety of energy compounds, ethanol and methane being the most prominent.

As can be seen, the conversion of cellulose to glucose offers the most versatility in the production of a food or energy source. Rosenbluth and Wilke (63) developed a process for hydrolyzing cellulose to glucose using the cellulase enzyme from Trichoderma viride. In their economic evaluation they found 58% of the plant cost was associated with the enzyme production stage. It was concluded that a major improvement could be made by either increasing the enzyme activity or by recovering the enzyme. Mandels and her co-workers (49) have produced a mutant strain of T. viride capable of producing three times the enzyme concentration of the organism used by Rosenbluth and Wilke. Ghose and Kostick (29) developed an enzyme recovery scheme using ultrafiltration membranes. However, the recovery of enzyme activity in this process was low. Only 46.7% of the filter paper activity was recovered.

It can be seen that improvements in the cellulose hydrolysis process can be made by improving the efficiency of the enzyme recovery stage. For this reason, the precipitation of cellulase enzyme from T. viride was investigated. The effect of temperature and protein concentration on the recoveries of C_1, C_x, and filter paper activities, and protein was examined. The stability of the dried cellulase precipitate during long term storage was also examined. Finally, the filtration characteristics were studied to determine the values of parameters needed to design large scale equipment.

BACKGROUND

The techniques for the concentration and purification of enzymes are numerous. Blatt (6) has categorized these techniques in the following classification:

1) Precipitative methods
   a) salt and/or solvent concentration
   b) isoelectric precipitation
   c) concentration by ultrafiltration
2) Solvent removal
   a) flash and vacuum evaporation
   b) lyophilization
   c) freeze-thaw procedures
3) Partition systems
   a) gel exclusion (Sephadex, Biogel, Lyphogel, etc.)
   b) adsorption chromatography
   c) membrane moderated procedures
      (1) pervaporation
      (2) dialysis (against solutes of high molecular weight)
      (3) electrodialysis
      (4) centrifugally accelerated ultrafiltration
      (5) pressure ultrafiltration.

Although these methods are available for laboratory scale procedures, not all of these techniques are applicable to large scale uses. The techniques that are used in industrial scale processes are salt and solvent precipitation, gel exclusion, and ultrafiltration.

Enzyme Recovery by Salting-out and Ultrafiltration

Concentration of enzymes by precipitation with inorganic salts was studied in 1889 by Hofmeister (38). In these experiments, Hofmeister crystallized ovalbumin with ammonium sulfate. Since that time salting-out of enzyme has become one of the most important techniques for protein purification. The literature references to this technique have been too voluminous to review. It was noted by Dunnill & Lilly (20), that in the Journal of Biological Chemistry during 1968 alone there were 50 articles using salting-out as an isolation technique. There have been significant developments in the understanding of this technique and applications to large scale use that bear review.

It was Cohn (12) that first suggested that the solubility of protein could be represented by the linear relationship

$$\log S = \beta - K c_s \qquad (1)$$

where S is the protein solubility, c_s is the concentration of the salt and β and K are constants for the particular protein-salt system. The various parameters affecting the salting-out characteristics of proteins have been examined by Cohn and Edsall (13), Green and Hughes (32), Czok and Bücher (17), and Foster, Dunnill and Lilly (26). The theoretical aspects of the precipitation of proteins in concentrated salt solutions have been examined by Dixon and Webb (18).

In 1958, Baumgarten et al. (4) reported the large scale purification of bovine pancreatic deoxyribonuclease which involved precipitation with ammonium sulfate at 2 - 5°C. 50 - 106 hours were required for the precipitation steps and the recovery of enzyme activity was 41%. Since 1967, Leuzinger and Baker (45), Dunnill et al. (19), Fisher et al. (25)

and Street et al. (69) have used precipitation with ammonium sulfate in the large scale purification of enzymes. Nyiri (56) recovered pectinases from a fermentation broth by salting-out. He also examined the problems associated with scaling-up from a pilot plant to a full scale plant.

These studies on the large scale recovery of enzymes by salting-out were done in batch processes. The major problem in operating a continuous salt precipitation process was the long contact time needed for complete precipitation. In 1972 Gray, Dunnill, and Lilly (31) isolated β-galactosidase using ammonium sulfate precipitation in a continuous process. They found that the enzyme recovery from the continuous process was greater than when the precipitate was allowed to equilibrate in a batch process.

Ammonium sulfate precipitation has been used to recover the cellulase enzyme systems of Myrothecium verrucaria (43,79,80), Stachybotrys atra (72), Polyporus palustris (37), and Thermomonospora curvata (70). From non-microbial sources, cellulase from green malt extracts (22), silverfish (Ctenoloprisma lineata) (44) and a marine wood-boring mollusc, (Teredo) (33) have been recovered by salting-out. Jermyn (40) recovered extracellular β-glucosidase from S. atra by salting-out with lead acetate.

Toyama (74) reported on the large scale production of cellulase by the koji process. The cellulase is produced by growing cultures of Trichoderma koningii on wheat bran (koji.) The cellulase is then extracted with water and precipitated with ammonium sulfate. The precipitate was allowed to equilibrate overnight before separation.

Ultrafiltration technology has existed for the past 40 years. Until recently, ultrafiltration membranes were composed of cellophane and were characterized by high pressure drops and low filtration rates. In 1965, Blatt et al. (7) reported the use of a new polyelectrolyte membrane that completely changed the importance of ultrafiltration as a purification technique. Blatt, working with human serum and blood fractions, found at least a 20 fold increase in filtration rates over the cellophane membranes. Recovery of protein activity was about 90%. Wang, Sonoyama, and Mateles (78) examined the effect of pressure, temperature, and salts on the filtration rates. The recovery of enzyme activity ranged from 50 to 91%.

Other researchers (8,9,77) have examined the purification of mixtures of enzymes. It was noted that there was a greater retention of low molecular weight proteins that are not normally retained. Still others (10,35) have evaluated the different ultrafiltration membranes and systems.

Ultrafiltration was first applied to the recovery of the cellulase of Trichoderma viride by Ghose and Kostick (29) in 1970. They achieved filtration rates from 7.1 to 14.2 gals/ft^2 day with membranes whose molecular weight cut off ranged from 10,000 to 30,000, respectively. In a 5-fold concentration of the enzymes, the maximum recoveries of enzyme activity were 46.7% and 56.7% for the filter paper and C_x activities respectively. The pressure in the ultrafiltration system was maintained at 42-44 psig. Mitra and Wilke (51) also concentrated the cellulase of T. viride by ultrafiltration. Using an operating pressure

of 20 psig, they examined the recovery of enzyme activity over a range of concentrations from 2-fold to 10-fold. The recovery of C_1 activity varied from 62.5% at a 2 fold concentration to 31.4% at a 10 fold concentration. At the same time, the recovery of C_x activity varied from 62.3% to 15.6%.

Enzyme Recovery by Solvent Precipitation

Organic solvents were first used to precipitate proteins by Mellanby (50) and Hardy and Gardiner (36) in 1908. In these early studies, the treatment with organic solvents was done at room temperature and resulted in denaturation of the proteins. As a result, organic solvents were not as extensively used as inorganic salts, which provided a milder treatment, in precipitating enzymes.

In 1936, Ferry, Cohn, and Newman (23) precipitated egg albumin in a 25% ethanol solution at -5°C and found little denaturation. This observation was extended by Cohn and his co-workers (11, 15, 24) to other proteins. In 1946, Cohn, Strong, Hughes, Mulford, Ashworth, Melin, and Taylor (16) extensively studied the method of protein fractionation with organic solvents. The effects of salts, protein, pH, and temperature on protein precipitation were examined and methods for the fractionation of human serum and plasma proteins were developed. In these methods ethanol was precooled and added very slowly to the precipitation vessel. The temperature in the vessel ranged from -5 to 0°C and the mixture was constantly stirred to prevent isolated build-up of ethanol. Subsequently, Cohn et al. (14) improved the methods for plasma fractionation with ethanol precipitation, and these methods are still being used today in fractionating blood plasma.

Askonas (3) applied the procedures developed by Cohn et al. (16) to the separation of enzymes from aqueous rabbit muscle extracts, using ethanol, methanol, n-propanol and acetone as precipitating agents. Askonas observed that acetone appeared superior to the other solvents for enzyme separation. It was believed to be due to acetone's lower dielectric constant at low temperatures. Enzyme recoveries were reported at near 100%.

The cellulase system from a number of organisms has been recovered by precipitation with organic solvents. Ethanol has been used in the purification of the cellulase from Myrothecium verrucia (64, 79, 80), T. koningii (74), and S. atra (72) and the intracellular β-glucosidase from S. atra (81). Acetone was used to recover the cellulase from wood-rotting fungus, Polyporus tulipiferae (52, 54), and Poria vaillantii (68). Stutzenberger (70) attempted unsuccessfully to recover the cellulase from T. curvata by alcohol and acetone precipitation.

Mandels, Kostick, and Parizek (47) used acetone precipitation as part of their analytical procedures while working on the cellulase of T. viride. Acetone was used to separate the protein from glucose solutions. The conditions during solvent precipitation were not reported but they reported 100% protein recovery.

Characteristics of the Cellulase System of Trichoderma viride

In 1950, observing that not all microorganisms having cellulolytic ability could hydrolyze native cellulose, Reese, Siu and Levinson (61) first postulated the existence of the C_1-C_x complex for the cellulase system. The C_1 component is believed to be essential in the breakdown

of the crystalline structure of native cellulose. The C_x component is believed to breakdown the amorphous and soluble forms of cellulose. The cellulases from a number of microorganisms have been examined, and the cellulase from T. viride was found to have the most active and stable C_1 component (48). Mutation experiments on T. viride have produced mutant strains having higher levels of cellulase activity (49,65).

The properties of the T. viride cellulase have been studied by a number of investigators and the results have been quite varied. The total number of components in this system has been reported to vary from 2 to 7 (46,55,57,58,66,57,73). Furthermore, the number of subunits having C_1 activity and the number of components having C_x activity ranged from 1 to 7. The molecular weights of the components reported in the literature have also varied considerably. The molecular weight of the C_1 component ranged from 48,000 to 61,000, and the molecular weight of the C_x component ranged from 12,600 to 76,600.

The roles of the C_1 and C_x components in the hydrolysis of cellulose have not been fully defined. Many workers have reported a synergistic relationship between the C_1 and C_x components (39,46,51,66,67). These researchers have reported that the C_1 component alone was not able to degrade cotton and only when it was combined with the C_x component did it possess the ability to do so. Other researchers (55,57,58) have reported that the isolated C_1 components also possessed C_x activity and were able to degrade cotton independent of other components. The mode of action of the cellulases has been summarized by Nisizawa (53). He concluded that the order of attack on cellulose is first by C_x which produces cellulose fragments of lower degree of polymerization followed

by the C_1 component which attacks the cellulose fragments.

THEORY

Salting-out

The theoretical interpretation of the solubility of zwitterions in salt solutions has been developed by Kirkwood (41). He assumed that the solvent is idealized as a structureless dielectric continuum and the deviation of the solution from ideal behavior is due to electrostatic intermolecular forces alone. The logarithm of the activity coefficient of a dipolar ion, i, may be written as

$$\log \gamma_i = C_i K_{Rii} + \sum_{k=1}^{\nu} K_{Rik} C_k , \qquad (2)$$

$$K_{Rik} = \frac{N}{1000\,\kappa T} \int_0^1 \int_\omega^v V_{ik} \exp[-W_{ik}(\lambda)/\kappa T]\, dv d\lambda , \qquad (3)$$

where ν = total number of ionic species

C_j = concentration of the jth component

V_{ik} = electrostatic work required to bring the ith and kth molecules together from infinite separation to a given configuration in a pure solvent

$W_{ik}(\lambda)$ = the average work required to bring the ith and kth molecules together from infinite separation to a given configuration in the actual solution

λ = fraction of the full value of all charges on molecule i.

It is assumed that the forces due to the interaction of 2 dipolar ions are small. Thus the first term in equation (2) can be dropped. It is evident that in the limit as concentration of all species approach

zero,

$$W_{ik}(\lambda) = \lambda\ V_{ik}\,. \tag{4}$$

Now equations (2) and (3) can be written as

$$\log \gamma_i = \sum_{k=1}^{\nu} K'_{Rik}\, C_k \tag{5}$$

$$K'_{Rik} = \frac{N}{1000} \int_{\omega}^{\infty} [1-\exp(-V_{ik}/\kappa T)]\, dv \tag{6}$$

$$K'_{Rik} = \underset{C_1, C_2, \cdots C_\nu \to 0}{\text{limit}} K_{Rik}. \tag{7}$$

At ordinary temperatures with solvents having relatively high dielectric constant, the exponential expression inside the integral of equation (6) can be expanded and keeping only the first two terms yield

$$K'_{Rik} = \frac{N}{1000} \int_{\omega}^{\infty} \left[\frac{V_{ik}}{\kappa T} - \frac{1}{2}\left(\frac{V_{ik}}{\kappa T}\right)^2\right] dv\,. \tag{8}$$

The dipolar ion is characterized as being in a cavity, ω_0, of lower dielectric constant, D_i, than the solvent in general. The electrostatic work, V_{ik} required to bring a kth ion having a charge of $z_k \epsilon$ from infinite separation to a point r_k from the dipolar ion is given by

$$V_{ik} = \frac{1}{2}\left[\sum_{\ell=1}^{s} \epsilon_\ell \underline{\psi}(r_\ell) + z_k \epsilon \psi(r_k)\right], \tag{9}$$

where $\underline{\psi}(r_\ell)$ is the electrostatic potential in the interior of the cavity at the location of charge ϵ_ℓ and $\psi(r_k)$ is the electrostatic potential at r_k, outside of the cavity. Laplace's equation, $\nabla^2\psi = 0$, holds for both potentials, inside and outside of the cavity, and at the surface of the

cavity the following boundary conditions exist

$$\underline{\psi} = \psi \tag{10}$$

$$D_i \bar{n} \cdot \nabla \underline{\psi} = D \bar{n} \cdot \nabla \psi \tag{11}$$

where $\bar{n}$ is a unit vector normal to the surface.

Nothing thus far has been said about the structure of the dipolar ion. A model must be assumed in order to evaluate the electrostatic work force. Consider a spherical dipolar ion with a radius b, and whose dipolar moment, μ, is located at the center. The cavity of lower dielectric constant, ω_0, is also a sphere of radius b. The electrostatic work term has thus become

$$V_{ik} = \frac{3 z_k \epsilon \mu \cos\theta}{(2D+D_i) r^2} + \frac{z_k^2 \epsilon^2 b^3}{2r^4} \frac{D-D_i}{D} \sum_{n=0}^{\infty} \frac{(n+1)}{(n+2)D+(n+1)D_i} \left(\frac{b}{r}\right)^{2n} \tag{12}$$

where r is the distance between the centers of the ion and dipolar ion and θ is the angle between the vectors, $\bar{r}$ and $\bar{\mu}$, the dipole moment. The ratio of dielectric constant D_i/D is small and can be neglected. Substituting equations (12) into equation (8) and integrating, the result is

$$K_{R_i} = \frac{2\pi N \epsilon^2}{2303 D \kappa T} \left[\frac{3\mu^2}{2aD\kappa T} - \frac{b^3 \alpha(\rho)}{a} \right] \tag{13}$$

where a is the radius of the ion, assumed to be the same for all ions in the solution and ρ is the ratio of the radii, b/a. The term $\alpha(\rho)$ is defined as

$$\alpha(\rho) = \frac{1}{3\rho^4} [(\rho^3 - 2)\log(1+\rho) - (\rho^3 + 2)\log(1-\rho) - 2\rho^2]. \tag{14}$$

The activity coefficient of the dipolar ion is given by the equation

$$\log \gamma_i = - K_{Ri}\Gamma/2 \tag{15}$$

$$\log(S/S_0) = \frac{2\pi N\epsilon^2}{2303D\kappa T}\left[\frac{3\mu^2}{2aD\kappa T} - \frac{b^3\alpha(\rho)}{a}\right]\frac{\Gamma}{2} \tag{16}$$

$$\Gamma/2 = \frac{1}{2}\Sigma C_k z_k^2 \tag{17}$$

where the activity coefficient, γ_i, is equal to the ratio of solubilities S and S_0, of the dipolar ion in the presence and absence of the salt. The first term in equation (16) represents the salting-in effect and the second term the salting-out effects.

Now consider an ellipsoidal dipolar ion, with charges $+\epsilon$ and $-\epsilon$ located at the foci. The logarithm of the solubility becomes

$$\log S/S_0 = \frac{2\pi N\epsilon^4 g(\lambda_0)R}{2303(D\kappa T)^2}\,\Gamma/2\ . \tag{18}$$

Due to the complexity of the electrostatic work term, only the salting-in term was derived. It would be reasonable to suppose that by analogy, the salting-out term would also be proportional to the ionic strength and inversely proportional to the dielectric constant.

From the two cases presented above, it can be seen that the logarithm of the solubility of a dipolar ion would be proportional to the ionic strength and inversely proportional to the dielectric constant. It is extremely difficult to extend the theoretical treatment from the solubility of a single dipolar ion to the solubility of a protein, which consists of a large number of dipolar ions. However, the general relationship of the solubility with the ionic strength is consistent with the empirical equation first proposed by Cohn (12), and his equation can now be written to include the effect of

organic solvents as

$$\log S = \beta - \frac{K_s \Gamma/2}{D} . \tag{19}$$

Filtration

Figure 1 shows schematically the cross section of a filter cake and filter medium. Consider a thin layer of cake, dL, and the mass in it. Assuming that this layer consists of small solid spheres, the fluid velocity would be sufficiently slow to ensure laminar flow. The pressure drop across this layer can be described by the Kozeny-Carmen equation

$$\frac{dp}{dL} = \frac{k\mu u(1-\epsilon)^2 (s_p/v_p)^2}{g_c \epsilon^3} , \tag{20}$$

where p = pressure,

μ = viscosity,

u = linear velocity of the filtrate, based on the filter area,

s_p = surface area of single particle,

v_p = volume of single particle,

ϵ = porosity of the cake,

k = constant,

g_c = conversion factor.

The mass in the layer is related to the thickness of the layer by

$$dm = (1-\epsilon)\rho_p A dL \tag{21}$$

where ρ_p is the density of the particles in the cake and A is filter area.

The specific cake resistance, α, can now be defined as

$$\alpha = \frac{k(s_p/v_p)^2 (1-\epsilon)}{\epsilon^3 \rho_p} . \tag{22}$$

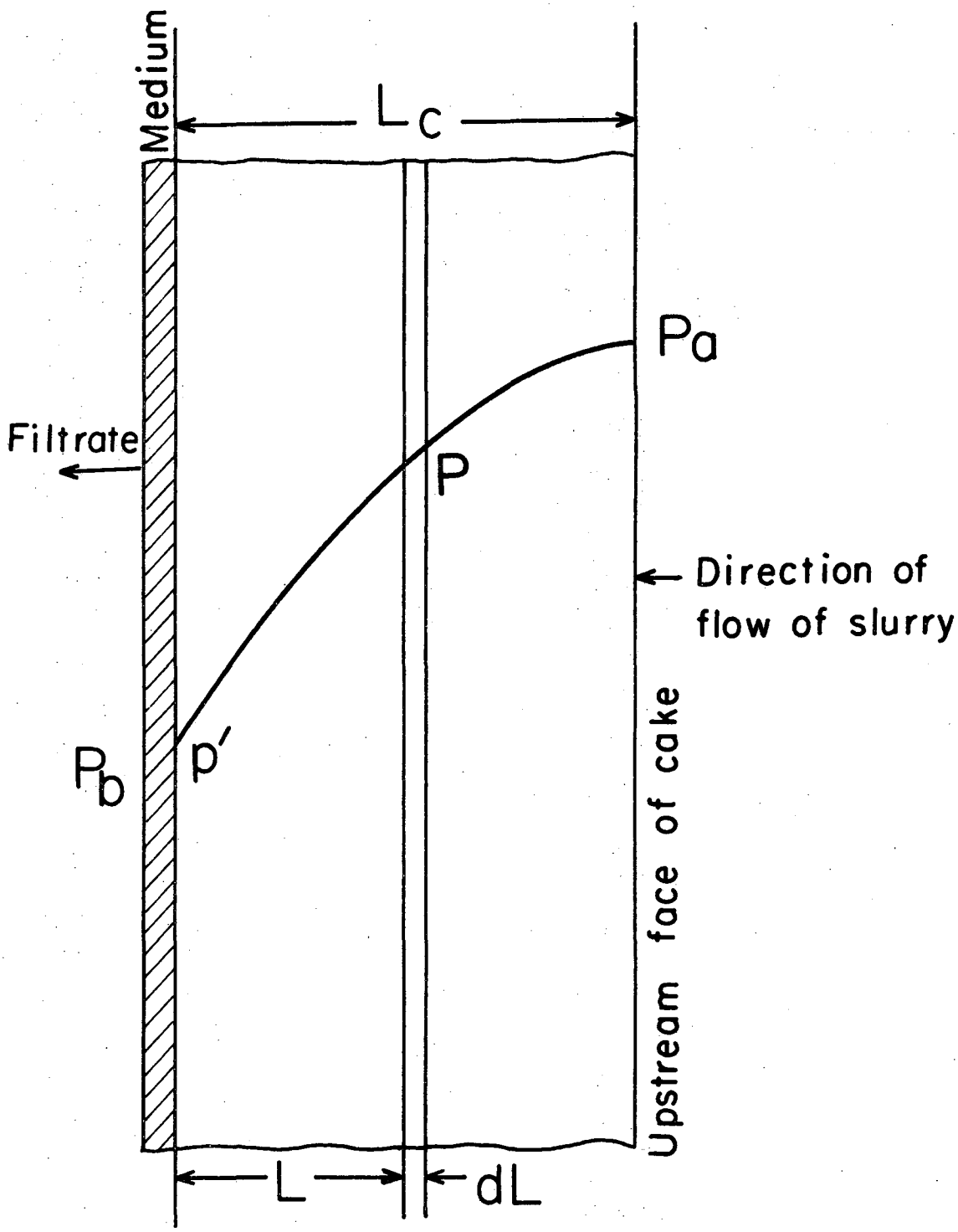

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Fig. 1. Section through filter medium and cake, including the pressure gradient.

Substituting equations (21) and (22) into equation (20) one gets

$$dp = \frac{\alpha \mu u}{A g_c} dm. \tag{23}$$

Now the pressure loss is associated with the drag forces on the particles in the bed and is equal to $p_a - p$, where p_a is the upstream pressure and p is the pressure at the thin layer of cake. The result is

$$dp = - d(p_a - p) = \frac{\mu u \alpha}{A g_c} dm \quad . \tag{24}$$

The specific cake resistance is a function of $(p_a - p)$. Assuming that this function is known, equation (24) can then be integrated

$$\int_0^{(p_a - p)} \frac{d(p_a - p)}{\alpha} = \frac{\mu u}{A g_c} \int_0^{m_c} dm \quad . \tag{25}$$

The specific cake resistance, α, is now an average value for the cake and m_c is the total mass of the cake.

The pressure drop through the filter medium can be defined by analogy by the equation

$$\frac{(p_b - p)}{R_m} = - \frac{\Delta p_m}{R_m} = \frac{\mu u}{g_c} \quad . \tag{27}$$

Thus the total pressure drop through the filter is

$$-\Delta p = - (\Delta p_c + \Delta p_m) = \frac{\mu u}{g_c} \left(\frac{\alpha m_c}{A} + R_m \right). \tag{28}$$

The linear velocity, u, is given by the equation

$$u = \frac{dV/dt}{A} \tag{29}$$

where V is the volume of filtrate collected in time, t. Substituting equation (29) into equation (28) and rearranging the terms, one gets

$$\frac{1}{A}\frac{dV}{dt} = \frac{(-\Delta p)\, g_c}{\mu\left(\frac{\alpha m_c}{A} + R_m\right)} . \tag{30}$$

The total mass of the cake, m_c, is equal to V·c, where c is the concentration of particles per unit volume of filtrate. Substituting for m_c in equation (30), the final result is

$$\frac{1}{A}\frac{dV}{dt} = \frac{(-\Delta p)\, g_c}{\mu\left(\frac{\alpha Vc}{A} + R_m\right)} . \tag{31}$$

In the case where the filter is operated at a constant Δp, equation (31) can be integrated as follows:

$$\int_0^t dt = \frac{\mu}{A(-\Delta p)g_c} \int_0^V \left(\frac{\alpha Vc}{A} + R_m\right) dV, \tag{32}$$

$$t = \frac{\mu}{A(-\Delta p)g_c} \left[\frac{\alpha c V^2}{2A} + R_m V\right] . \tag{33}$$

Now a word must be said about the dependence of α on Δp. If α is independent of Δp, the cake is said to be incompressible; if α varies greatly with pressure, then the cake is compressible. The dependence of α on Δp is usually determined by experimental observations. The data can be fitted to a number of empirical equations. Two common equations are

$$\alpha = \alpha_0 (-\Delta p)^s \tag{34}$$

and

$$\alpha = \alpha_0[1 + \beta(-\Delta p)^s] . \tag{35}$$

EXPERIMENTAL

Analytical Procedures

A) Enzyme activity

The cellulase enzyme system consists of two components, C_1 and C_x, needed in the breakdown of all types of cellulosic material. Mandels and Weber (48) have developed assay methods which measure these components individually and also their combined action. These assay procedures were followed in analyzing the experimental results.

1) C_1 - cotton. This test was used to determine the activity of the C_1 component. 50 mg of absorbent cotton was placed in a test tube. 1.0 ml of 0.05 M sodium citrate buffer pH 4.8 was added followed by 1.0 ml of enzyme solution. After incubating the sample for 24 hours at 50° C, the reducing sugar produced was measured by the dinitrosalicylic acid (DNS) method.

2) C_x-β(1-4) Glucanase. The activity of the C_x component was determined by this test. 0.5 ml of enzyme solution was added to 0.5 ml of 0.05 M sodium citrate buffer pH 4.8 containing 1.0% carboxymethylcellulose (CMC) 50T (Hercules Powder Company). The sample was incubated at 50°C for 30 minutes. The amount of reducing sugar produced was determined by the DNS method.

3) Filter Paper (FP). This test determined the overall cellulase enzyme activity. It measured the combined action of both the C_1 and C_x

components. 50 mg of Whatman No. 1 filter paper (1×6 cm) was placed in a test tube. 1.0 ml of enzyme solution and 1.0 ml of 0.05 M sodium citrate buffer pH 4.8 was then added. The sample was incubated for 1 hour at 50°C. The reducing sugar was then measured by the DNS method.

One unit of activity in each case corresponded to 1 mg of reducing sugar produced by the undiluted enzyme solution.

B) Protein assay

Protein was measured by the modified Biuret method of Koch and Putnam (42). Although not as sensitive as the conventional Lowry method, the modified Biuret method was more accurate and precise. 100 ml of Biuret reagent was prepared by mixing 46 ml of 10 N NaOH, 49 ml of 5.2 gm/l $CuSO_4 \cdot 5H_2O$ solution, and 5 ml of NH_4OH (28-30% NH_3). The reagent had a shelf life of 2-3 weeks. 1.0 ml of sample was mixed with 4.0 ml of reagent. After standing at room temperature for 0.5 hour, the reaction mixture was transferred to a 1×1 cm glass cuvet and the absorbance at 330 nm was measured against a reagent blank. The absorbance readings were converted to protein concentration by the standard curve shown in Fig. 2. The standard curve was based on bovine serum albumin (Mann Research Laboratories). It was assumed that the enzyme samples had the same light-scattering correction factor as a bovine serum albumin solution. The reagent blank was prepared by mixing 4.0 ml of reagent with 1.0 ml of distilled water.

C) Reducing sugar assay

The reducing sugar was measured by the dinitrosalicylic acid (DNS) method (71). 1.0 ml of sample was added to 3.0 ml of DNS reagent. The reaction mixture was placed in a boiling water bath for exactly 5 minutes,

followed by a cold water bath for 5 minutes. The mixture was then diluted to 25 ml with distilled water. 3.0 ml of the diluted mixture was placed in a 1 × 1 cm cuvet and the absorbance at 600 nm was measured against a distilled water blank. The reducing sugar concentration was obtained from the standard curve in Fig. 3. Glucose was used as the reducing sugar in preparing the standard curve.

Experimental Apparatus and Methods

A) Enzyme precipitation

Enzyme precipitation experiments were conducted in an oven (Braun-Knecht-Heinmann Co.), which was used to maintain a constant temperature throughout each run. A centrifuge (International Equipment Co. Model CL) was placed inside the oven and was used to separate the precipitate from the supernatant. The enzyme samples and organic solvents were dispensed into 50 ml glass stoppered flasks.

B) Filtration

The filtration experiments were conducted under vacuum. A single stage vacuum pump (W. M. Welch Manufacturing Co.) with a Westinghouse Thermoguard AC motor was used to generate the vacuum. Constant pressure was maintained by a cartesian manostat (Emil Greiner Co.). A Millipore Filter holder was used; it had a filtration area of 9.6 cm^2. Whatman No. 42 filter paper with a diameter of 5.0 cm was used as the filter medium. Unprinted Wall Street Journal paper, ball-milled to less than 200 mesh, was used as filter aid.

C) Production of cellulase enzyme

A slant culture of *Trichoderma viride* QM 9414 was obtained from the U. S. Army Natick Laboratories. The culture was transferred

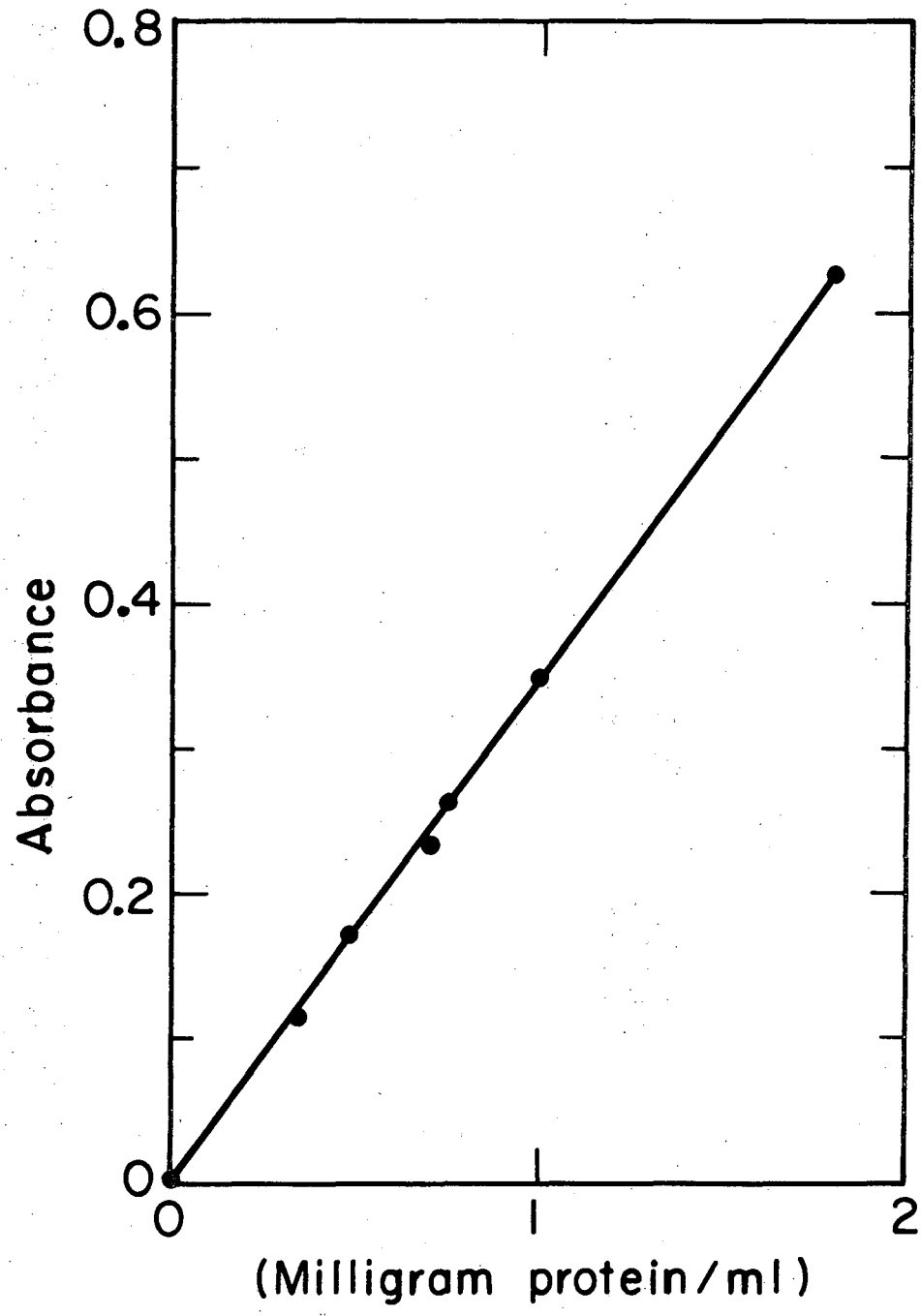

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Fig. 2. Standard curve for the protein assay by the modified Biuret method with bovine serum albumin as standard.

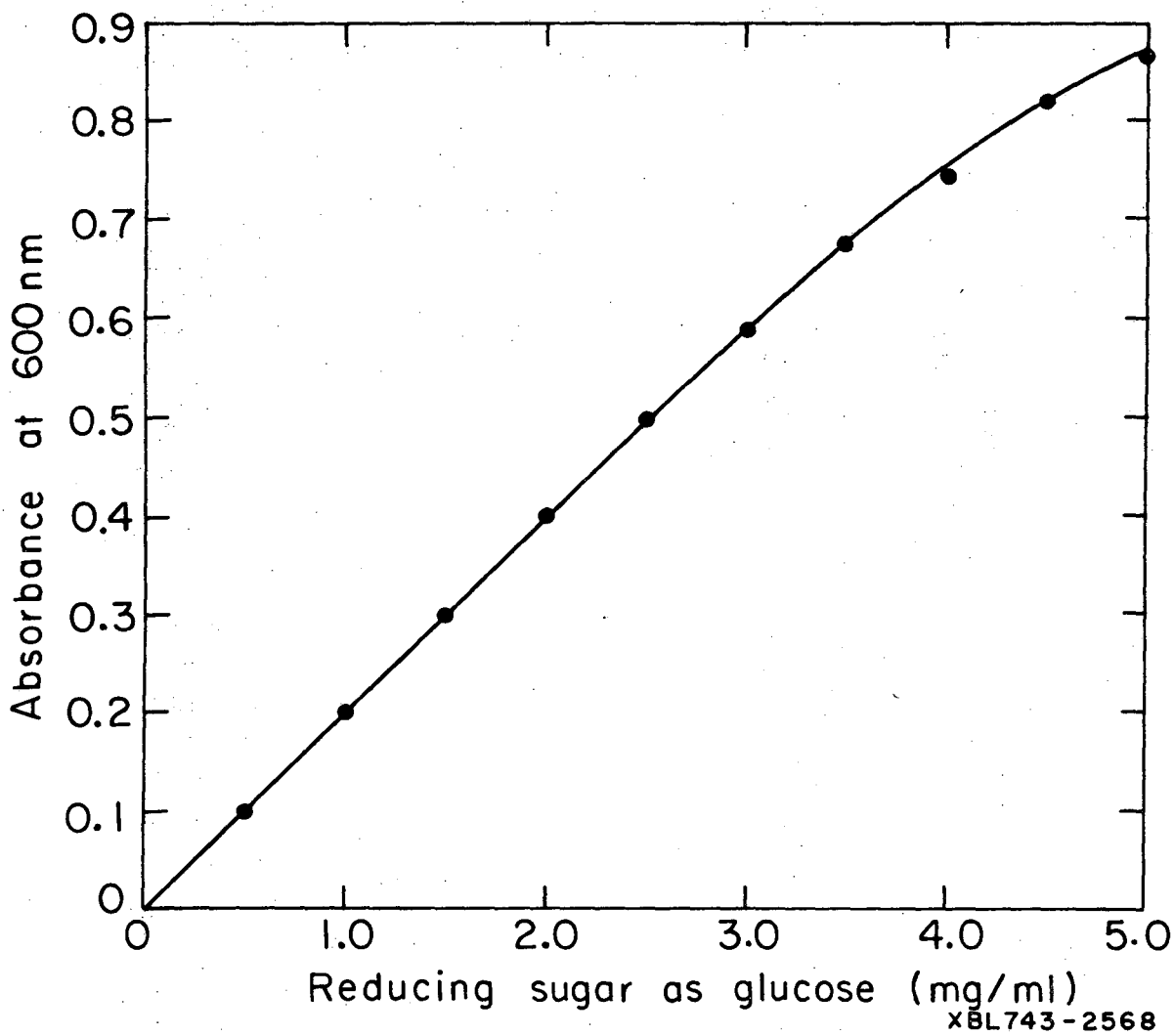

Fig. 3. Standard curve for the reducing sugar assay by the DNS method with glucose as the standard.

once to potato dextrose agar slants. The cellulase enzyme solution was produced by growing the fungus in batch culture.

The medium used in growing the fungus was developed by Mandels and Weber (42). The composition of the medium is shown in Table I. The only modification of the medium was that 0.5% Solka Floc SW40 (Brown Co.) was used instead of 1%. 9 l of medium was dispensed in a 14 l New Brunswick glass fermentor. The fermentor was then sterilized for 20 minutes at 121° C and 15 psig. After cooling, the fermentor was inoculated with 200 ml of a 5 day old shake flask culture of T. viride, grown on cellulose.

The fermentor was stirred with a 4 bladed turbine impeller (4-5/8 inch diameter) at an agitation rate of 150 r.p.m. The fermentor was aerated at a rate of 0.25 volumes of air per volume of fermentation liquid per minute (v.v.m.). The temperature and pH were controlled at 30°C and 5.0, respectively. Antifoam (AF60, General Electric Co.) was added as needed.

After 4.5 days, the fermentation was stopped and the culture was harvested. The T. viride mycelium and the unconsumed cellulose were separated from the cellulase enzyme solution by centrifugation followed by filtration. The filtrate was buffered at pH 5.0 with 0.050 M sodium citrate. Contamination of the enzyme solution was prevented by adding 0.01% Merthiolate (Eli Lilly Co.) and the solution was stored at 2-4° C. The initial enzyme activity was about 3.1 Filter Paper units and a slight decrease in activity was noted during the long storage.

Table I. Medium Composition for the Production of Cellulase by *Trichoderma viride* in Submerged Culture

Constituent	Concentration (g/l)
KH_2PO_4	2.0
$(NH_4)_2SO_4$	1.4
$(NH_2)_2CO$	0.3
$MgSO_4 \cdot 7H_2O$	0.3
$CaCl_2$	0.3
Proteose Peptone (Difco)	0.5
Solka Floc SW 40 (Brown Co., Berlin, N.H.)	5.0
Trace Minerals:	(mg/l)
$FeSO_4 \cdot 7H_2O$	5.0
$MnSO_4 \cdot H_2O$	1.56
$ZnSO_4 \cdot 7H_2O$	1.4
$CoCl_2$	2.0

Experimental Procedures

A) Temperature effects on acetone precipitation

In this set of experiments, the effect of temperature on the recovery of enzyme activity (C_1, C_x, and FP) and protein was examined. The temperature was varied from 30°C to 50°C in 5°C intervals. The cellulase enzyme solution was first removed from the refrigerator and allowed to warm to room temperature. 10 ml samples were then dispensed into 50 ml glass stoppered erlenmeyer flasks. A given amount of acetone was also dispensed at room temperature into separate stoppered erlenmeyer flasks. The quantity of acetone used to precipitate the enzyme varied from 10 ml to 35 ml in graduations of 5 ml.

The flasks of acetone and enzyme solutions were then placed into the oven which was set at the given operating temperature and allowed to reach thermal equilibrium. The acetone was then mixed with the cellulase solution, and the mixture was allowed to sit for 10 minutes to allow the precipitate to equilibrate. The mixture was then transferred to capped 50 ml polypropylene centrifuge tubes.

The precipitates were separated by centrifugation for 5 minutes at 800 rpm, at the given temperature of the experiment. The supernatant was decanted off, and the precipitate was redissolved in 0.05 M sodium citrate buffered at pH 4.8 to a final volume of 10 ml, the original volume of the cellulase solution. The C_1, C_x and filter paper activities of the recovered enzyme were measured. In each experimental run, the enzyme activities of the original enzyme solution were measured to compensate for any loss of enzyme activity during storage. The protein concentration of the redissolved cellulase enzyme solution was also measured.

B) Protein effects of acetone precipitation

In this set of experiments, the protein concentration of the cellulase solutions was varied to determine its effect on acetone precipitation. The total protein and cellulase protein concentrations of the fermentation broths were varied. The total protein of the enzyme solution was increased by addition of bovine serum albumin (BSA). The protein concentration was increased by 50 and 100% of the protein concentration of the fermentation broth in these experiments.

The effect of cellulase concentration was also examined. In order to concentrate the cellulase, a quantity of cellulase enzyme solution was treated with acetone at a concentration of 3 volumes of acetone per volume of enzyme solution. The precipitate was then recovered by centrifugation. The precipitate was dissolved in 0.05 M sodium citrate buffered at pH 4.8 and brought to a final volume equal to half of the original volume. These operations were all conducted at room temperature.

The acetone precipitation procedure was exactly the same as the procedure in the previously mentioned temperature experiments. Again, the recoveries of enzyme activity (C_1, C_x, and FP) and protein were recorded.

C) Stability of cellulase during storage

The stability of the dried cellulase enzyme was examined. The same precipitation procedure as in the temperature experiments was followed. 10 ml portions of fermentation broth containing cellulase enzyme were treated with acetone (3.0 v/v). The resulting mixture

was centrifuged and the supernatant was discarded. The precipitated enzyme remaining in the centrifuge tubes was dried and stored at room temperature in a dessicator. During the ensuing weeks, the enzyme was dissolved in 10 ml of 0.05 M sodium citrate buffered at pH 4.8. The C_1, C_x, and FP activities were then measured.

One 10 ml portion of precipitated enzyme was not stored but was immediately dissolved in buffer. The enzyme activities were measured and served as the standard in determining the stability of the cellulase enzyme.

D) Equilibrium of the cellulase precipitate

In this set of experiments, the time required for the cellulase precipitate to equilibrate was examined. 10 ml of cellulase enzyme was mixed with 30 ml of acetone. The resulting mixture was allowed to stand from 1 to 20 minutes. The precipitate was then separated by centrifugation at 1000 rpm for 5 minutes. The supernatant was discarded and the precipitate was redissolved in 0.05 M sodium citrate buffer pH 4.8 to a final volume of 10 ml. The filter paper activity was then measured. The experiments were conducted at room temperature.

E) Filtration

The filtration characteristics of the precipitated cellulase enzyme were studied under two conditions, in the presence and in the absence of filter aid. Because of the tendency of the solids to settle during filtration, the normal filtration procedure based on a continuous build-up of filter cake was not followed. Instead the procedure was based on filtration through a filter cake of constant thickness.

The cellulase enzyme was precipitated at room temperature with 3.0 v/v acetone. The cake thickness was varied by varying the amount of cellulase solution to be filtered. In the experiments using filter aid, ball milled paper was added in a weight ratio of 9:1 (paper to precipitated enzyme). The volumes of cellulase enzyme treated with acetone were 25 ml, 40 ml, 50 ml, 60 ml, and 75 ml in the experiments without filter aid, and 25 ml, 50 ml, 75 ml, and 100 ml in the experiments using filter aid.

The slurry was placed in the funnel and the solids were allowed to settle. After the solids had settled, a constant pressure was applied. The volume of filtrate passing through the filter was recorded as a function of time. The same pressure differential was used for each set of cake thicknesses. Then the pressure differential was changed and the experiments were repeated. Three different pressure drops were used (10, 17, and 24 in. Hg).

RESULTS

Effect of acetone concentration on enzyme recovery

The recovery of C_1, C_x and FP activities at various acetone concentrations and at different temperatures are shown in Figs. 4, 5, and 6 respectively. The results at 50°C had a greater uncertainty, because the actual temperature was only within ±1°C of the desired operating temperature. The recoveries are based on the percentage of original activity recovered. Each of these figures indicate that little enzyme activity is recovered at acetone concentrations of 1.0 v/v or less. With acetone concentrations greater than 1.0 v/v, the recovery of enzyme activity increases rapidly and then levels off at acetone

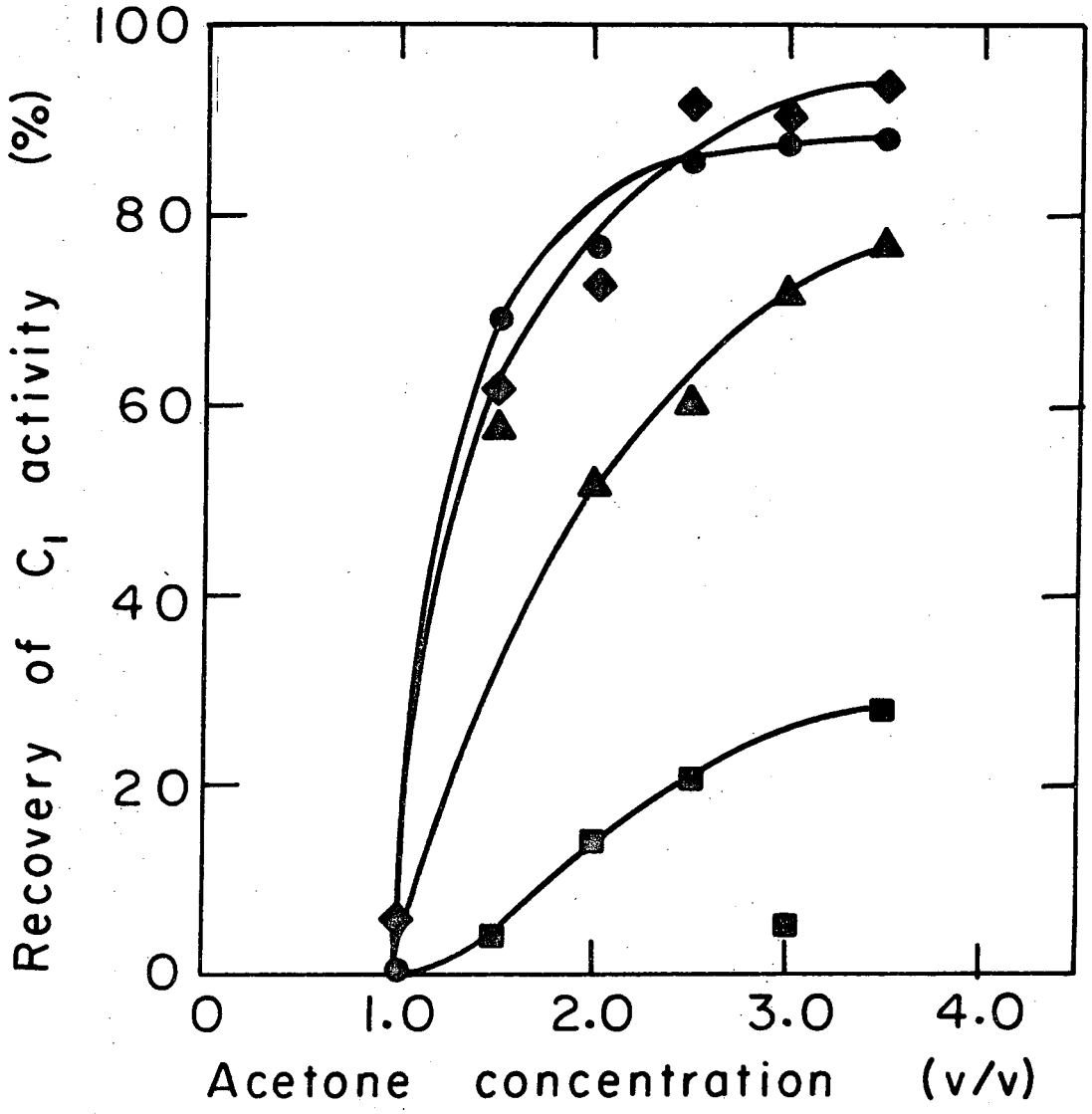

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Fig. 4. Effect of acetone concentration on the recovery of C_1 activity. The initial activity and the temperature were 6.25 units at 30°C(●), 6.43 units at 35°C(◆), 7.69 units at 40°C(▲), and 6.12 units at 50°C(■).

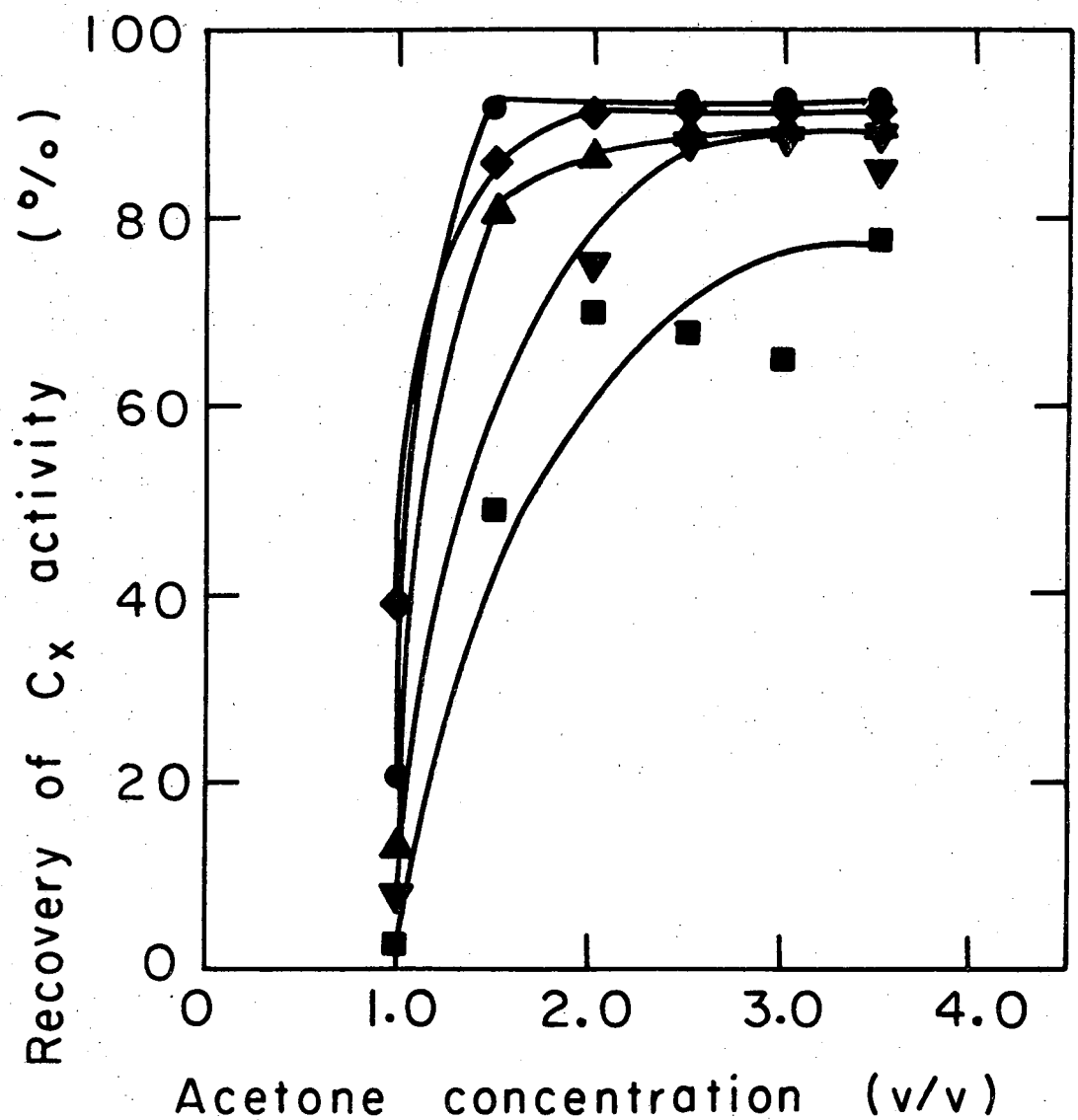

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Fig. 5. Effect of acetone concentration on the recovery of C_x activity. The initial activity and the temperature were 2.36 units at 30°C(●), 2.20 units at 35°C(◆), 1.92 units at 40°C(▲), and 1.85 units at 50°C(■).

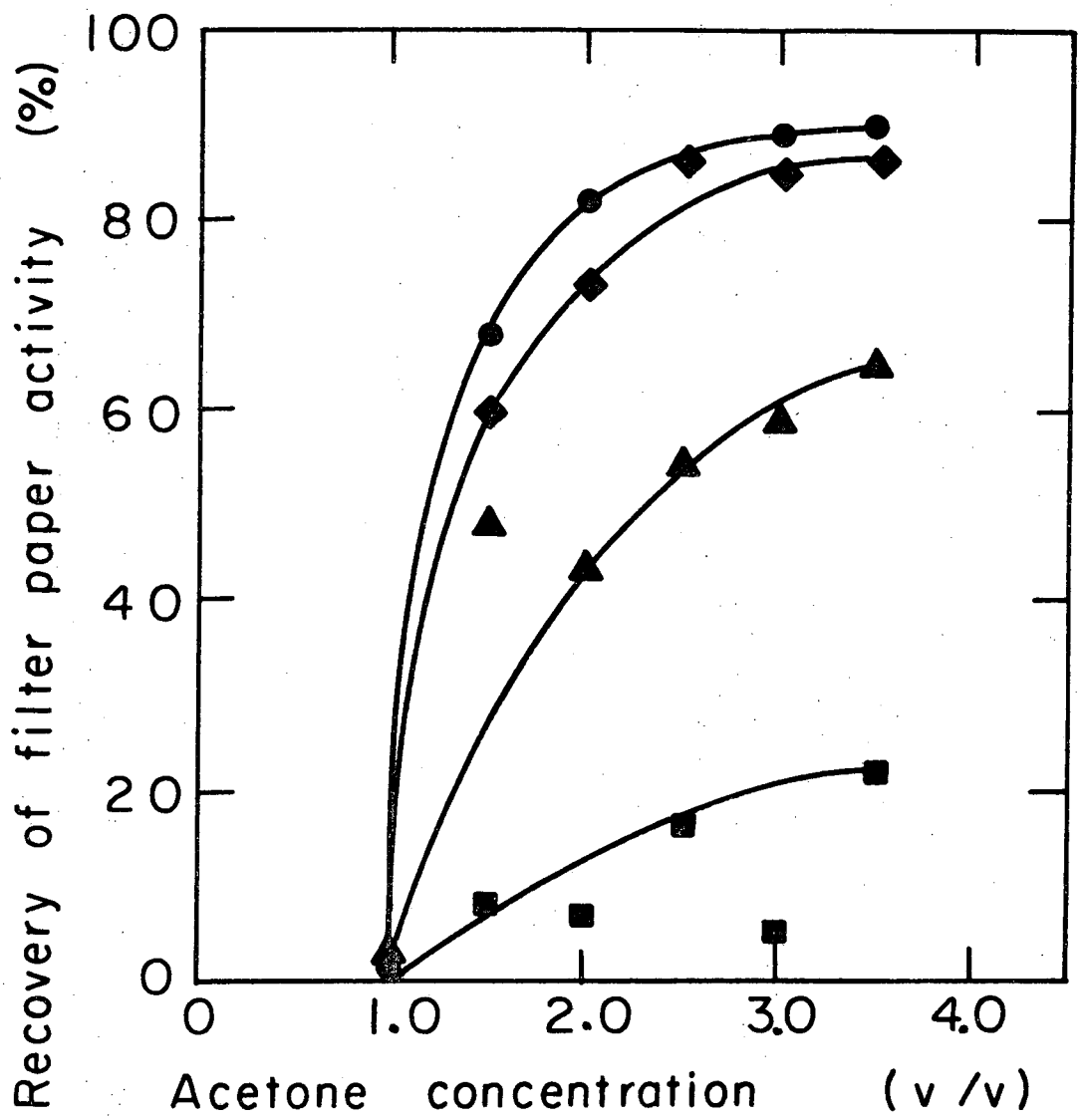

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Fig. 6. Effect of acetone concentration on the recovery of FP activity. The initial activity and the temperature were 3.25 units at 30°C(●), 3.08 units at 35°C(◆), 3.38 units at 40°C(▲), and 2.85 units at 50°C(■).

concentrations of 3.5 v/v. The initial slopes of the curves for each of the enzyme activities decreases with increasing temperature.

It can be seen from Figs. 4 and 6 that the recovery characteristics of the C_1 and FP activities are very similar. At 30°C, the recovery of both activities reaches 90% of the final recovery at 2.0 v/v acetone and greater than 98% at 3.0 v/v acetone. The recovery of C_x activity on the other hand exhibit different characteristics. At 30°C, 99% of the final recovery of C_x activity is achieved with an acetone concentration of only 1.5 v/v.

In Figs. 7, 8, and 9, the unrecovered fractions of C_1, C_x, and FP activities are plotted on semi-logarithmic scale against the dielectric constant of the solution. The dielectric constant for a given acetone-water solution was obtained from the data gathered by Akerlöf (2) given in the Appendix. Because denaturation of the enzyme occurs with increasing temperature, the recovery of C_1, C_x, and FP activities has been normalized by assuming that, at a given temperature, the maximum recovery of activity was assumed to be 100% recovery. The method of least-squares was used to obtain the best straight line for a set of data points. The results are summarized in Tables II, III and IV.

It can be seen from Figs. 7, 8, and 9 and Tables II, III and IV that the constants K_s and β, vary with temperature for each of the enzyme activities. The constants were plotted against temperature on a semi-logarithmic scale and are shown in Figs. 10, 11, and 12. The resulting

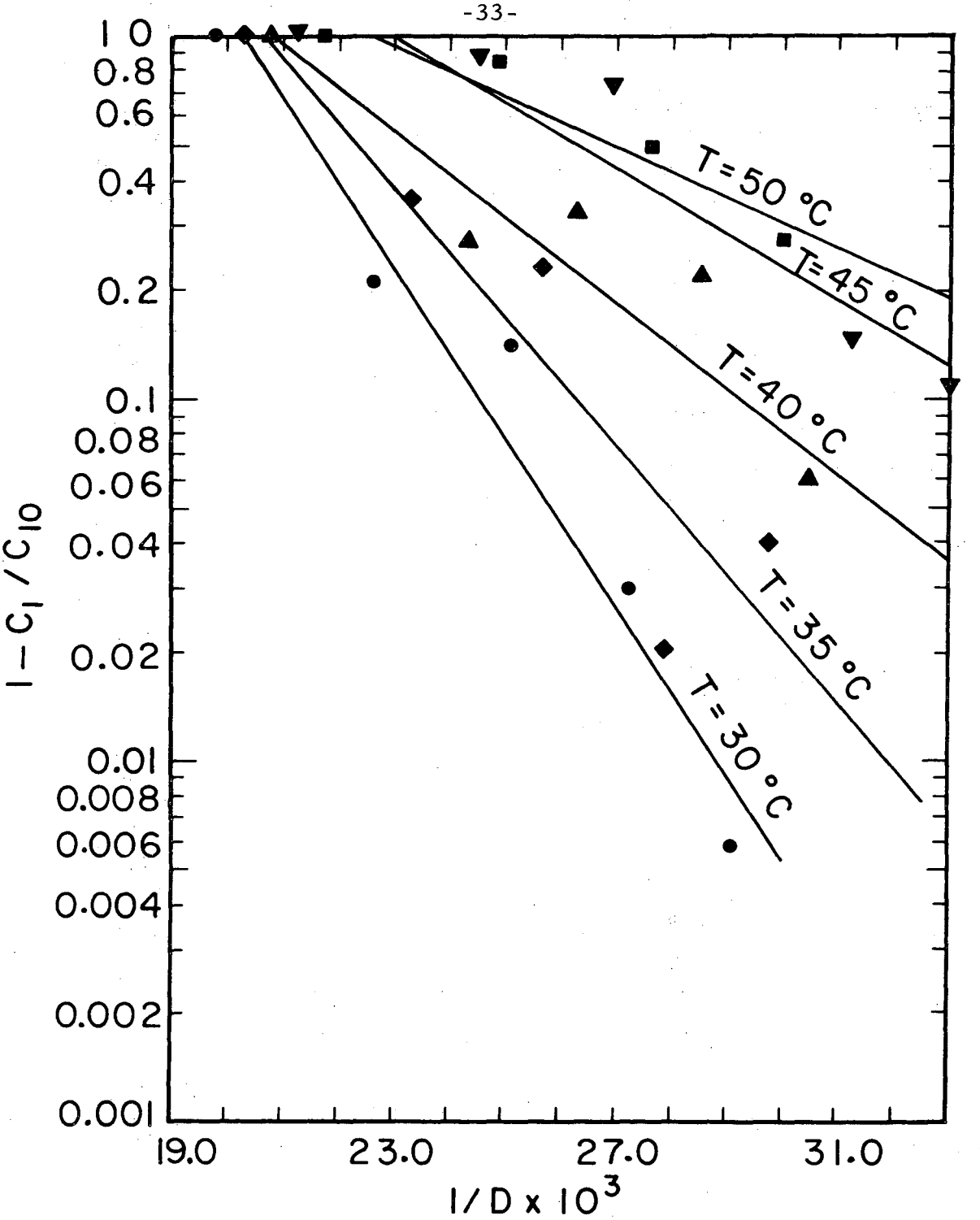

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Fig. 7. Effect of dielectric constant on the recovery of C_1 activity. The initial activity and the temperature were 6.25 units at 30°C(●), 6.43 units at 35°C(◆), 7.69 units at 40°C(▲), 6.8 units at 45°C(▼), and 6.12 units at 50°C(■).

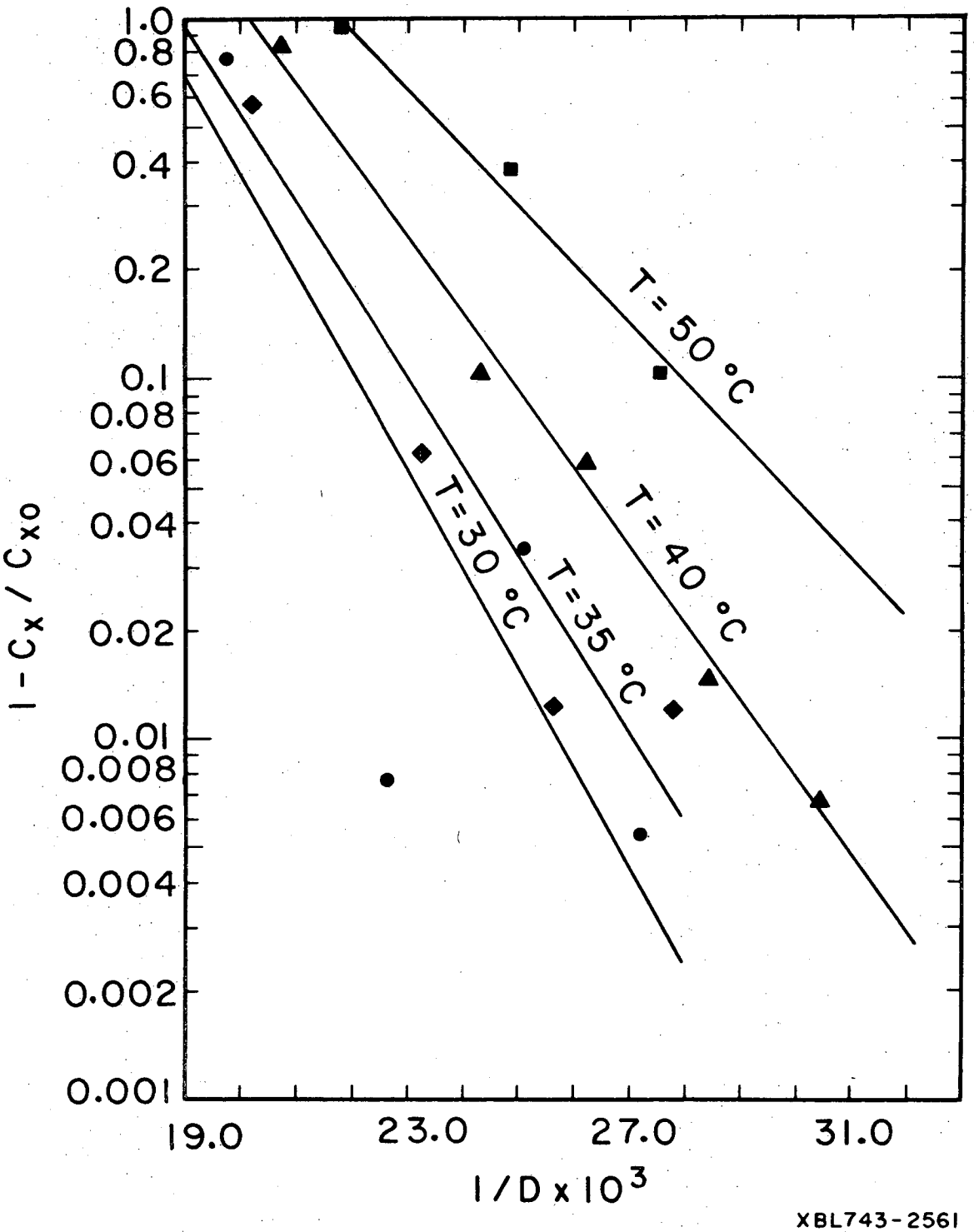

Fig. 8. Effect of dielectric constant on the recovery of C_x activity. The initial activity and the temperature were 2.36 units at 30°C(●), 2.20 units at 35°C(◆), 1.92 units at 40°C(▲), and 1.85 units at 50°C(■).

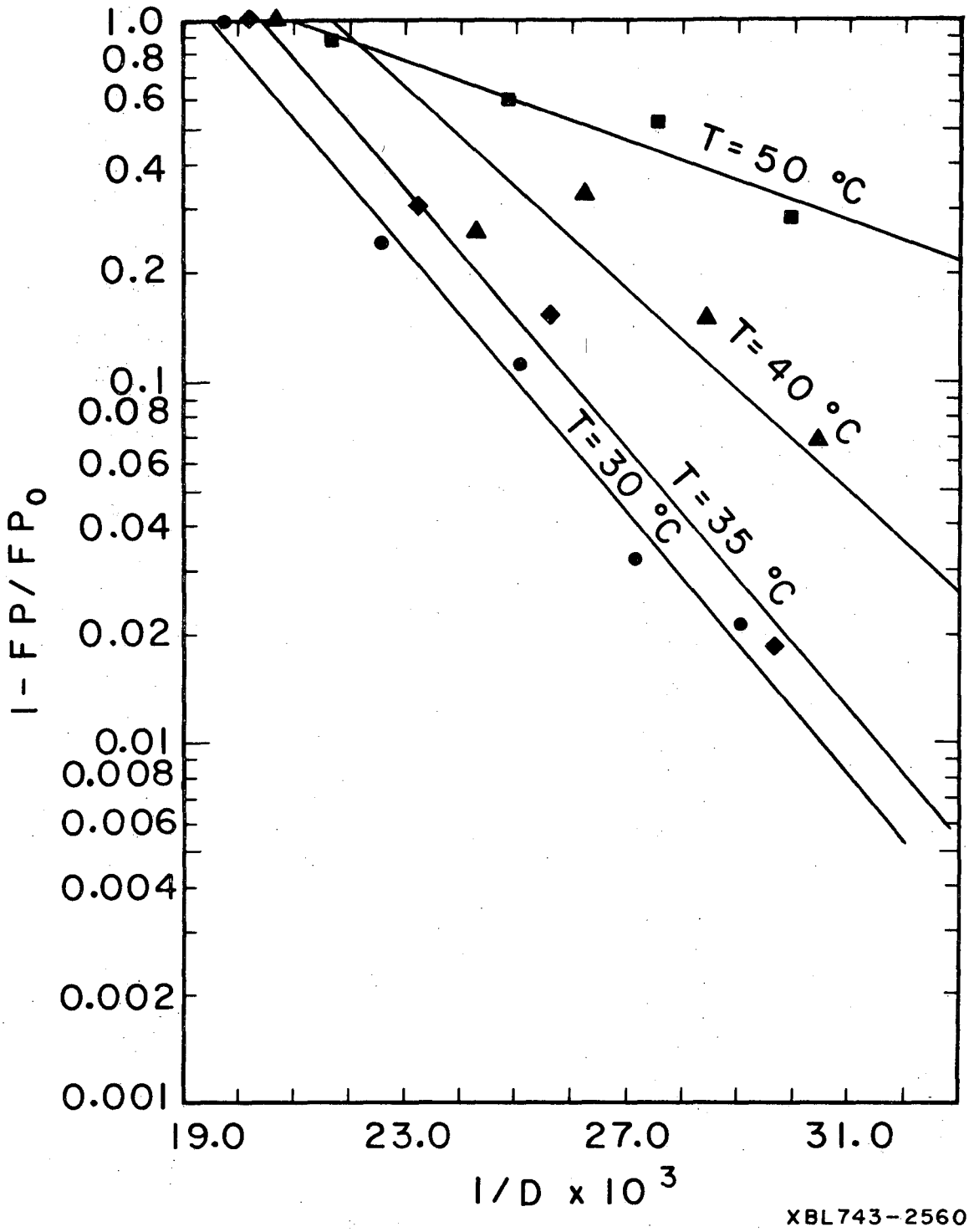

Fig. 9. Effect of dielectric constant on the recovery of FP activity. The initial activity and the temperature were 3.25 units at 30°C(●), 3.08 units at 35°C(◆), 3.38 units at 40°C(▲), and 2.85 units at 50°C(■).

Table II. Summary of the Salting-out Constants for the C_1 Activity at Different Temperatures

Temperature (°C)	β	K_s
30.0	4.77	235.6
35.0	3.65	177.28
40.0	2.36	114.24
45.0	2.06	89.95
50.0	1.58	69.93

Table III. Summary of the Salting-out Constants for the C_x Activity at Different Temperatures

Temperature (°C)	β	K_s
30.0	5.0	271.42
35.0	4.6	243.601
40.0	4.31	213.913
50.0	3.48	159.913

Table IV. Summary of the Salting-out Constants for the FP Activity at Different Temperatures

Temperature (°C)	β	K_s
30.0	3.55	181.505
35.0	3.68	180.02
40.0	2.21	108.55
50.0	1.16	55.228

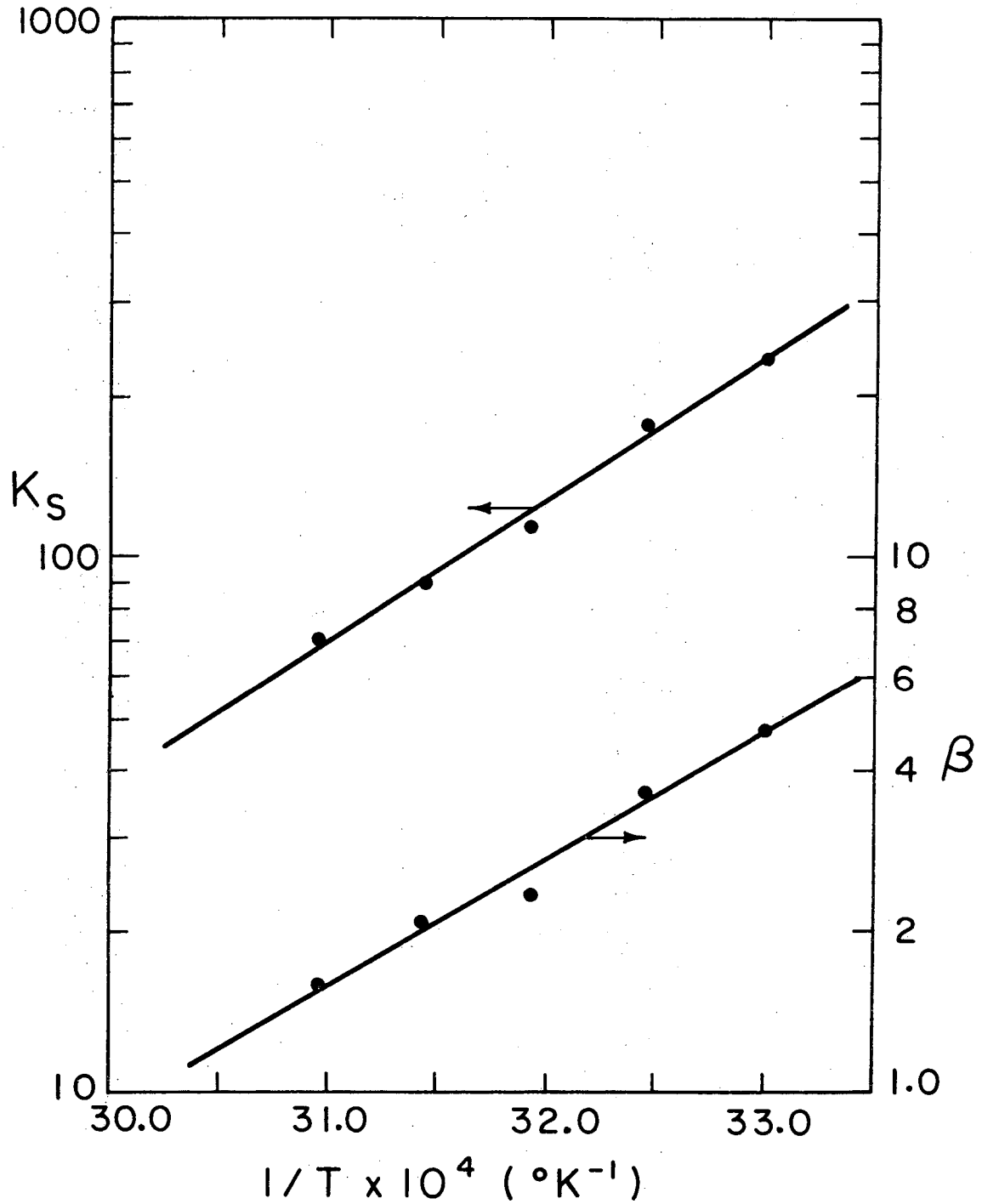

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Fig. 10. Variation of the constants of the salting-out equation for C_1 recovery with the reciprocal of the temperature.

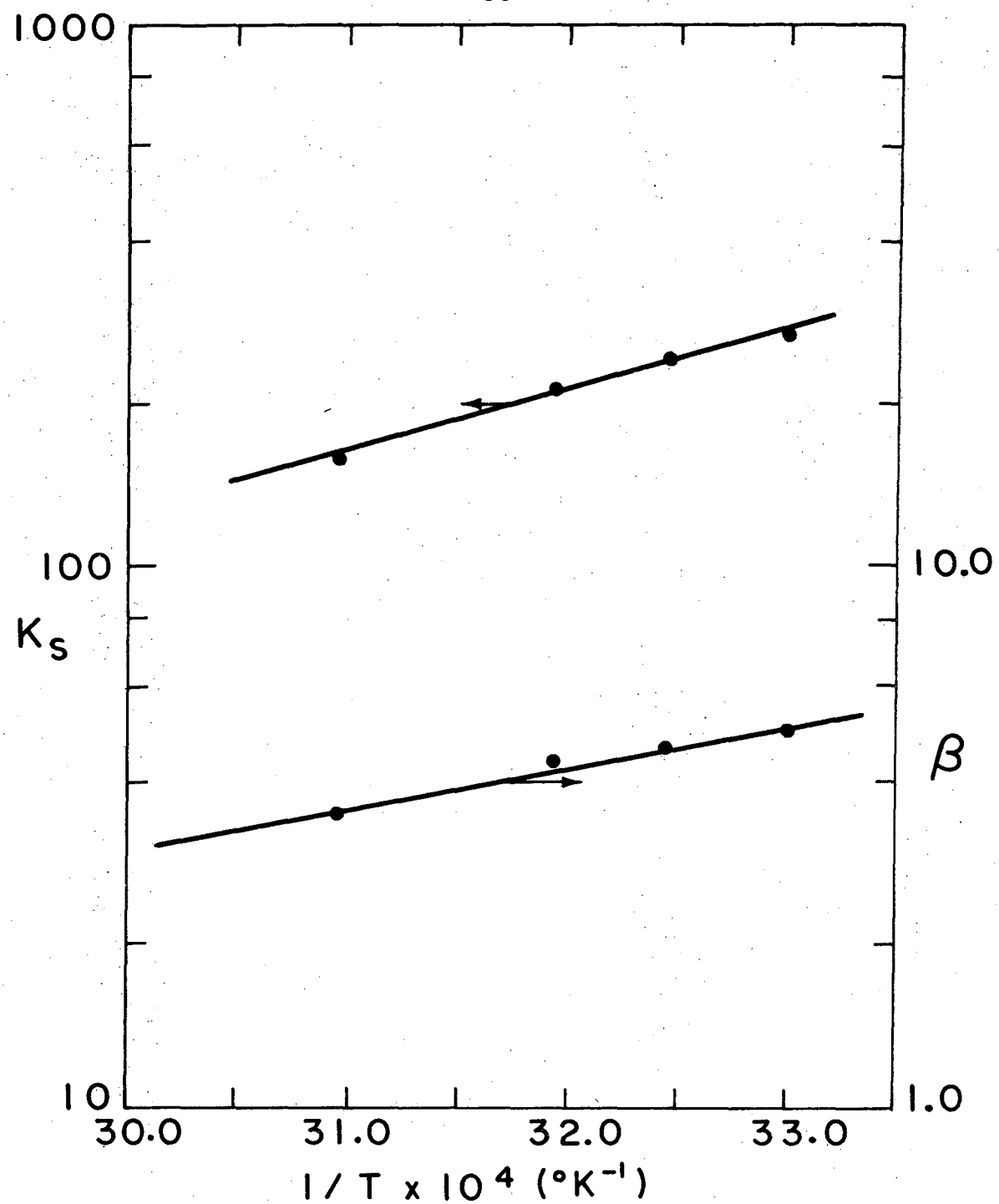

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Fig. 11. Variation of the constants of the salting-out equation for C_x recovery with the reciprocal of the temperature.

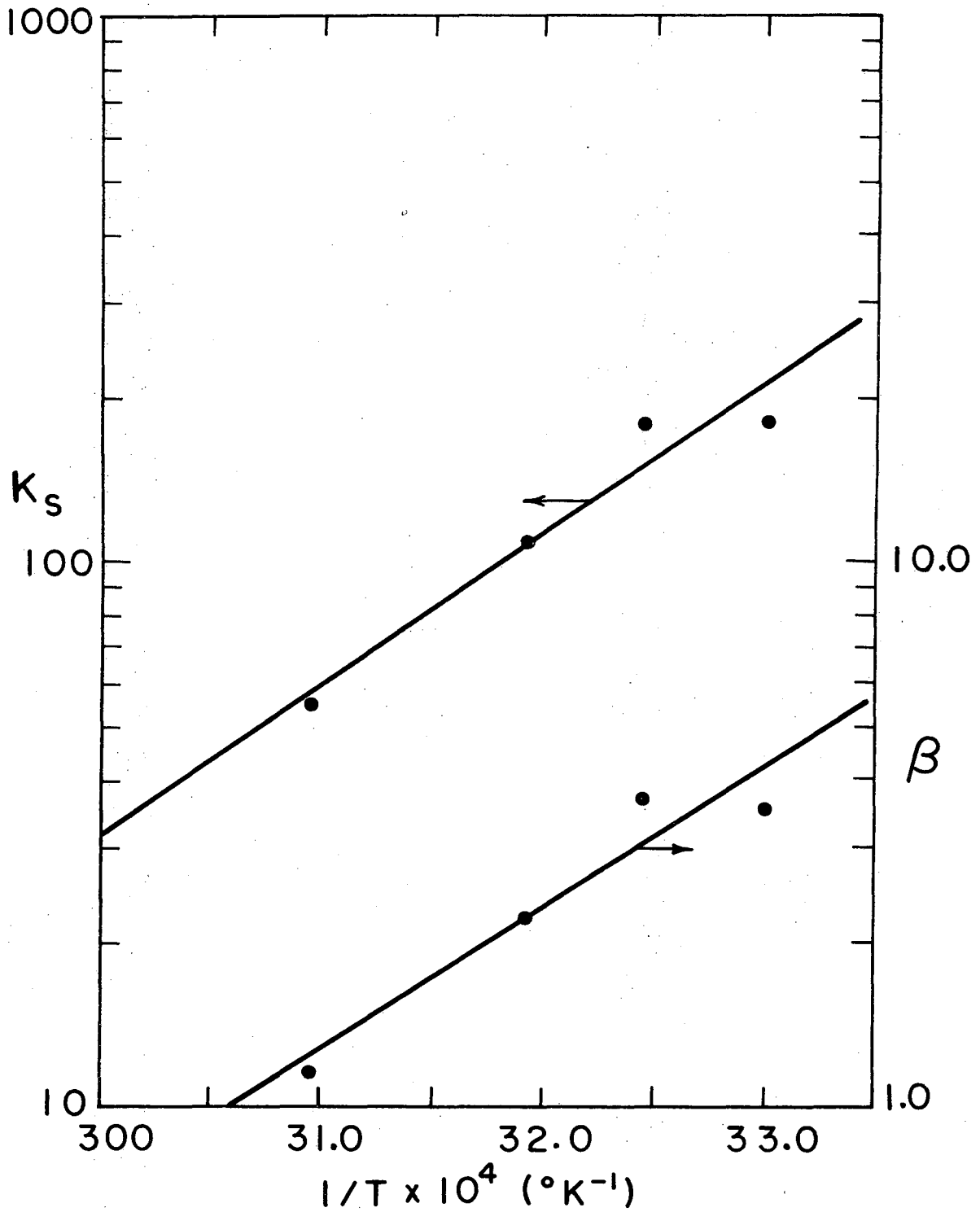

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Fig. 12. Variation of the constants of the salting-out equation for FP recovery with the reciprocal of the temperature.

straight lines indicate an exponential relationship of the constants with temperature. The results are summarized in Table V.

The amount of protein precipitated as a function of acetone concentration is shown in Fig. 13. The data from the different operating temperatures are shown in the figure. The general shape of the curve is similar to the recovery of C_1 and FP activities. The solubility of the protein is plotted against the dielectric constant of the solution in Fig. 14. It can be seen that the data can be approximated by a single straight line on the semi-logarithmic graph.

Effect of temperature on enzyme recovery

The effect of temperature on the recovery of C_1, C_x, and FP activities at an acetone concentration of 3.0 v/v is shown in Figs. 15 and 16. The loss of enzyme activity is due to denaturation of the enzyme. Denaturation is a first-order process with respect to the activity of the enzyme. The rate constants for denaturation for each of the enzyme activities are plotted against temperature and are shown in Figs. 17, 18, and 19. The activation energy and constant, k, for C_1, C_x, and FP activities are listed in Table VI. The effect of temperature on K_s and β for each enzyme activity was shown in the previous section. From Figs. 13 and 14, it can be seen that temperature has little effect on protein recovery.

Protein effects on acetone precipitation

The effect of total protein on the recovery of C_1, C_x, and FP activities is shown in Figs. 20, 21, and 22. The addition of protein does not greatly affect the recovery of C_x activity. On the other hand,

Table V. Summary of the Effect of Temperature on the Constants of the Salting-out Equation for the C_1, C_x, and FP Activities

Enzyme Activity	β		K_s	
	Intercept	Slope	Intercept	Slope
C_1	-7.146	+2369.59	-6.36	+2646.86
C_x	-1.844	+ 772.5	-1.31	+1137.93
FP	-7.9	+2584.2	-6.8	+2767.67

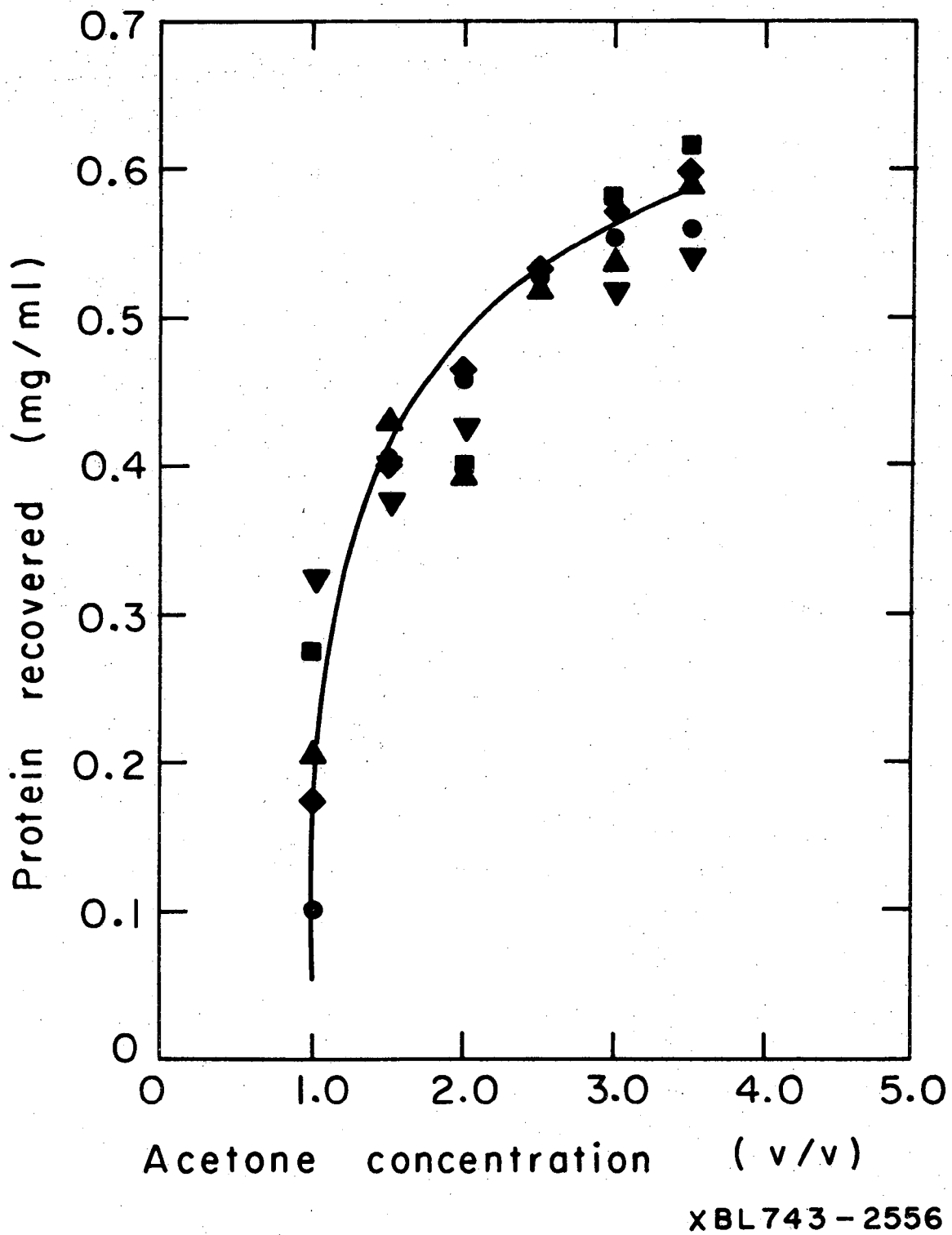

Fig. 13. Effect of acetone concentration on the recovery of protein. Included are the data from 30°C(●), 35°C(◆), 40°C(▲), 45°C(▼), and 50°C(■). The soluble protein concentration was 1.2 mg/ml.

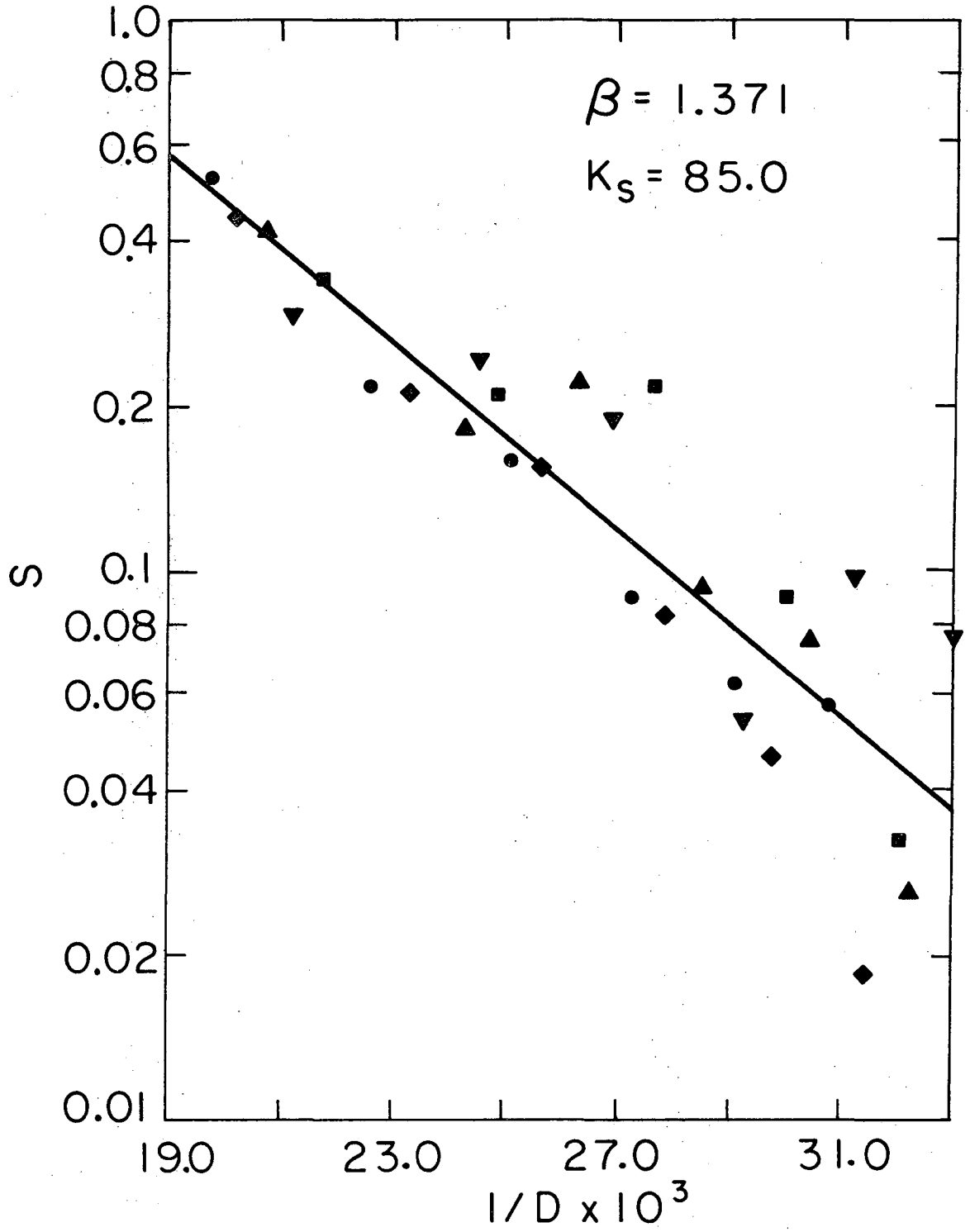

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Fig. 14. Effect of dielectric constant on the recovery of protein. Included are data from 30°C(●), 35°C (◆), 40°C(▲), 45°C(▼), and 50°C(■). The crude cellulase protein concentration was 0.615 mg/ml.

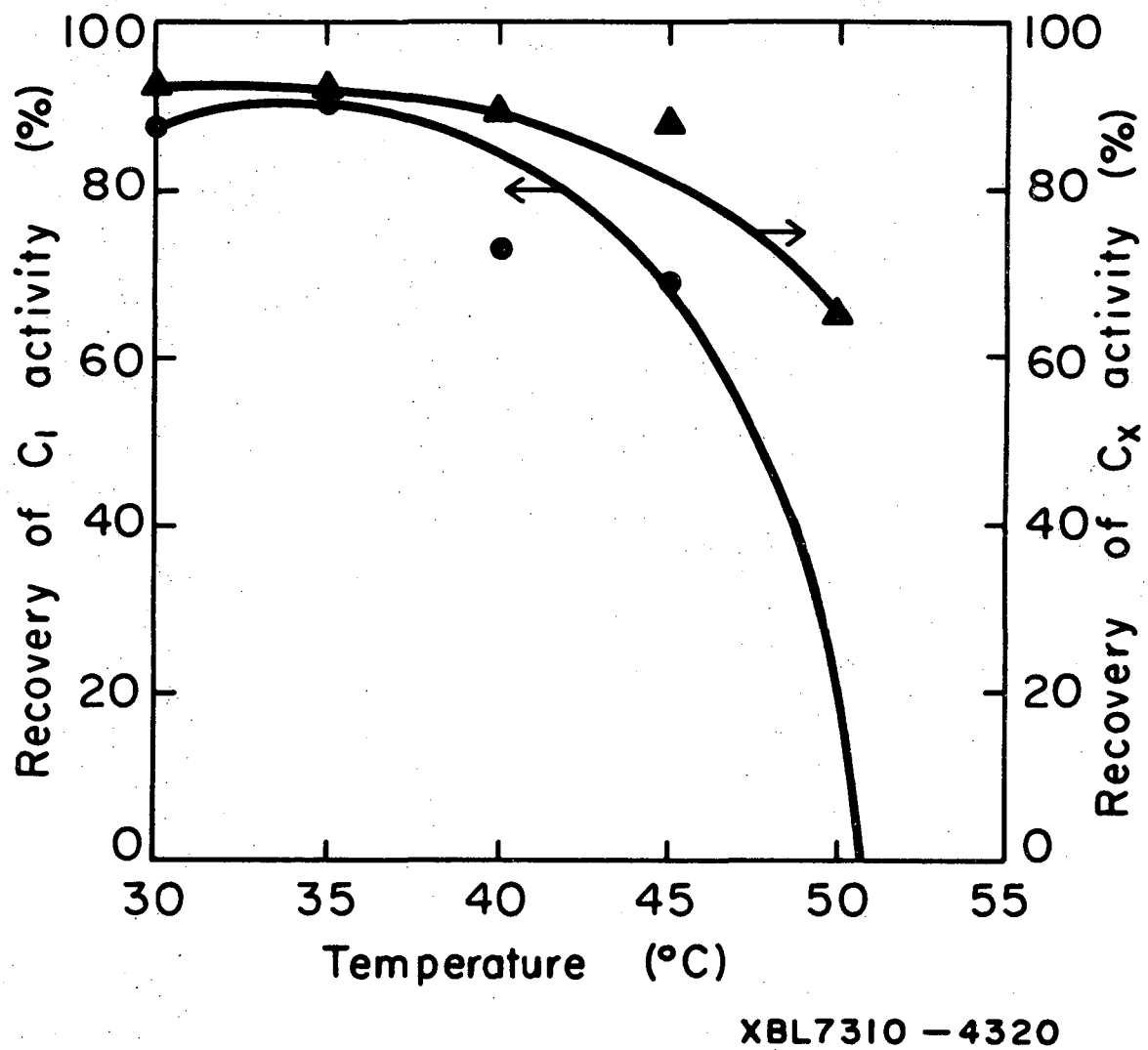

Fig. 15. Effect of temperature on the recovery of C_1 and C_x activities at an acetone concentration of 3.0 v/v. The initial activities were 6.25 C_1 units and 2.36 C_x units at 30°C, 6.43 C_1 units and 2.20 C_x units at 35°C, 7.69 C_1 units and 1.92 C_x units at 40°C, and 6.12 C_1 units and 1.85 C_x units at 50°C.

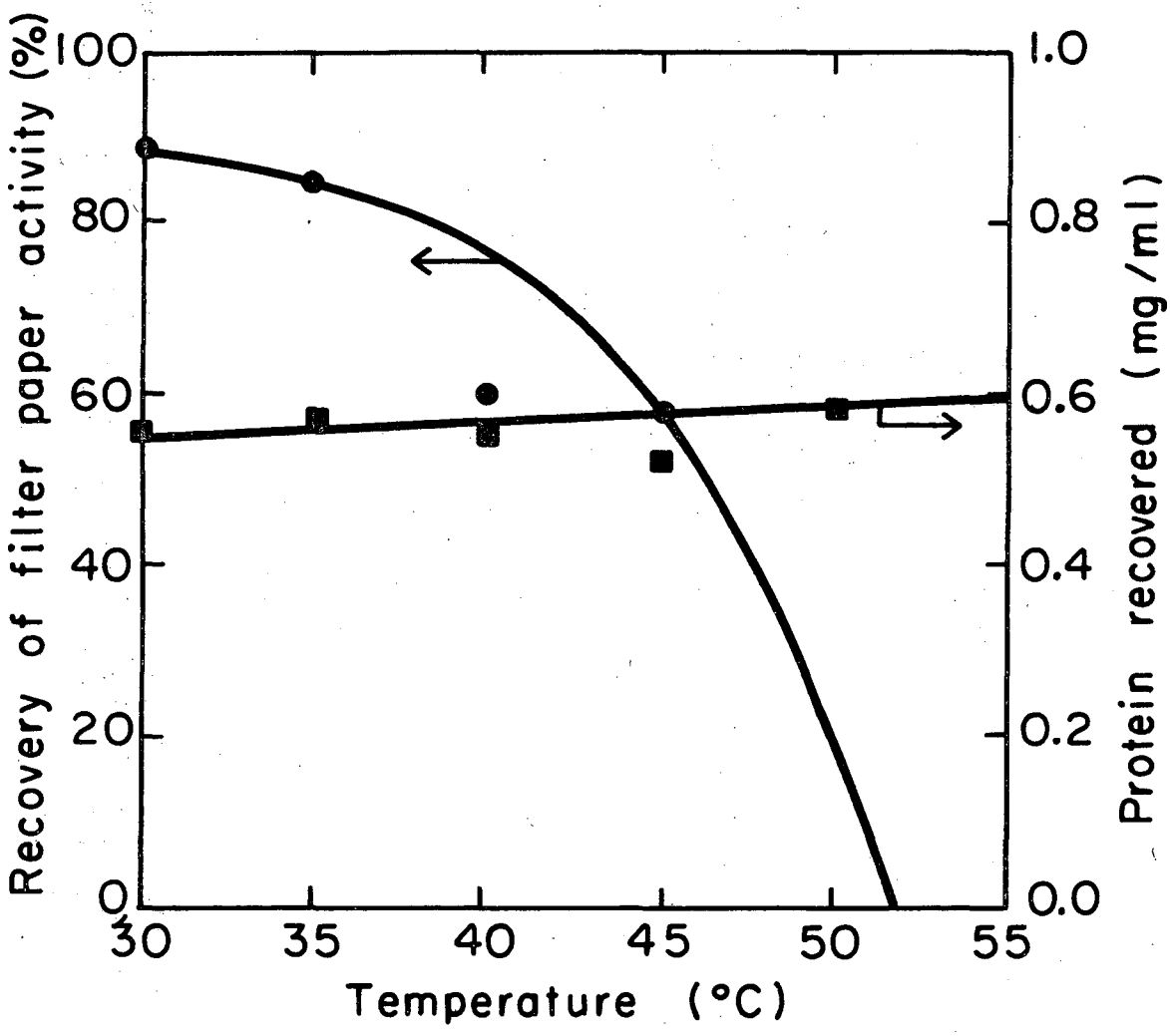

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Fig. 16. Effect of temperature on the recovery of FP activity and protein at an acetone concentration of 3.0 v/v. The initial FP activity was 3.25 units at 30°C, 3.08 units at 35°C, 3.38 units at 40°C, and 2.85 units at 50°C, and the soluble protein concentration was 1.2 mg/ml.

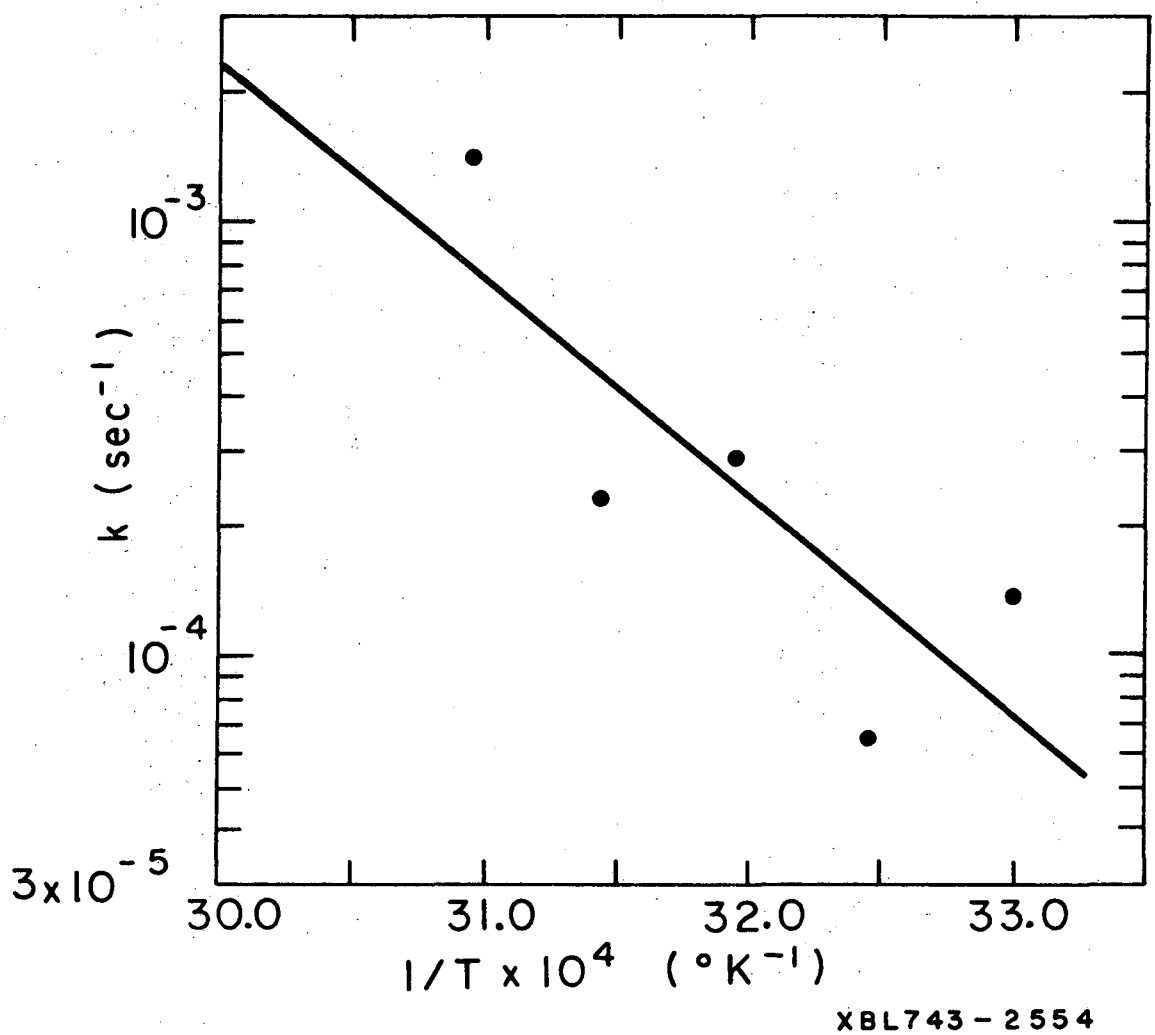

Fig. 17. Temperature dependency of the denaturation rate constant for C_1 activity during precipitation at an acetone concentration of 3.0 v/v.

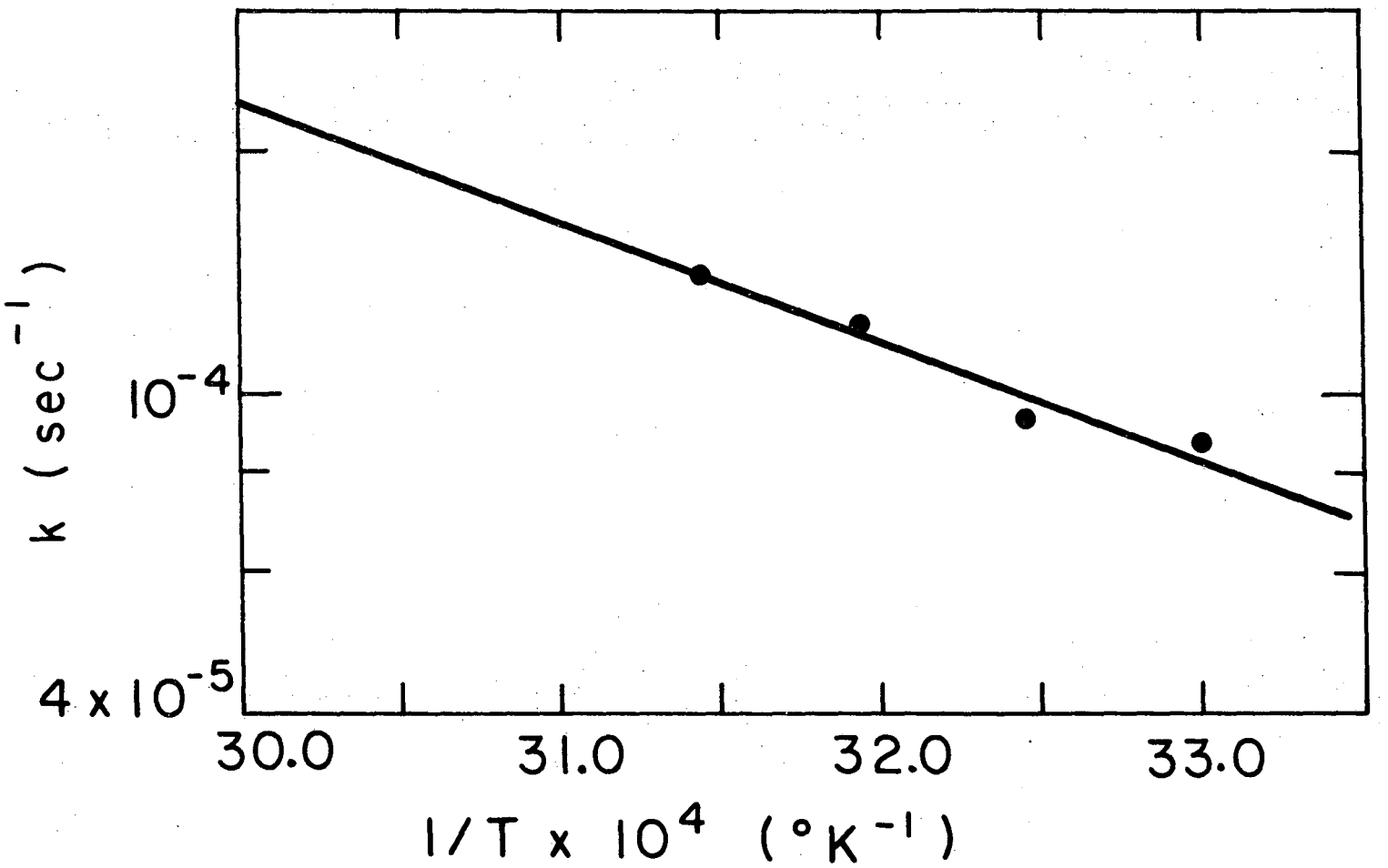

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Fig. 18. Temperature dependency of the denaturation rate constant for C_x activity during precipitation at an acetone concentration of 3.0 v/v.

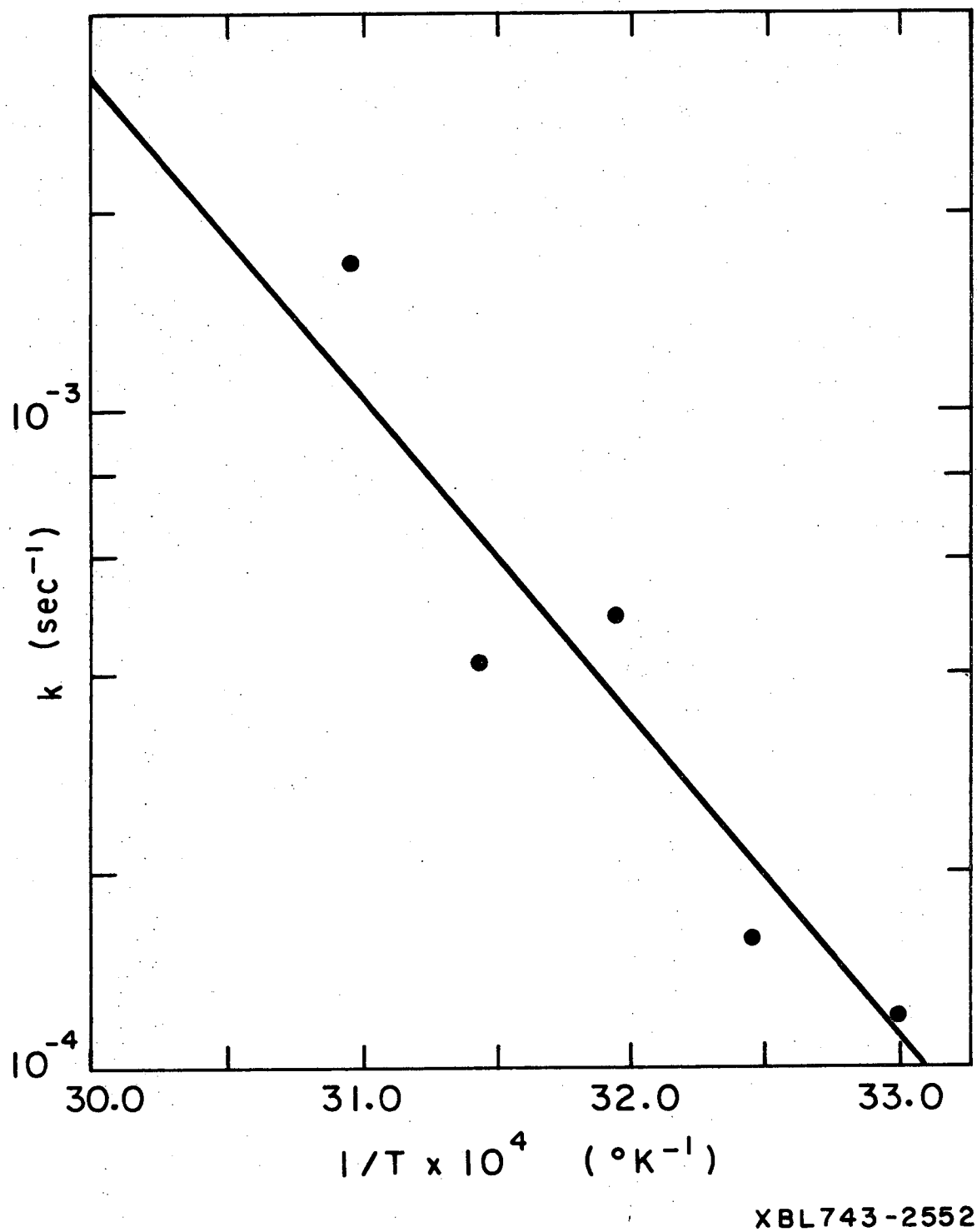

Fig. 19. Temperature dependency of the denaturation rate constant for FP activity during precipitation at an acetone concentration of 3.0 v/v.

Table VI. The Arrhenius Pre-exponential Factor and the Activation for the Denaturation Rate Constant of the Cellulase Enzymes during Precipitation at an Acetone Concentration of 3.0 v/v.

Enzyme Measurement	k_0	ΔE_a (cal/gm mole)
C_1	2.968×10^{12}	23034.24
C_x	6.153	6753.8
FP	8.5777×10^{11}	22012.55

Table VII. Effect of Concentration on Cellulase Activity

Item	Culture filtrate	2-fold Concentration	% Increase
C_1 Activity	6.37	7.838	23.0
C_x Activity	4.0	3.75	-7.25
FP Activity	2.55	3.675	44.1
Cellulase Protein	0.568	1.136	100.0

the recovery of C_1, and FP activities appear to be slightly affected by the addition of bovine serum albumin (BSA). The effect of total protein on protein recovery is shown in Fig. 23. It can be seen that the amount of protein recovered increased as the concentration of BSA increased. The additional protein recovered is not a constant but increases as the concentration of acetone increases.

When cellulase enzyme was concentrated 2-fold, the increase in C_1 and FP activity was less than 50% and there was no increase in C_x activity. The results are shown in Table VII. The recovery characteristics of this concentrated enzyme are shown in Figs. 24 and 25. It can be seen that the maximum amount of recovered activity increases. There is also a large increase in enzyme recovery between 1.0 and 1.5 v/v acetone. The solubility of the 2-fold concentrated enzyme is seen in Fig. 26. The line on the figure is taken from Fig. 14 for unconcentrated cellulase. In comparison with the protein solubility of the unconcentrated cellulase solution at 30°C, the data appear to be within the confidence limits of the line.

Stability of cellulase during storage

The cellulase precipitate was stored in a desiccator at room temperature. The effect of these storage conditions was examined and the results are shown in Fig. 27. In the analysis of the data, total recovery of enzyme activity is based on the activity of the once precipitated enzyme that was not dried but redissolved immediately. The C_1 and C_x activities appear to be quite stable during storage, although there was an initial decrease in C_1 activity during the first week after precipitation. For FP activity, there maybe a slight decreasing trend in the recovery of activity.

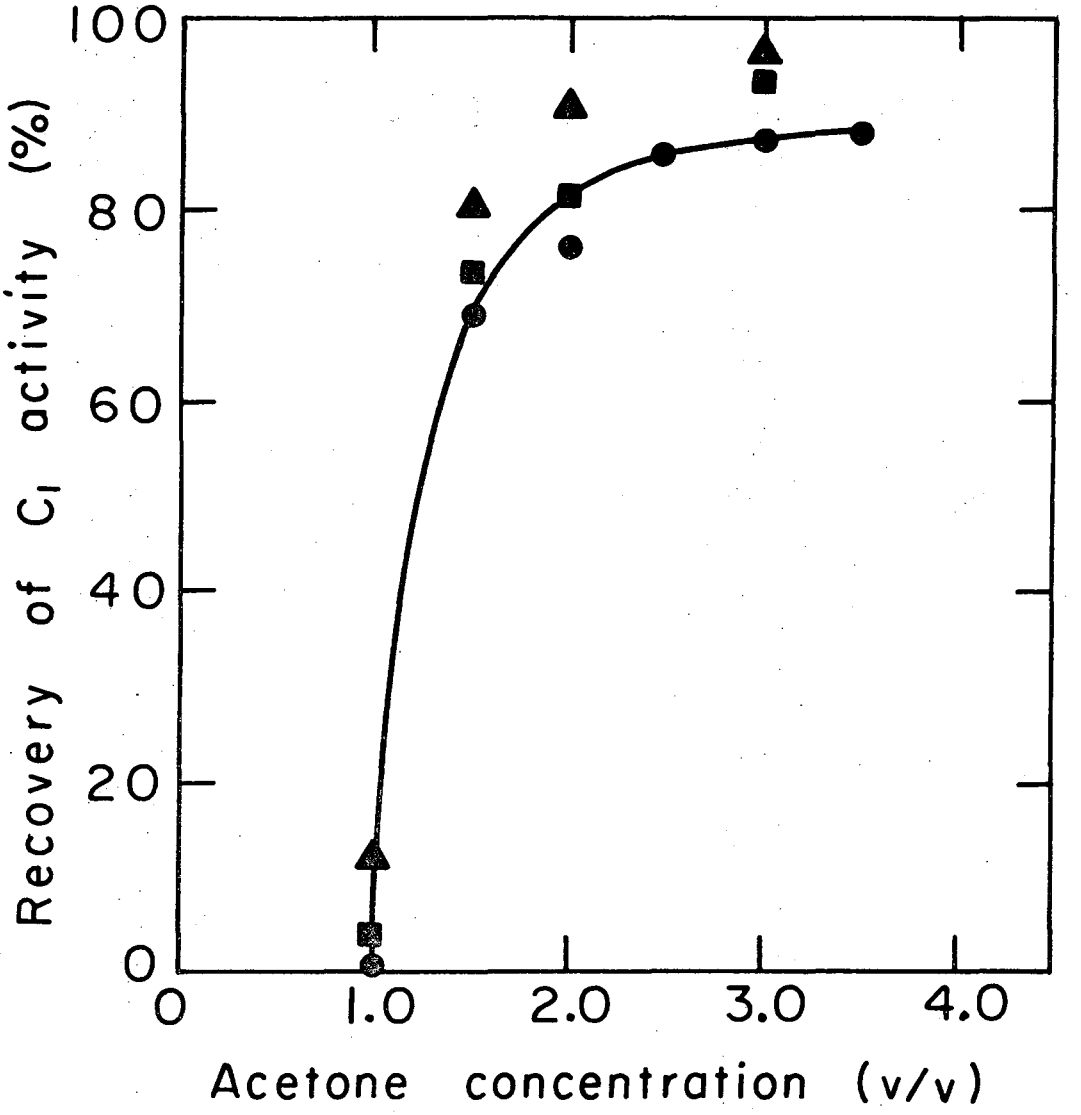

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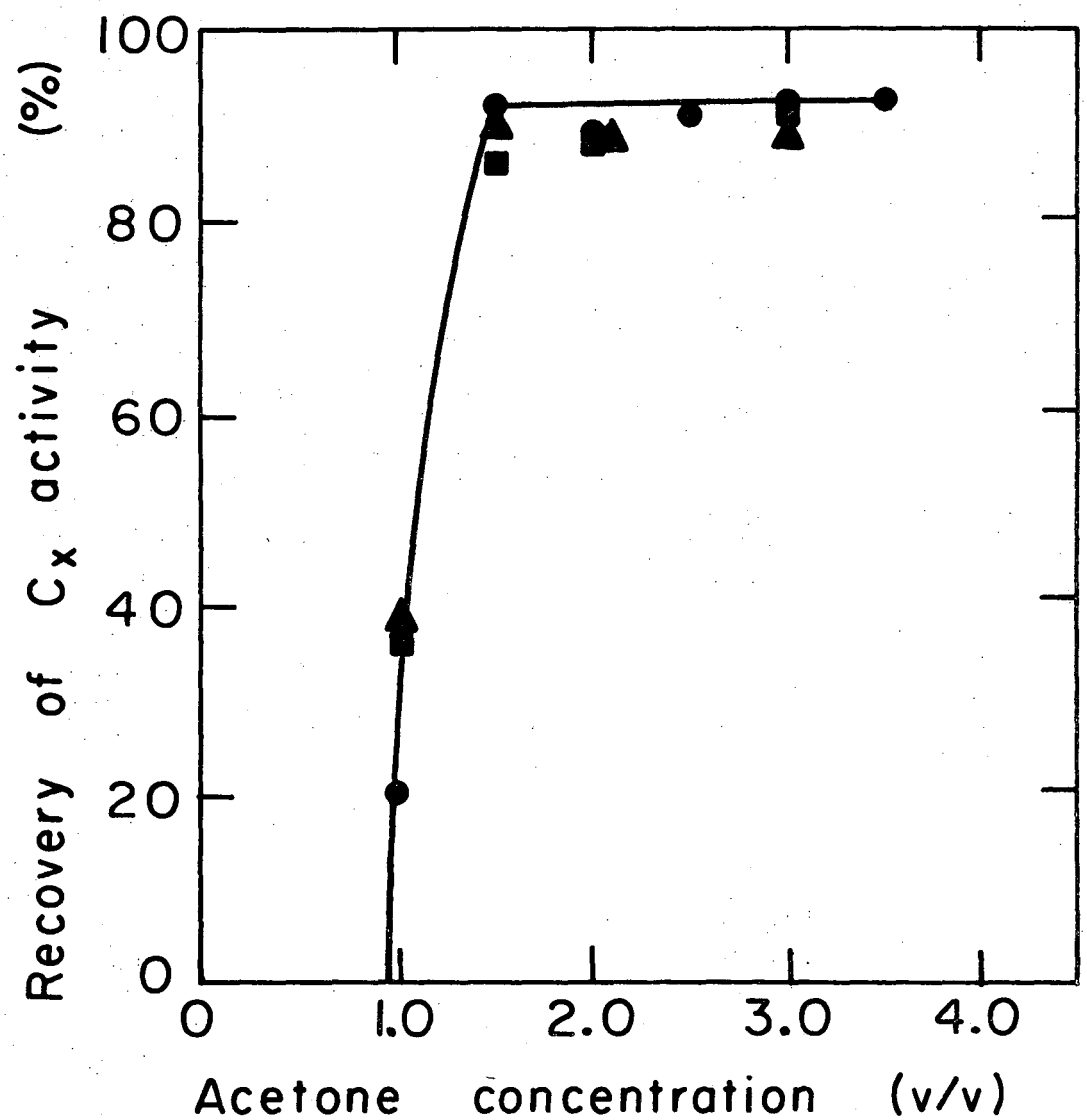
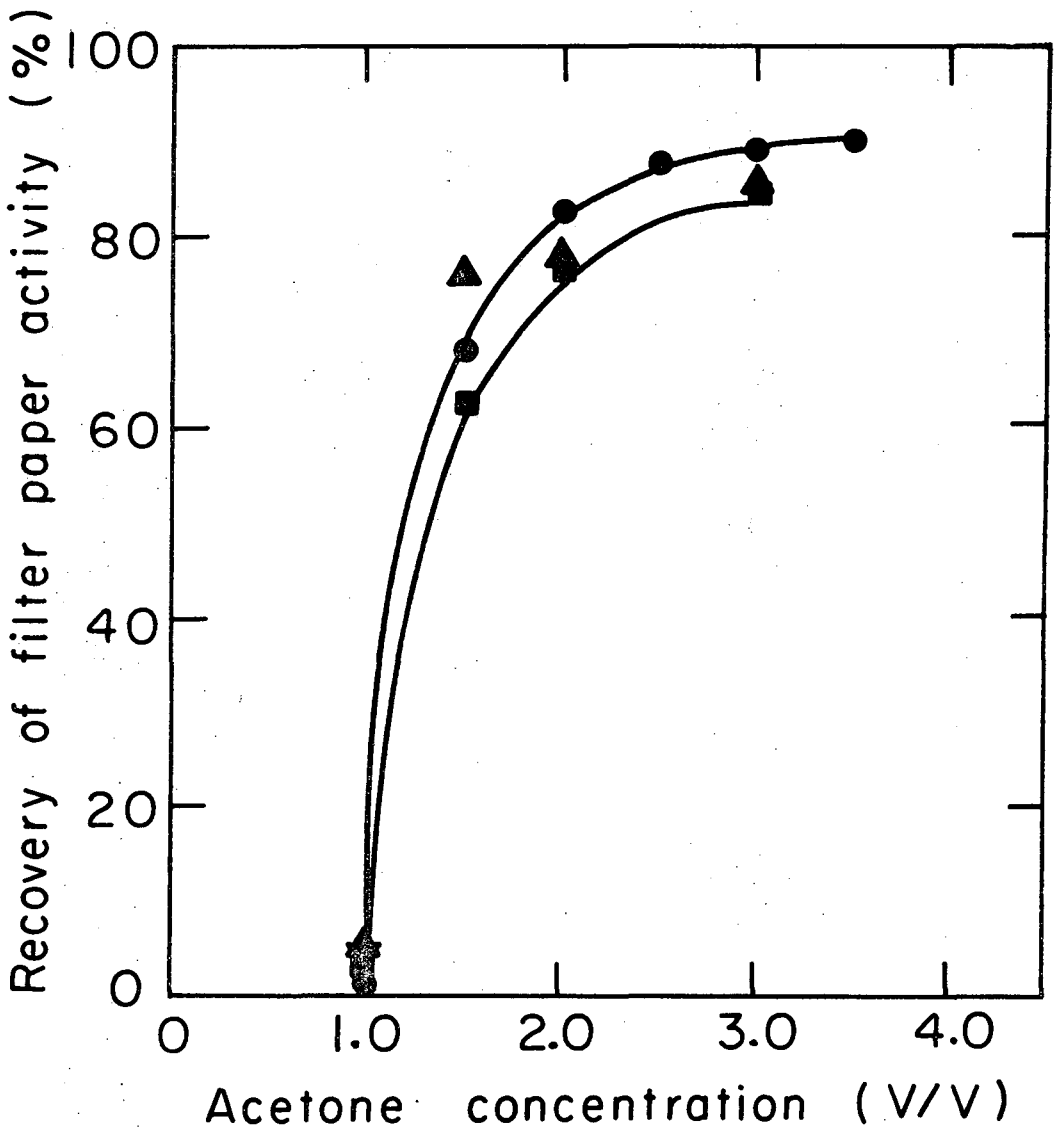
Fig. 20. Effect of total soluble protein concentration on the recovery of C_1 activity at 30°C. No additional protein (●), 0.58 mg/ml BSA added (■), and 1.14 mg/ml BSA added (▲). The initial C_1 activities were 6.25 units, 6.24 units, and 6.16 units, respectively.



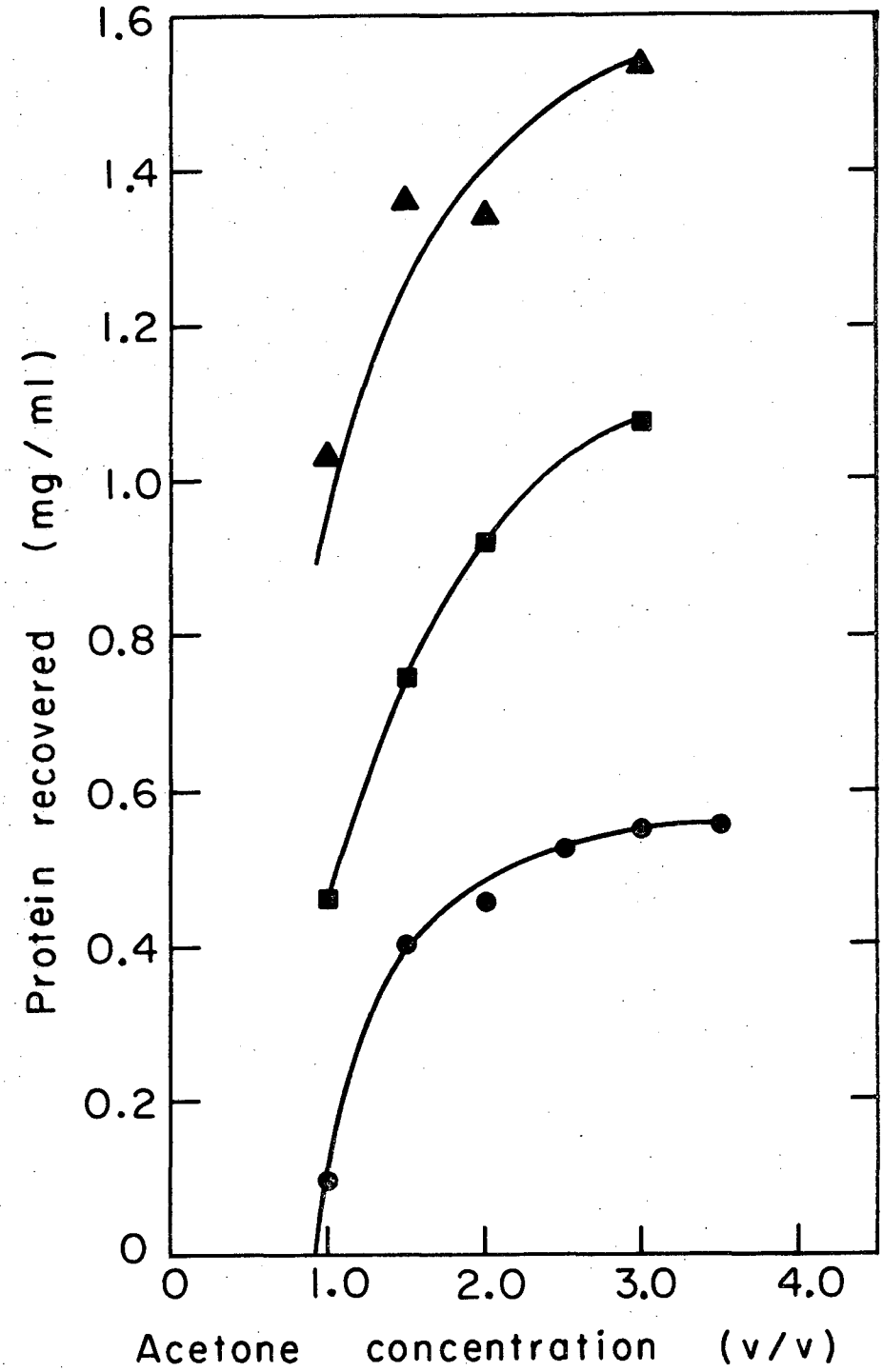
XBL743-2550

Fig. 21. Effect of total soluble protein concentration on the recovery of C_x activity at 30°C. No additional protein (●), 0.58 mg/ml BSA added (■), and 1.14 mg/ml BSA added (▲). The initial C_x activities were 2.36 units, 1.95 units, and 1.82 units, respectively.



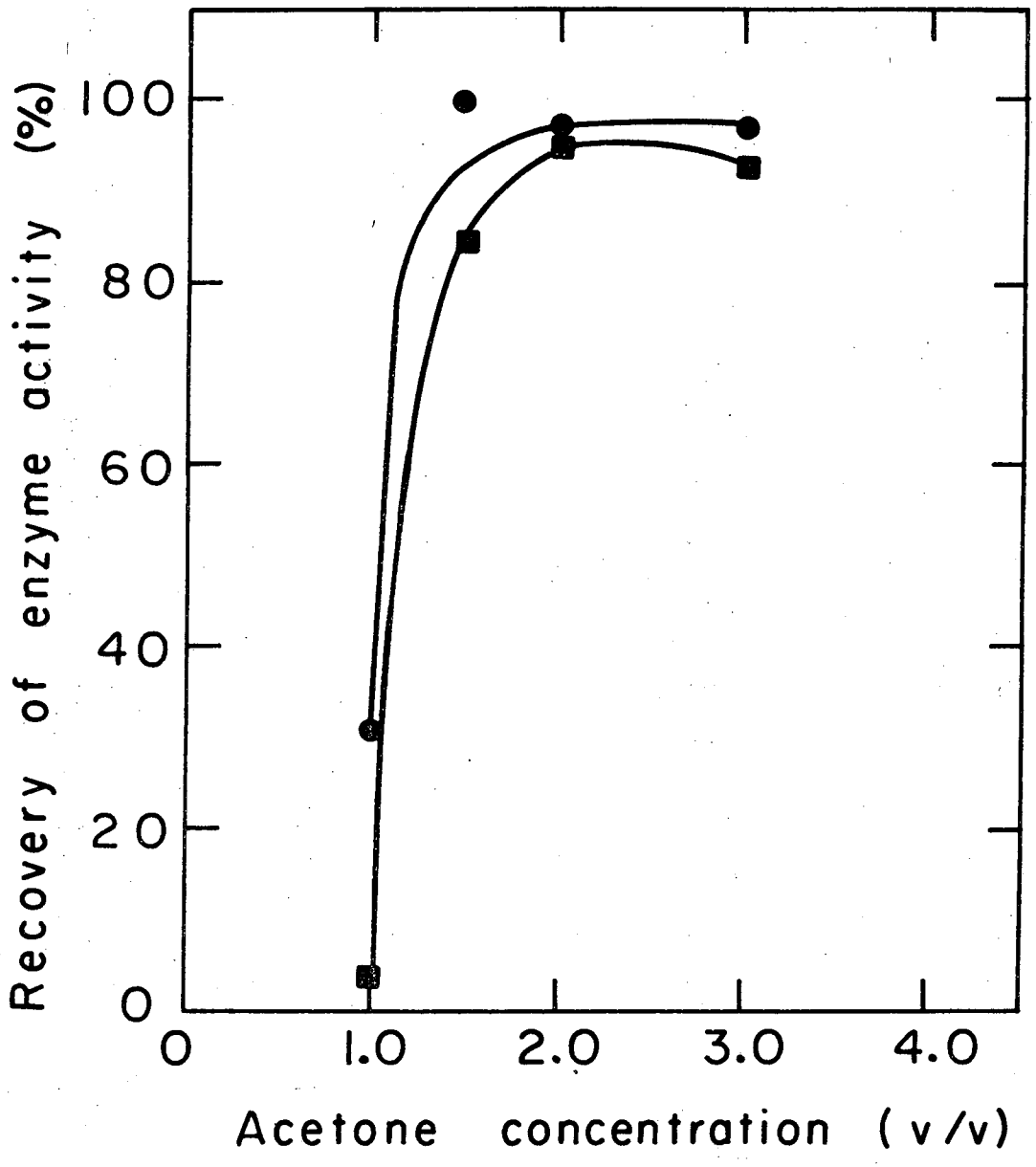
XBL743-2549

Fig. 22. Effect of total soluble protein concentration on the recovery of FP activity at 30°C. No additional protein (●), 0.58 mg/ml BSA added (■), and 1.14 mg/ml BSA added (▲). The initial FP activities were 3.25 units, 2.78 units, and 2.84 units, respectively.



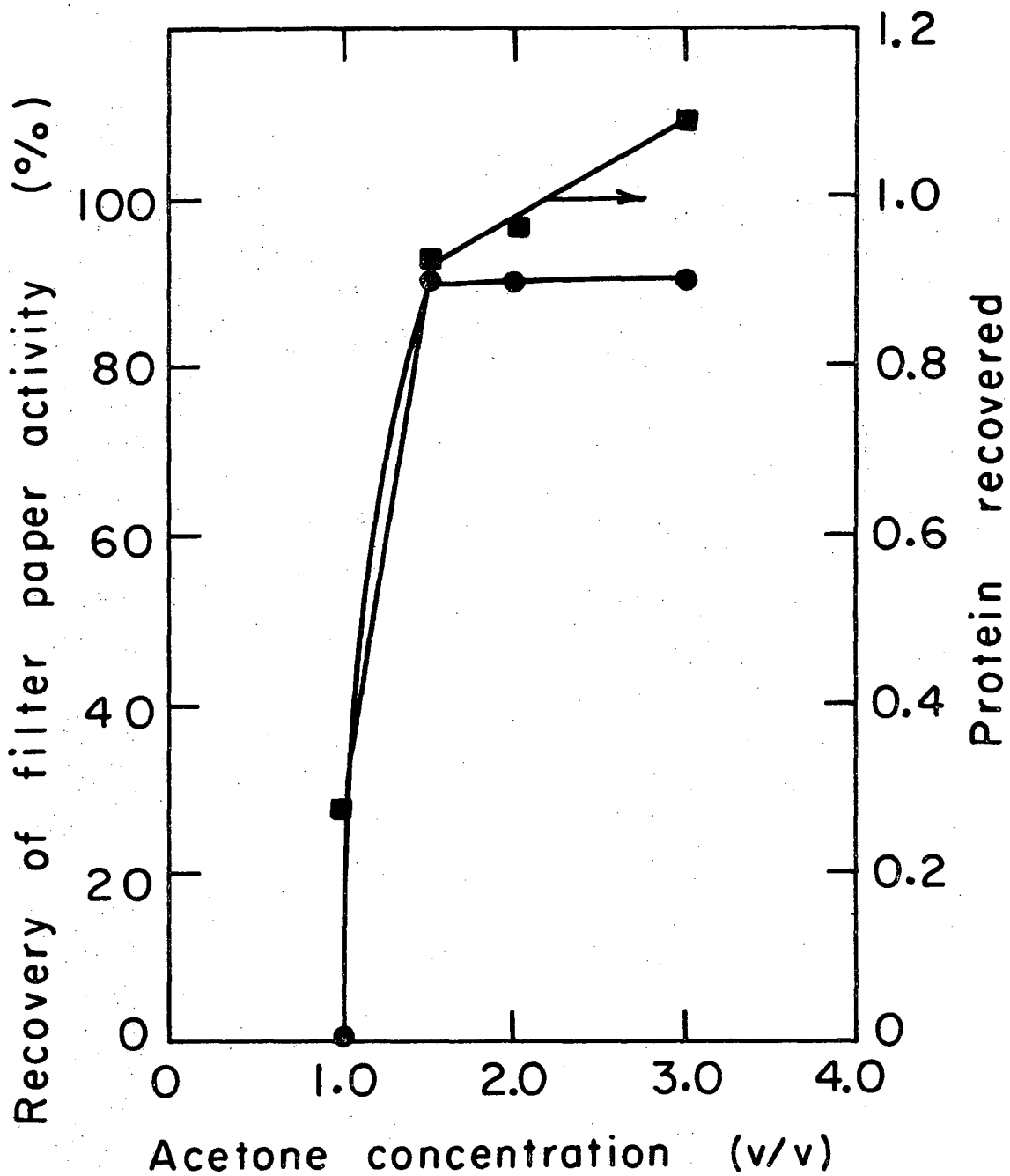
XBL743-2548

Fig. 23. Effect of total soluble protein concentration on the recovery of protein at 30°C. No additional protein (●), 0.58 mg/ml BSA added (■), and 1.14 mg/ml BSA added (▲). The initial soluble protein concentration was 1.2 mg/ml.



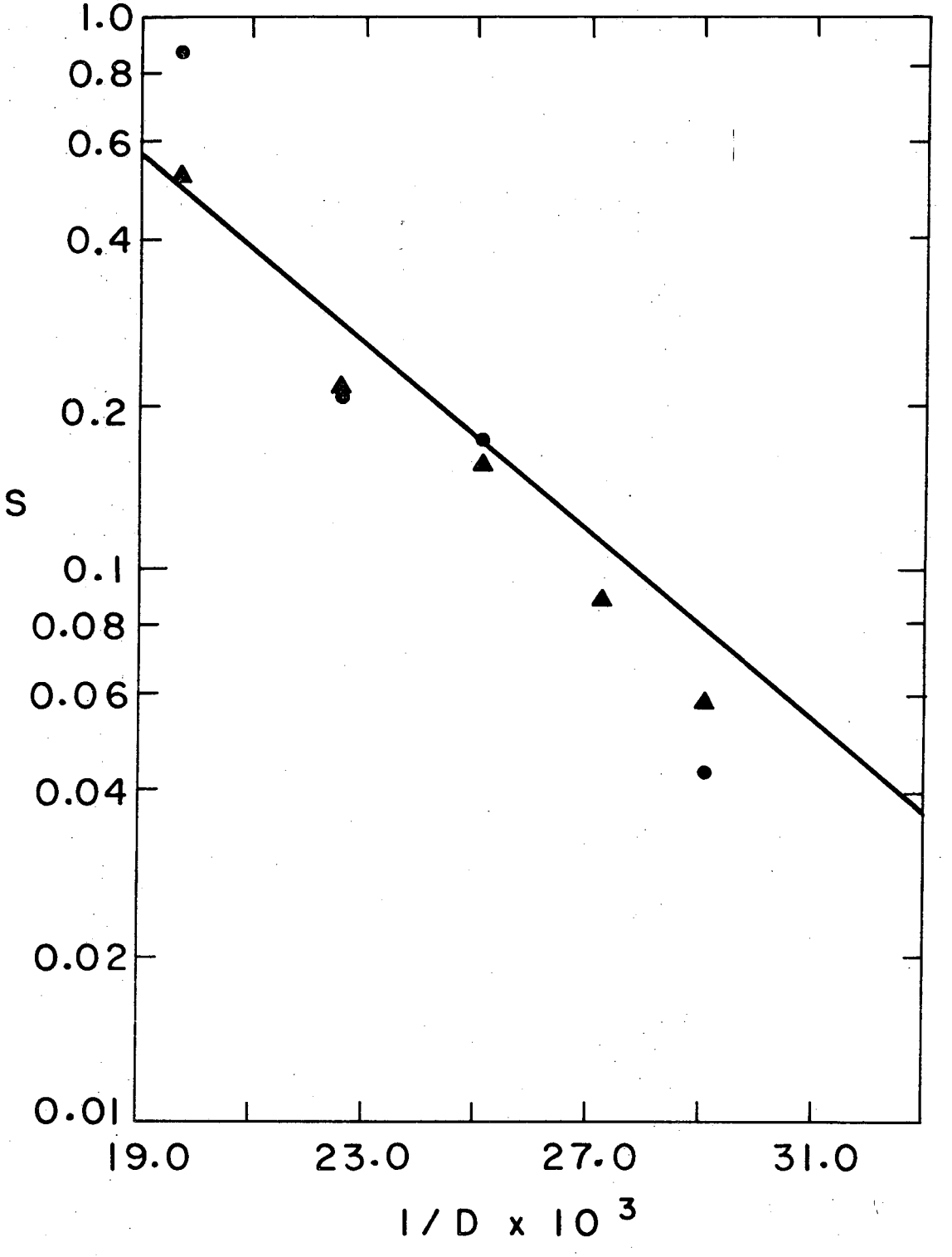
XBL743-2547

Fig. 24. Effect of cellulase enzyme concentration on the recovery of activity at 30°C. C_1 activity (■) and C_x activity (●) recovered from a 2-fold concentrated enzyme solution with initial activities of 7.84 C_1 units and 1.88 C_x units.



XBL743-2546

Fig. 25. Effect of cellulase enzyme concentration on the recovery of FP activity and protein at 30°C. A 2-fold concentrated enzyme solution contained 3.68 FP units and 1.14 mg/ml of protein, initially.



XBL743-2545

Fig. 26. Effect of cellulase enzyme concentration on its solubility.

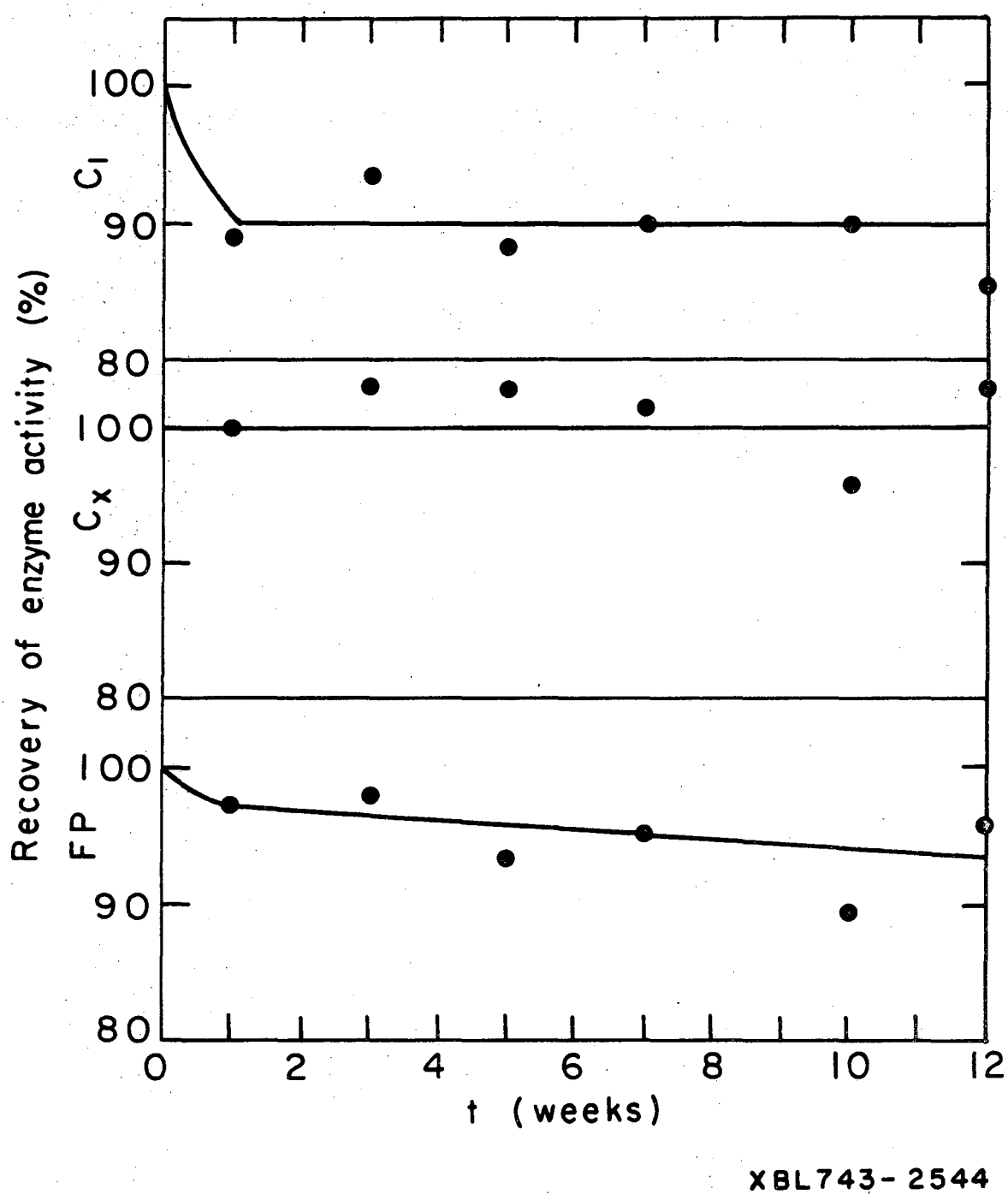


Fig. 27. Stability of the cellulase enzyme precipitate. The initial enzyme activities were 7.17 C_1 units, 1.75 C_x units and 2.62 FP units.

Equilibrium of the cellulase precipitate

The time required for the cellulase precipitate to equilibrate was examined and the results are shown in Fig. 28. The centrifugation time has been included in the time the precipitate was allowed to stand. The precipitate was seen to form immediately with the addition of acetone. Settling of the precipitate was fairly rapid and after 2 minutes no further clearing of the mixture was observed. The pellet formed by the precipitate which was allowed to set for 2 minutes or more was more compact than the precipitate that was allowed to sit for only 1 minute.

Filtration

The results of the filtration experiments are shown in Figs. 29, 30, and 31. In Figs. 29 and 30, the reciprocal of the asymptotic volume throughput rate is plotted against the weight of filter cake, which is expressed as the volume of fermentation broth. The filter holder used in these experiments did not have a constant diameter but had a smaller diameter at the bottom. Because of this, it was difficult to build up an even filter cake. As a result, the data on thicker filter cakes have been stressed in Fig. 29. The cellulase protein concentration in the enzyme solution was 0.553 mg/ml. In the experiments using filter aid, 4.977 mg/ml of ball milled paper was added as filter aid. The effect of pressure on the specific cake resistance with and without filter aid is shown in Fig. 31. Cake thicknesses, moisture contents, and compressibility coefficients are listed in Table VIII.

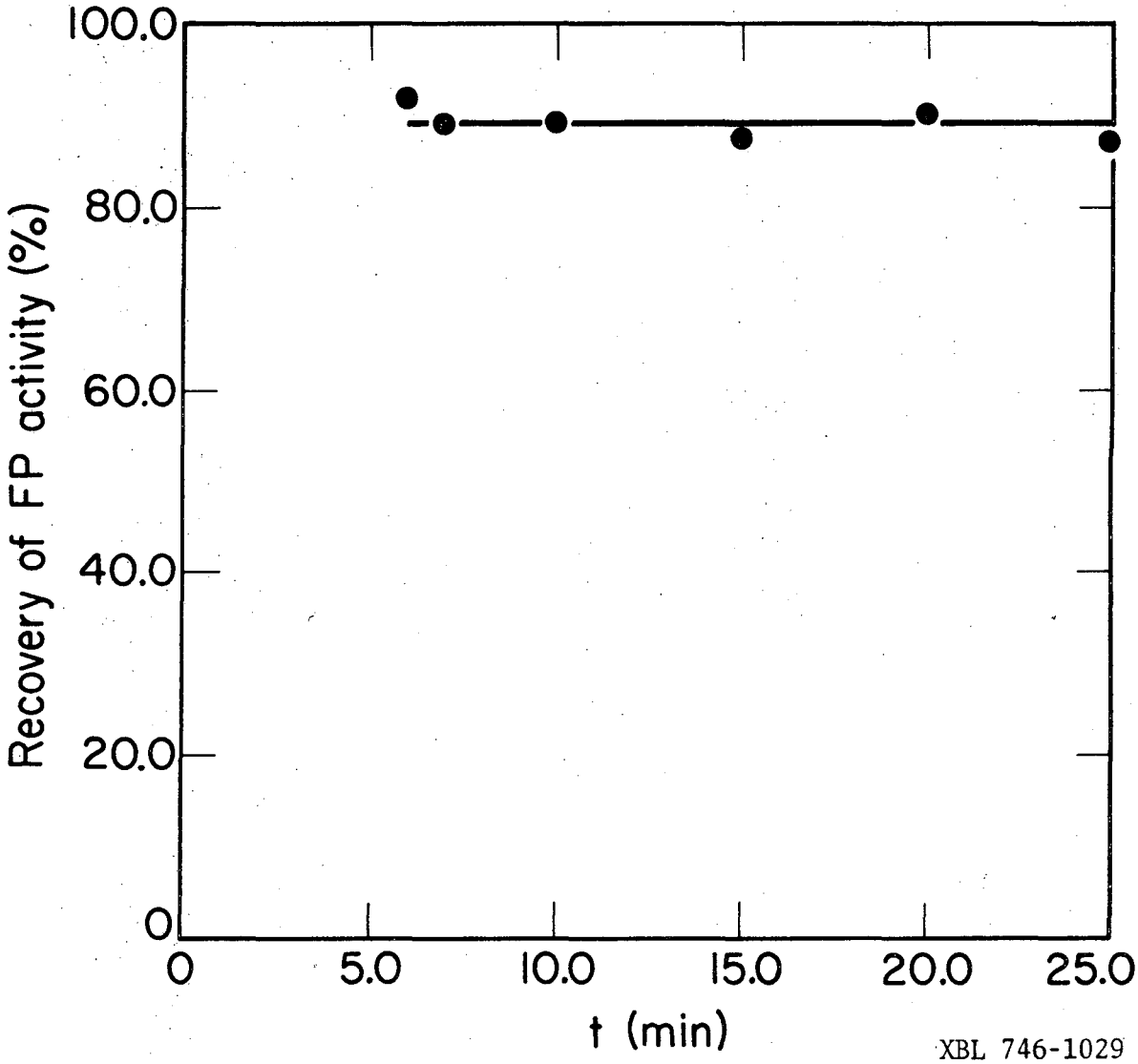
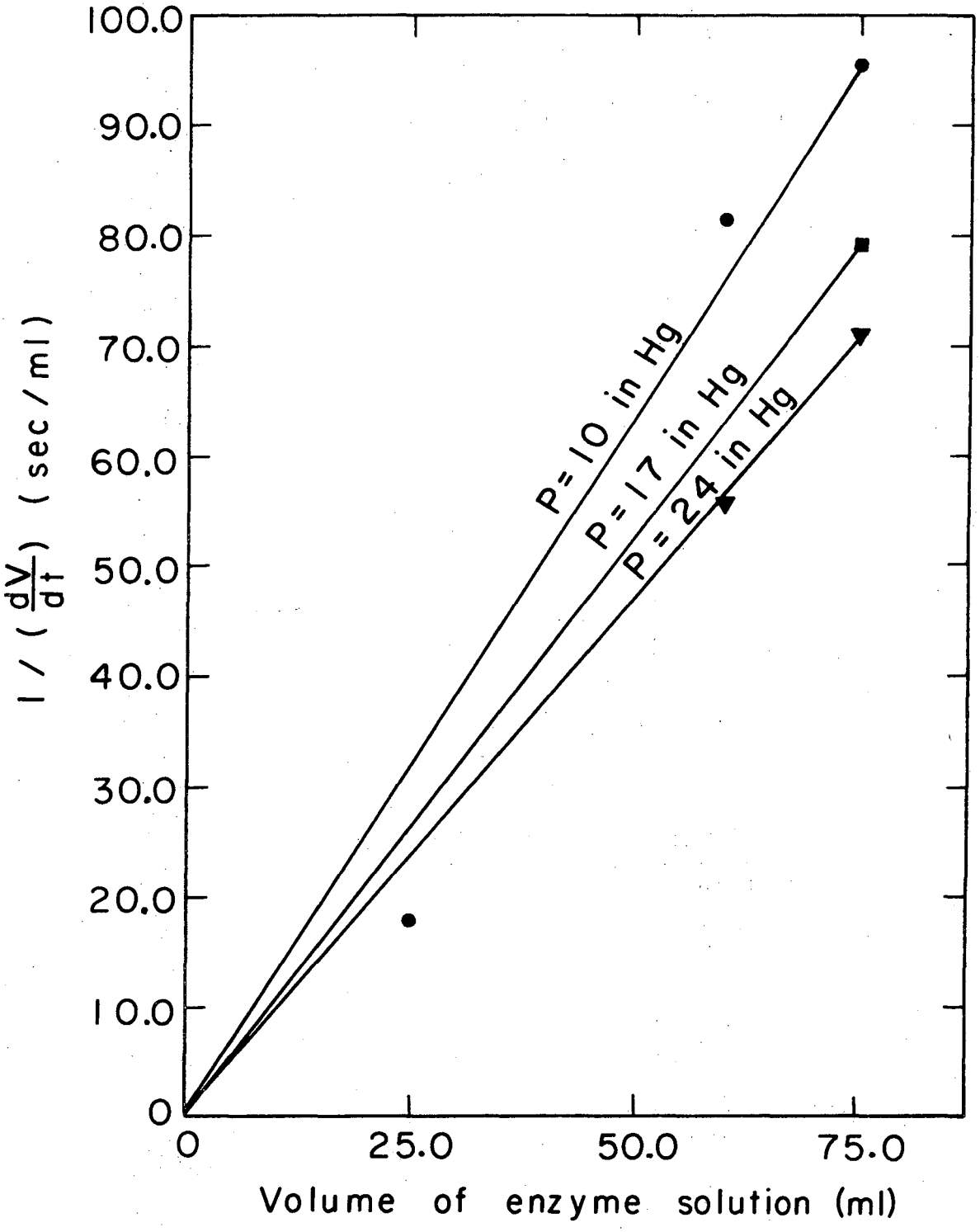
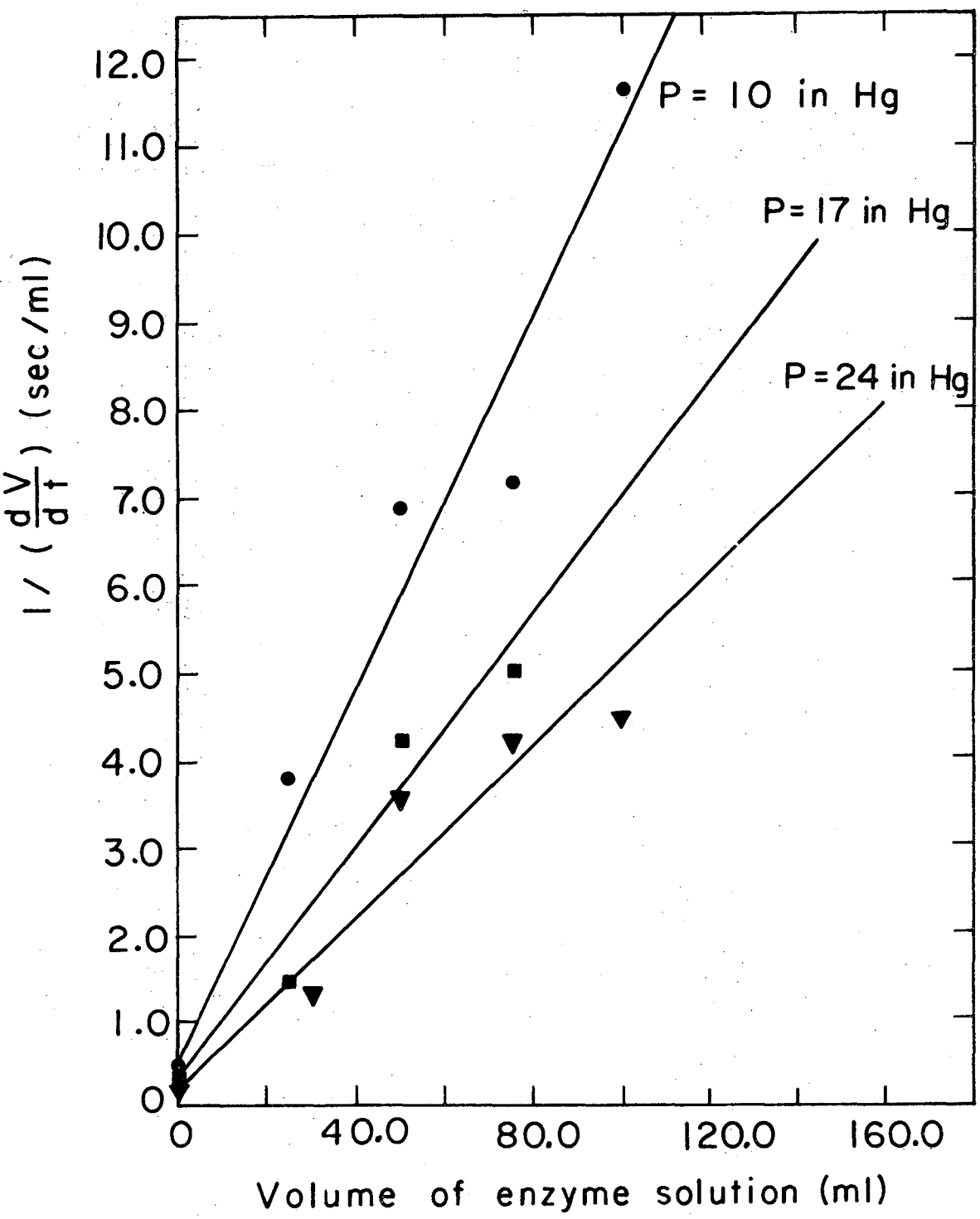


Fig. 28. Effect of contact time on the recovery of FP activity at room temperature. The initial FP activity was 2.8 units.



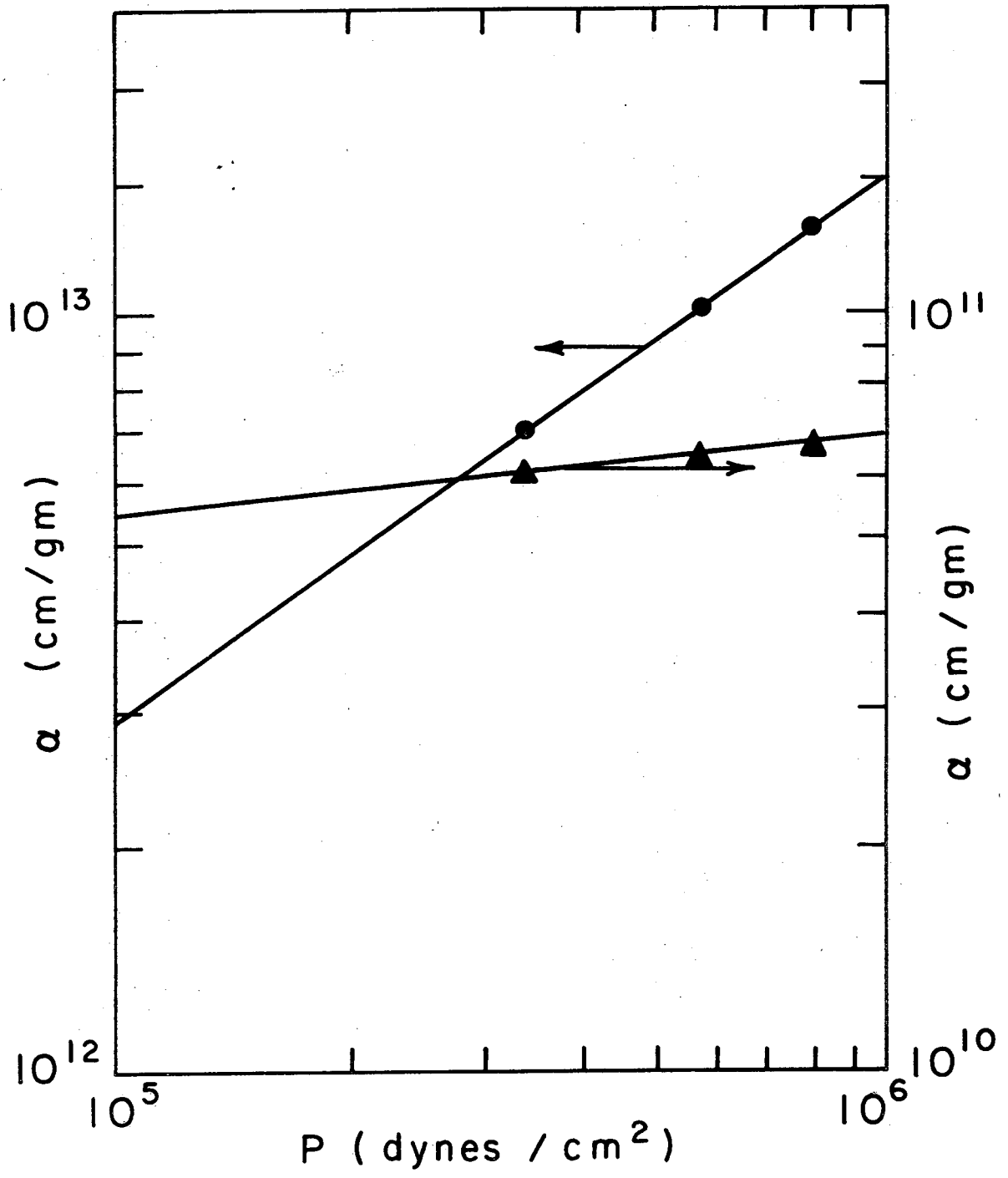
XBL743-2543

Fig. 29. Dependency of filtration rate on the thickness of the cake and pressure drop without filter aid.



XBL743-2542

Fig. 30. Dependency of the filtration rate on the thickness of the cake and pressure drop. Cake consists of protein precipitate and ball-milled paper as filter aid in a 1:9 ratio.



XBL743-2541

Fig. 31. Dependency of the specific cake resistance on pressure drop. No filter aid (●), and with a 1:9 ratio of precipitate to filter aid (▲).

Table VIII. Summary of the Characteristics of the Filter Cakes.

Item	No filter aid	With filter aid
Cake density(dry), gm/cm^3	0.05272	0.2739
Percent moisture	64.75	45.2
s	0.71	0.11
α_0	8.291×10^8	1.51×10^{10}

DISCUSSION OF RESULTS

Effect of acetone concentration on enzyme recovery

The effect of acetone concentration on the recovery of cellulase enzyme is summarized in Figs. 4, 5, and 6 indicating the range in which the cellulase system may be recovered. Little enzyme is recovered at an acetone concentration of 1.0 v/v and most of the enzyme is recovered at 3.5 v/v. In comparing the results shown in Figs. 4 and 5, it can be seen that most of the C_x component is recovered at a lower acetone concentration. This implies that the acetone concentration which corresponds to the maximum recovery of C_1 activity will also yield the maximum recovery of C_x activity.

In comparing Figs. 4 and 6, it can be seen that the recoveries of C_1 and FP activities closely approximate each other, and the results from other sections also reflect this similarity. These results are expected since the C_1 component is more soluble than the C_x component, and it is required for the breakdown of the crystalline structure of filter paper and other more crystalline forms of cellulose. The strong dependence

of the recovery of the FP activity on the C_1 component allows one to treat the cellulase system as a single enzyme.

The results shown in Fig. 6 at the lower temperatures agree qualitatively with the data of Mandels, Kostick, and Parizek (47). A detailed comparison of their results with this work could not be made since the conditions during their precipitation were not given. However, they did report 100% protein recovery with an acetone concentration of 3.0 v/v.

From a theoretical standpoint, it is the change in dielectric constant corresponding to the addition of organic solvent that results in the precipitation of the enzyme. The theory predicts that the solubility of the protein should vary inversely with the dielectric constant. The data from Grönwall (34) generally agree with the theory. The results from this work also agree with the theory. The solubility of cellulase, defined in terms of the unrecovered fraction of the enzyme activity, can be characterized by the following equations:

for C_1:

$$\log(1-{}^{C_1}/C_{1o}) = 7.145\times10^{-8}\exp(5456.18/T)-4.365\times10^{-7}\exp(6094.62/T)/D \quad (36)$$

for C_x:

$$\log(1-{}^{C_x}/C_{xo}) = 0.01432\exp(1778.75/T) - 0.04898\exp(2620.18/T)/D \quad (37)$$

for FP:

$$\log(1-{}^{FP}/FPo) = 1.259\times10^{-8}\exp(5950.34/T)-1.585\times10^{-7}\exp(6372.795/T)/D \quad (38)$$

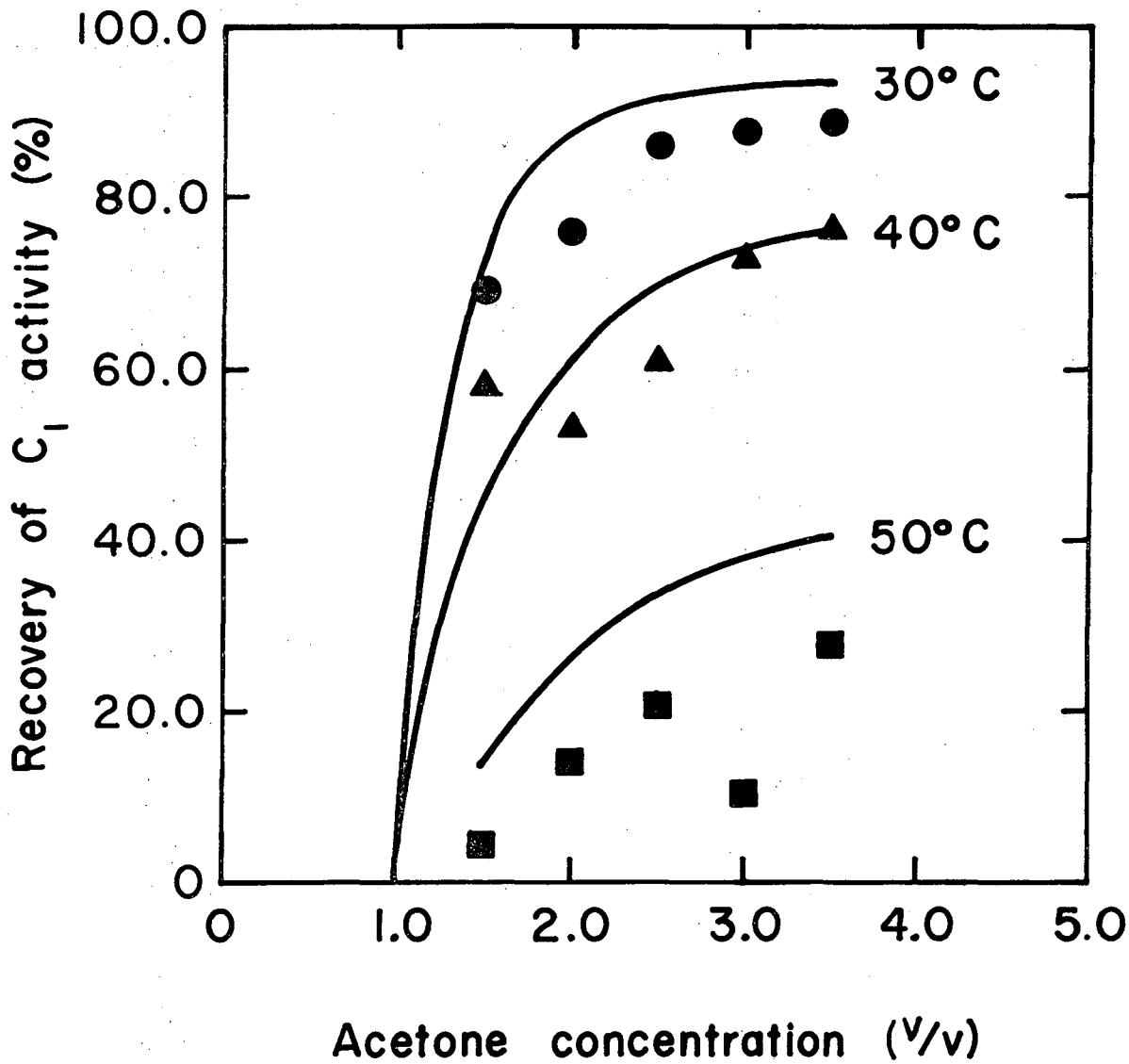
where the fraction recovered has been normalized to account for denaturation. The constants in equations (36), (37), and (38) were determined

from the slopes and intercepts of the lines in Figs. 10, 11, and 12, respectively, as computed by a least squares analysis.

In Figs. 32, 33, and 34, the predicted recoveries of C_1, C_x and FP activities are compared with the experimental data. The normalized recoveries from equation (36), (37), and (38) are multiplied by the fraction of activity that is not denatured by the temperature effects to get the actual predicted recovery. The prediction of C_x recovery is excellent whereas the prediction of C_1 and FP recoveries is fair. In all three cases, the predicted recoveries at 50°C were much higher than the experimental results. This may be due to other factors that may denature the enzyme. One possibility is that the enzyme precipitate may be more susceptible to shear forces caused by centrifugation at the higher temperatures. Also, there was an uncertainty of ±1°C in the actual temperature.

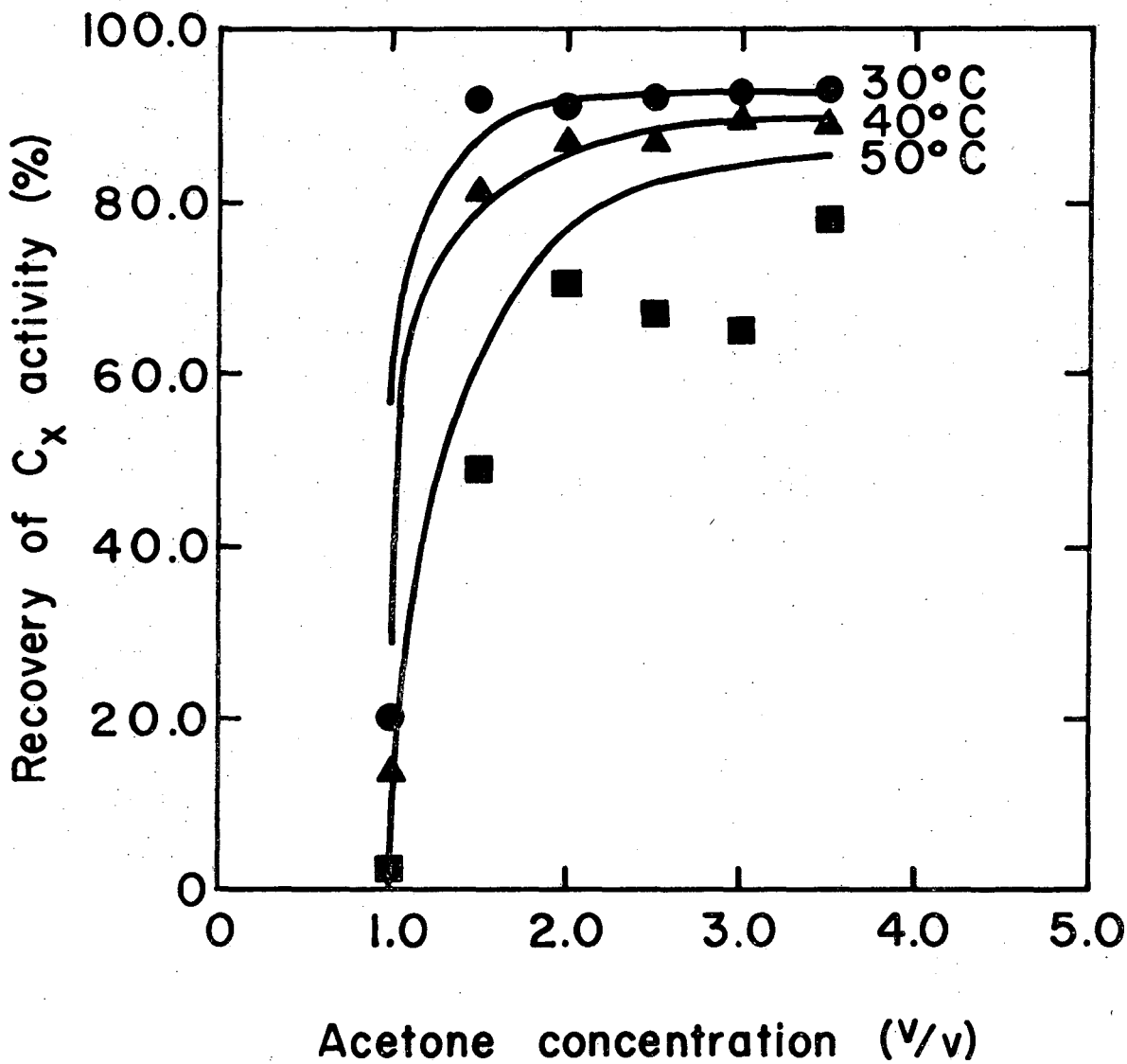
In comparing Figs. 7 and 8, it can be seen that it would be possible to separate the C_1 and C_x components. This effect is expected since salting-out with organic solvents or inorganic salts is widely used to fractionate and purify proteins. This knowledge may be of little value since a number of researchers (46,67) have demonstrated the synergism between the C_1 and C_x components. The separation of these components would result in the loss of the ability to degrade cotton and a reduction in the ability to degrade less crystalline forms of cellulose.

The effect of temperature on the constants, β and K_s as seen in equations (36), (37), and (38) and Figs. 7,8, and 9, is not an expected one. The results from salting-out experiments using inorganic salts generally indicate that K_s is independent of temperature and β varies



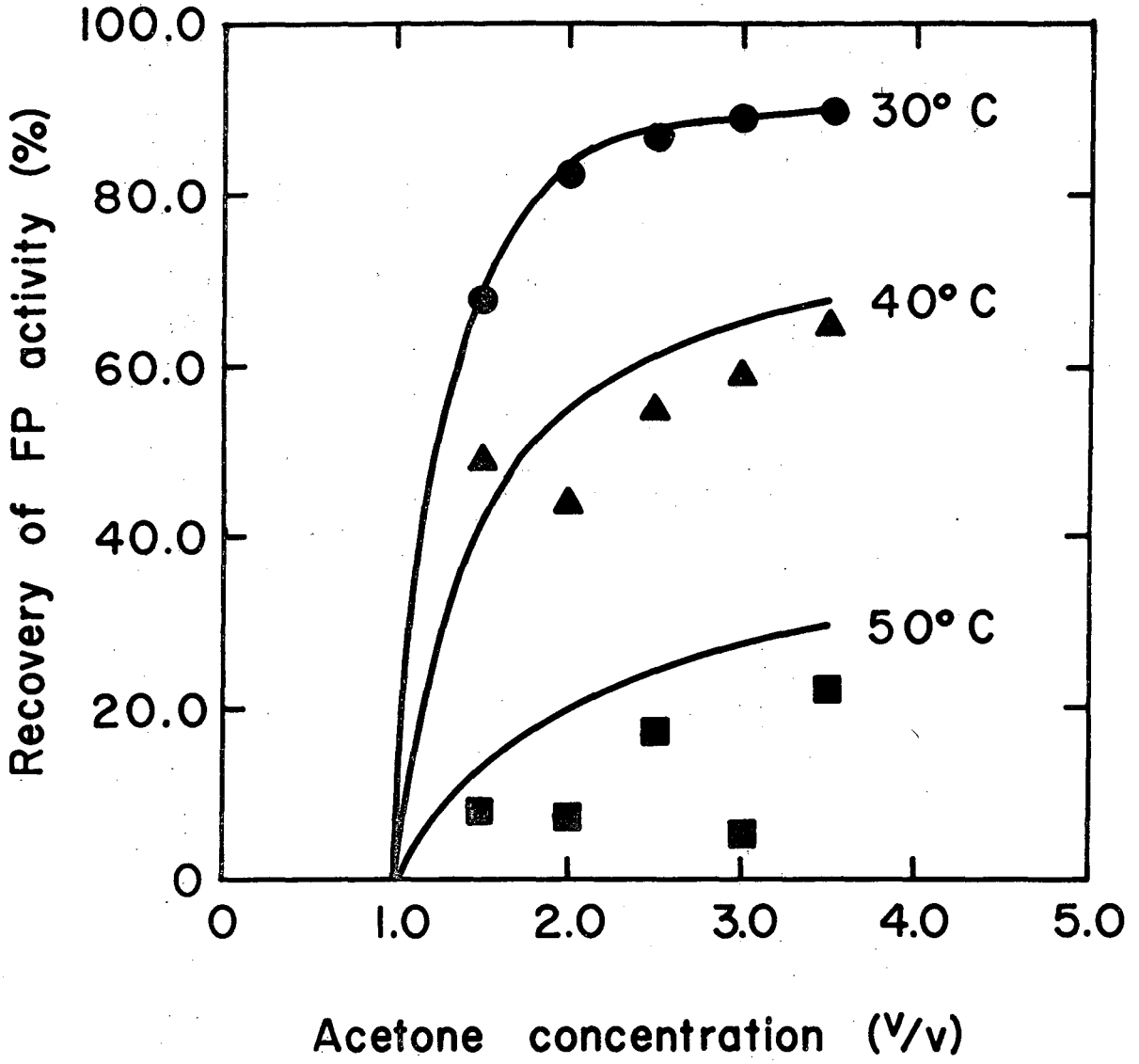
XBL 744-747

Fig. 32. Comparison of the salting-out equation for C_1 activity with the experimental results.



XBL 744-748

Fig. 33. Comparison of the salting-out equation for C_x activity with the experimental results.



XBL 744-749

Fig. 34. Comparison of the salting-out equation for FP activity with the experimental results.

inversely with temperature. However, these results are based on the solubility of the protein and not on the recovery of enzyme activity. It is believed that the exponential relationship of these constants with the reciprocal of the temperature is due to the normalization of the recovery fractions. This belief is substantiated by the results shown in Fig. 14.

Figure 14 is a plot of the solubility of the cellulase protein against the reciprocal of the dielectric constant of the solution. Included are the data from several temperatures. The data may be best correlated by a single line with a constant slope and a single intercept. Thus the solubility of the cellulase protein can be described by the following equation

$$\log S = 1.371 - 85.0/D \ . \qquad (39)$$

A closer examination of the effect of temperature on the slope and intercept of the cellulase protein solubility was limited by the nature of the fermentation broth. This broth contained not only cellulase protein but other proteins as well. It was not possible to determine the initial concentrations of the C_1 and C_x components.

It is apparent that acetone is acting as a non-specific precipitant. It is known that the cellulase enzyme system is composed of at least two and perhaps as many as five components. The results mentioned earlier indicated the recovery of two components. Still, the solubility curve appears to be that of a single protein. Roche and Derrien (62), working with a mixture of enzymes, found each enzyme to precipitate more or less independently of the other, and two solubility curves were obtained for a mixture of two proteins. Also, when bovine serum albumin was added to the cellulase solution, it precipitated out in the

same range of acetone concentrations (see Fig. 23). It was not possible from the data to discern the number of enzymes that comprised the C_x component.

Effect of temperature on enzyme recovery

Denaturation of an enzyme is the primary reason that solvents are not widely used as a purifying or fractionating agent. The cellulase enzyme is also denatured when treated with acetone. Enzyme denaturation is a first-order rate process and the Arrhenius plot of the rate constant for each of the enzyme activities is shown in Figs. 17, 18, and 19. The pre-exponential factors and the activation energies are listed in Table VI. The kinetics of denaturation have not been studied under these conditions.

Several workers have examined the inactivation of the cellulase enzyme from T. viride. Mandels and Weber (48) studied the stability of the enzyme up to 50°C and found no inactivation. Ghose and Kostick (28) reported a loss of Filter Paper activity at 60°C and hypothesized that the loss was due to the inactivation of the C_x component. Ghose (27) examined the inactivation of the C_x component between 40 and 60°C. However, the data were not sufficient to determine the activation energy for the denaturation of this component.

Effect of proteins on enzyme recovery

Bovine serum albumin was added to the cellulase solution to determine what effects extraneous protein might have on the recovery of cellulase. As shown in Figs. 20, 21, and 22, BSA has little effect on the recovery of C_1, C_x and FP activities. This corresponds with the results of Roche and Derrien (62).

The effect of cellulase protein on the recovery of cellulase was also examined. As shown in Table VII although the precipitate concentration was doubled, the increase in enzyme activity was less than 50%. The recovery of enzyme activity, however, was greater, especially between 1.0 and 2.0 v/v acetone. These results indicate that the saturation limit of the cellulase enzyme may have been reached.

The effect of cellulase protein concentration on the solubility is shown in Fig. 26. Within experimental uncertainty, the solubility of the protein is unaffected by the initial concentration of the protein. Only the acetone concentration at which precipitation begins would be affected by the initial cellulase protein concentration. This experimental uncertainty is due, in part, to the fact that in the unconcentrated cellulase solution, the amount of cellulase protein is not exactly known. This result is in agreement with the theoretical expectations of salting-out as discussed by Dixon and Webb (18).

Stability and equilibrium of the precipitated cellulase

The stability of the cellulase enzyme is shown in Fig. 27. It is evident that the enzyme was quite stable during the 3 months that this experiment was conducted. The initial decrease in the activity of the C_1 component in the first week can be attributed to an error in the standard since no further decrease in the activity is seen. These results are in agreement with the work of Reese and Mandels (59) who have kept fungal cellulases in powdered form for 8 years with no loss in enzyme activity.

The results of the experiment on the equilibrium time required for the precipitate to stabilize indicate that the time required to complete the precipitation process is on the order of 2 to 3 minutes or less. The

laboratory experiments were limited by the mechanical manipulation required to separate the precipitate rather than by the time required for the precipitate to form.

Filtration

The filtration experiments yielded the specific cake resistance for the protein precipitate with and without filter aid. The compressibility factor was also obtained. Centrifugation is normally used in the laboratory for the separation of protein precipitates, and the filtration characteristics of protein precipitates have not been previously reported. Dunnill and Lilly and their co-workers (20, 21) have published a number of reports on the large scale isolation of enzymes, but in each of these processes centrifugation was used to separate the precipitate from the solution.

Grace (30) found that the specific cake resistances for a number of inorganic precipitates ranged from 10^{10} to 10^{12} cm/gm. The specific cake resistance for the protein precipitate is an order of magnitude higher, meaning that it is much more difficult to filter. He also found the specific cake resistance for Solka Floc (BW-200) to be 2×10^{8} cm/gm. The specific cake resistance for the protein precipitate with filter aid is much higher than this value. This corresponds to the observations of Dunnill and Lilly (21) who noted that even with the addition of filter aid, filtration of enzyme precipitates was very slow. However, no values were presented in conjunction with their observations.

The average filtration rate, which is the total volume of filtrate divided by the total time required to collect this volume of filtrate per unit filter area, for the filtration of the cellulase protein only was

0.998 gal/ft^{2}hr. Using filter aid, the average filtration rate was 84.23 gal/ft^{2}hr. These values were for a final cake thickness of 1/8 inch and a constant operating pressure of 50 psig.

CONCLUSIONS

It has been shown that acetone precipitation is an effective method of recovering the cellulase of Trichoderma viride from the fermentation broth. Careful control of the operating temperature, acetone concentration, and contact time during the precipitation process increases the recovery of the enzyme activity. The significance of the processing variables is summarized below.

The cellulase of T. viride is very stable at temperatures as high as 50°C. This stability is also seen in the treatment with acetone. Normally, precipitation with organic solvents is done at 0°C or lower. In the case of T. viride cellulase, however, high recoveries of enzyme activity are obtained at room temperature or higher. The enzyme recovery process can be operated at 30°C, the optimum growth temperature. This would eliminate any refrigeration requirements that are associated with conventional enzyme precipitation processes.

The inactivation of the enzyme due to the precipitation with acetone exhibited first-order kinetics. This is seen in the recovery of C_1, C_x, and FP activities. The inactivation rate constants of all three enzyme activities conform to Arrhenius' law. The C_1 and FP activities are more adversely affected by an increase in the operating temperature.

The recovery of cellulase activity increases with increasing acetone concentrations. At 30°C, most of the cellulase activity is recovered with acetone concentrations of 2.0 v/v or greater. Fractionation of the

C_1 and C_x components occurs with the C_x component precipitating out first. The concentration of acetone does not in itself inactivate the enzyme, and no special precautions need be taken in recovering the entire enzyme system.

The solubility of the cellulase protein in aqueous acetone solutions can be described by the salting-out equation as a function of the dielectric constant. The solubility of the protein is independent of temperature and cellulase protein concentration and is not affected by other protein in the solution. The time required for the precipitate to equilibrate was found to be less than 6 minutes.

The specific resistance, α, of the cellulase precipitate is very large. The addition of filter aid greatly improves the filtration rates, although the specific resistance remains large.

From the foregoing results it would appear that acetone precipitation can be the basis for a practical industrial process for cellulase recovery from *Trichoderma viride* filtrates.

ACKNOWLEDGEMENTS

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NOMENCLATURE

Symbol	Definition
A	filter area, cm^2
c	concentration of solids, gm/cm^3
c_s	concentration of salt, g/ℓ
C_i	concentration of the ith component, $moles/\ell$
D, D_i	dielectric constant
g_c	conversion factor
k	constant in Kozeny-Carmen equation
k	rate constant for inactivation of enzyme, sec^{-1}
k_0	Arrhenius preexponential factor in Arrhenius' equation
K, K_s	constant in salting-out equation
K_{Ri}, K_R'	interaction constant of ion-dipolar ion due to Coulomb forces
L	cake thickness, cm
m, m_c	mass of cake, gm
N	Avogardro's number, molecule-mole
p, P	pressure, atm, psi, kg/m^2
R_m	resistance of the medium, cm^{-1}
s	compressibility factor
s_p	surface area of single particle, cm^2
S	protein solubility, g/ℓ
t	time
u	velocity, cm/sec
v	volume, cm^3

v_p	volume of single particle, cm^3
V	volume of filtrate, cm^3
V_{ik}	electrostatic work term, erg
$W_{ik}(\lambda)$	average work term, erg
α	specific cake resistance, cm/gm
β	constant in salting-out equation
$\Gamma/2$	ionic strength, moles/l
γ	activity coefficient
ϵ	proton charge, esu
ϵ	porosity of cake
κ	Boltzmann's constant, erg/molecule °K
μ	chemical potential
μ	viscosity, poise
ν	number of ionic species
ρ	density, gm/cm^3
$\Psi(r_1)$	electrostatic potential

APPENDIX I

Experiment No.: 1.1-Acetone Concentration and Temperature Analysis

Temperature: 30°C

Special Conditions: None

pH: 4.8

Volume of Enzyme Solution: 10 ml

Acetone Concentr. (v/v)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (300 nm)	Recovered Protein Protein (g/l)
1.0	0.01	0.1	0.015	0.15	0.05	0.5	0.03	0.086
	0.01	0.1	0.01	0.1	0.065	0.65	0.035	0.101
	0.01	0.1	0.005	0.05	0.04	0.4	0.035	0.101
	0.01	0.1	0.005	0.05	0.045	0.45	0.035	0.101
1.5	0.23	2.3	0.415	4.16	0.226	2.26	0.135	0.388
	0.23	2.3	0.445	4.48	0.22	2.2	0.135	0.388
	0.245	2.45	0.432	4.32	0.215	2.15	0.145	0.417
	0.245	2.45	0.468	4.72	0.23	2.3	0.14	0.403
2.0	0.24	2.4	0.445	4.48	0.186	1.86	0.155	0.446
	0.222	2.22	0.472	4.78	0.20	2.0	0.155	0.446
	0.252	2.52	0.495	5.02	0.20	2.0	0.165	0.474
	0.256	2.56	0.448	4.5	0.181	1.81	0.160	0.46
2.5	0.244	2.44	0.525	5.34	0.192	1.92	0.18	0.52
	0.24	2.4	0.505	5.12	0.184	1.84	0.18	0.52
	0.292	2.92	0.545	5.56	0.236	2.36	0.185	0.532
	0.286	2.86	-----	----	0.236	2.36	0.185	0.532
3.0	0.246	2.46	0.56	5.72	0.18	1.8	0.19	0.544
	0.236	2.36	0.528	5.38	0.19	1.9	0.195	0.561
3.5	0.246	2.46	0.56	5.72	0.186	1.86	0.195	0.561
	0.246	2.46	0.535	5.44	0.184	1.84	0.193	0.555
Initial Concentr.	0.338	3.38	0.6	6.18	0.246	2.46	0.40	1.15
	0.358	3.58	0.645	6.56	0.2	2.0	0.414	1.19
	0.274	2.74	0.625	6.32	0.23	2.3	0.407	1.17

Experiment No.: 1.2-Acetone Concentration and Temperature Analysis

Temperature: 35°C

Special Conditions: None

pH: 4.8

Volume of Enzyme Solution: 10 ml

Acetone Concentr. (v/v)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (300 nm)	Recovered Protein Protein (g/l)
1.0	0.01	0.1	0.065	0.65	0.1	1.0	0.0425	0.122
	0.01	0.1	0.015	0.15	0.07	0.7	0.08	0.23
1.5	0.182	1.82	0.395	3.95	0.195	1.95	0.14	0.4026
	0.182	1.82	0.35	3.5	0.185	1.85	0.14	0.4026
2.0	0.215	2.15	0.44	4.44	0.20	2.0	0.16	0.46
	0.23	2.3	0.435	4.35	0.20	2.0	0.16	0.46
2.5	0.255	2.55	0.54	5.5	0.20	2.0	0.185	0.532
	0.27	2.7	0.56	5.72	0.2	2.0	0.185	0.532
3.0	0.26	2.6	0.555	5.66	0.205	2.05	0.2	0.575
	0.255	2.55	0.525	5.34	0.2	2.0	0.195	0.5608
3.5	0.285	2.85	0.655	6.78	0.18	1.8	0.21	0.6039
	0.27	2.7	0.645	6.68	0.18	1.8	0.205	0.5895
Initial Concentr.	0.31	3.1	0.595	6.1	0.22	2.2	0.42	1.208
	0.295	2.95	0.59	6.04	0.22	2.2	0.42	1.208
	0.32	3.2	0.685	7.14	0.2	2.0	----	-----

Experiment No.: 1.3-Acetone Concentration and Temperature Analysis

Temperature: 40°C

Special Conditions: None

pH: 4.8

Volume of Enzyme Solution: 10 ml

Acetone Concentr. (v/v)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (300 nm)	Recovered Protein Protein (g/l)
1.0	0.01	0.1	0.005	0.05	0.025	0.25	0.065	0.1869
	0.005	0.05	0.005	0.05	0.025	0.25	0.075	0.2157
1.5	0.16	1.6	0.445	4.48	0.155	1.55	0.165	0.4745
	0.165	1.65	0.435	4.38	0.155	1.55	0.135	0.388
2.0	0.15	1.5	0.42	4.2	0.165	1.65	0.13	0.3738
	0.145	1.45	0.395	3.95	0.16	1.6	0.145	0.417
2.5	0.19	1.9	0.445	4.48	0.175	1.75	0.185	0.532
	0.18	1.8	0.485	4.9	0.165	1.65	0.1775	0.5104
3.0	0.185	1.85	0.565	5.78	0.173	1.73	0.18	0.5176
	0.22	2.2	0.535	5.44	0.17	1.7	0.195	0.5608
3.5	0.225	2.25	0.58	5.92	0.17	1.7	0.205	0.5895
	0.21	2.1	0.58	5.92	0.175	1.75	0.205	0.5895
Initial Concentr.	0.35	3.5	0.725	7.64	0.195	1.95	0.435	1.251
	0.325	3.25	0.734	7.75	0.19	1.9	0.425	1.222

Experiment No.: 1.4-Acetone Concentration and Temperature Analysis

Temperature: 45°C

Special Conditions: None

pH: 4.8

Volume of Enzyme Solution: 10 ml

Acetone Concentr. (v/v)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (300 nm)	Recovered Protein Protein (g/l)
1.0	-----	-----	0.0	0.0	0.02	0.2	0.13	0.3738
	0.005	0.05	0.0	0.0	0.015	0.15	0.095	0.2732
1.5	0.03	0.3	0.085	0.85	0.12	1.2	0.135	0.3882
	0.02	0.2	0.1	1.0	0.11	1.1	0.125	0.3595
2.0	0.06	0.6	0.15	1.5	0.135	1.35	0.145	0.417
	0.055	0.55	0.16	1.6	0.145	1.45	0.15	0.431
2.5	0.17	1.7	0.545	5.56	0.165	1.65	0.2	0.575
	0.2	2.0	0.54	5.5	0.165	1.65	0.19	0.5464
3.0	0.155	1.55	0.395	3.95	0.165	1.65	0.18	0.5176
	0.16	1.6	0.515	5.2	0.165	1.65	0.18	0.5176
3.5	0.155	1.55	0.475	4.8	0.16	1.6	0.195	0.5608
	0.145	1.45	0.51	5.2	0.16	1.6	0.18	0.5176
Initial Concentr.	0.265	2.65	0.66	6.86	0.182	1.82	0.43	1.237
	0.275	2.75	0.65	6.74	0.192	1.92	0.43	1.237

Experiment No.: 1.5-Acetone Concentration and Temperature Analysis

Temperature: 50°C

Special Conditions: None

pH: 4.8

Volume of Enzyme Solution: 10 ml

Acetone Concentr. (v/v)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (300 nm)	Recovered Protein Protein (g/l)
1.0	0.01	0.1	0.005	0.05	0.005	0.05	0.095	0.2732
	0.005	0.05	0.0	0.0	0.005	0.05	0.095	0.2732
1.5	0.0015	0.15	0.0015	0.15	0.105	1.05	0.135	0.3882
	0.035	0.35	0.035	0.35	0.075	0.75	0.145	0.417
2.0	0.005	0.05	0.095	0.95	0.125	1.25	0.135	0.3882
	0.03	0.3	0.075	0.75	0.135	1.35	0.14	0.4026
2.5	0.045	0.45	0.105	1.05	0.125	1.25	0.175	0.5032
	0.045	0.45	0.14	1.4	0.125	1.25	0.19	0.5464
3.0	0.015	0.15	0.035	0.35	0.125	1.25	0.21	0.604
	0.015	0.15	0.03	0.3	0.115	1.15	0.195	0.5608
3.5	0.07	0.7	0.165	1.65	0.14	1.4	0.2125	0.6111
	0.055	0.55	0.17	1.7	0.15	1.5	0.215	0.6183
Initial Concentr.	0.285	2.85	0.615	6.32	0.18	1.8	0.405	1.165
	-----	----	0.58	5.92	0.19	1.9	0.415	1.193

Experiment No.: 2.1-Total Protein Analysis

Temperature: 30°C

Special Conditions: 0.58 g/l BSA added to enzyme solution.

pH: 4.8

Volume of Enzyme Solution: 10 ml

Acetone Concentr. (v/v)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (330 nm)	Recovered Protein Protein (g/l)
1.0	0.005	0.05	0.025	0.25	0.07	0.7	0.16	0.46
1.5	0.175	1.75	0.45	4.54	0.17	1.7	0.27	0.776
	0.17	1.7	0.46	4.64	0.165	1.65	0.25	0.719
2.0	0.21	2.1	0.5	5.06	0.175	1.75	0.315	0.906
	0.21	2.1	0.51	5.16	0.17	1.7	0.325	0.935
3.0	0.22	2.2	0.56	5.7	0.175	1.75	0.37	1.06
	0.245	2.45	0.58	5.94	0.18	1.8	0.38	1.09
Initial	0.275	2.75	0.605	6.2	0.19	1.9	----	----
Concentr.	0.28	2.8	0.615	6.28	0.2	2.0	----	----

Experiment No.: 2.2-Total Protein Analysis

Temperature: 30°C

Special Conditions: 1.149 g/l BSA added to enzyme solution.

pH: 4.8

Volume of Enzyme Solution: 10 ml

Acetone Concentr. (v/v)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (330 nm)	Recovered Protein Protein (g/l)
1.0	0.025	0.25	0.075	0.75	0.065	0.65	0.355	1.021
	0.02	0.2	0.075	0.75	0.075	0.75	0.365	1.05
1.5	0.205	2.05	0.5	5.06	0.165	1.65	0.485	1.395
	0.21	2.1	0.485	4.9	0.165	1.65	0.465	1.337
2.0	0.22	2.2	0.55	5.6	0.16	1.6	0.48	1.38
	0.22	2.2	0.545	5.56	0.165	1.65	0.46	1.323
3.0	0.24	2.4	0.585	6.0	0.165	1.65	0.54	1.553
	0.25	2.5	0.58	5.94	0.16	1.6	0.53	1.524
Initial Concentr.	0.285	2.85	0.605	6.2	0.185	1.85	0.795	2.286
	0.275	2.75	0.6	6.14	0.18	1.8	0.775	2.229

Experiment No.: 2.3-Total Protein Analysis

Temperature: 30°C

pH: 4.8

Volume of Enzyme Solution: 10 ml

Special Conditions: Cellulase concentrated 2-fold by precipitation with 3.0 v/v acetone and redissolving in half the original volume of citrate buffer.

*Indicate 3-fold dilution of sample before measuring the reducing sugar.

Acetone Concentr. (v/v)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (330 nm)	Recovered Protein Protein (g/l)
1.0	0.0	0.0	0.02	0.2	0.055	0.55	0.1	0.286
	0.0	0.0	0103	0.3	0.06	0.6	0.09	0.259
1.5	0.33	3.3	0.23*	6.9	0.19	1.9	0.325	0.935
	0.33	3.3	0.21*	6.3	-----	-----	0.32	0.92
2.0	0.315	3.15	0.245*	7.35	0.185	1.85	0.335	0.963
	0.35	3.5	0.25*	7.5	0.18	1.8	0.335	0.963
3.0	0.335	3.35	0.245*	7.35	0.185	1.85	0.385	1.107
	0.325	3.25	0.245*	7.35	0.18	1.8	0.375	1.078
Initial Concentr.	0.375	3.75	0.26*	7.8	0.19	1.9	0.395	1.136
	0.36	3.6	0.263*	7.89	0.185	1.85	0.395	1.136
Native Enzyme Solution	0.25	2.5	0.6	6.14	0.2	2.0	0.41	1.179
	0.26	2.6	0.63	6.5	0.2	2.0	0.41	1.179

Experiment No.: 3.1-Precipitate Stability Analysis

Temperature: Room Temperature

pH: ----

Volume of Enzyme Solution: 10 ml

Special Conditions: Enzyme was precipitated with 3.0 v/v acetone and stored at room temperature in a desiccator.

Time Weeks	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (330 nm)	Recovered Protein Protein (g/l)
0	0.27	2.7	0.675	7.04	0.175	1.75	----	----
	0.255	2.55	0.7	7.34	0.175	1.75	----	----
1	0.26	2.6	0.62	6.38	0.175	1.75	----	----
	0.25	2.5	0.62	6.38	0.175	1.75	----	----
3	0.255	2.55	0.63	6.5	0.18	1.8	----	----
	0.26	2.6	0.665	6.9	0.18	1.8	----	----
5	0.24	2.4	0.6	6.14	0.175	1.75	----	----
	0.25	2.5	0.63	6.5	0.185	1.85	----	----
7	0.24	2.4	0.64	6.66	0.18	1.8	----	----
	0.26	2.6	0.61	6.26	0.175	1.75	----	----
10	0.24	2.4	0.63	6.5	0.165	1.65	----	----
	0.23	2.3	0.62	6.38	0.17	1.7	----	----
12	0.265	2.65	0.605	6.2	0.185	1.85	----	----
	0.24	2.4	0.59	6.04	0.175	1.75	----	----

Experiment No.: 4.1-Precipitate Equilibrium Analysis

Temperature: Room Temperature

pH: ----

Volume of Enzyme Solution: 10 ml

Special Conditions: Enzyme precipitated with 3.0 v/v acetone. The time of contact was varied.

Time (minutes)	FP Activity Absorbance (600 nm)	FP Activity Reducing Sugar(g/l)	C_1 Activity Absorbance (600 nm)	C_1 Activity Reducing Sugar(g/l)	C_x Activity Absorbance (600 nm)	C_x Activity Reducing Sugar(g/l)	Recovered Protein Absorbance (330 nm)	Recovered Protein Protein (g/l)
1	0.262	2.62	-----	-----	-----	-----	-----	-----
	0.25	2.5	-----	-----	-----	-----	-----	-----
2	0.255	2.55	-----	-----	-----	-----	-----	-----
	0.245	2.45	-----	-----	-----	-----	-----	-----
5	0.245	2.45	-----	-----	-----	-----	-----	-----
	0.255	2.55	-----	-----	-----	-----	-----	-----
10	0.245	2.45	-----	-----	-----	-----	-----	-----
20	0.24	2.4	-----	-----	-----	-----	-----	-----
	0.25	2.5	-----	-----	-----	-----	-----	-----
Initial Concentr.	0.275	2.75	-----	-----	-----	-----	-----	-----
	0.285	2.85	-----	-----	-----	-----	-----	-----

Experiment No.: 5.1-Filtration Studies

Temperature: Room Temperature

pH: 4.8

Acetone Concentration: 3.0 v/v

Special Conditions: No Filter Aid

Volume of Enzyme Solution:

Pressure Drop: 10 in. Hg.

40 ml		50 ml		70 ml		75 ml	
Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)
0	0	0	0	0	0	0	0
0.34	<25	6.38	25	16.46	25	5.53	<25
4.84	50	18.67	50	43.87	50	22.89	50
12.46	75	32.65	75	73.81	75	52.57	75
21.28	100	48.28	100	104.00	100	84.7	100
32.9	125	65.84	125	134.7	125	118.61	125
45.4	150	84.00	150	166.88	150	154.56	150
		102.35	175	197.38	175	190.69	175
		119.99	200	226.61	200	222.79	200
						252.68	225
						280.83	250
						304.03	275

Experiment No.: 5.2-Filtration Studies

Temperature: Room Temperature

pH: 4.8

Acetone Concentration: 3.0 v/v

Special Conditions: No Filter Aid

Volume of Enzyme Solution:

Pressure Drop: 17 in. Hg.

25 ml		40 ml		50 ml		75 ml	
Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)
0	0	0	0	0	0	0	0
1.93	25	0.76	<25	5.8	<25	20.90	50
5.16	50	2.15	50	17.12	50	47.90	75
9.01	75	----	75	29.67	75	78.42	100
		5.99	100	43.38	100	109.84	125
		7.69	125	56.42	125	141.20	150
				70.92	150	174.38	175
				86.86	175	207.3	200
						240.89	225
						273.93	250

Experiment No.: 5.3-Filtration Studies

Temperature: Room Temperature

pH: 4.8

Acetone Concentration: 3.0 v/v

Special Conditions: No Filter Aid

Volume of Enzyme Solution:

Pressure Drop: 24 in. Hg.

40 ml		50 ml		60 ml		75 ml	
Time (minutes	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)
0	0	0	0	0	0	0	0
0.93	25	3.65	<25	9.15	25	10.47	25
1.98	50	10.12	50	21.71	50	27.81	50
3.27	75	17.94	75	38.14	75	48.88	75
4.54	100	26.60	100	56.23	100	71.35	100
5.85	125	-----	125	75.44	125	95.11	125
7.57	150	45.86	150	96.67	150	-----	150
		55.33	175	-----	175	146.95	175
		65.65	200	139.13	200	173.9	200
						201.71	225
						229.37	250

Experiment No.: 5.4-Filtration Studies

Temperature: Room Temperature

pH: 4.8

Acetone Concentration: 3.0 v/v

Special Conditions: Ball milled newsprint added as filter aid in 9:1 ratio.
Pressure Drop: 10 in. Hg.

Volume of Enzyme Solution:

25 ml		50 ml		75 ml		100 ml	
Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)
0	0	0	0	0	0	0	0
0.62	<25	1.73	<50	0.43	25	7.39	50
1.80	50	3.33	75	-----	50	11.15	75
3.27	75	5.3	100	3.04	75	----	100
		7.33	125	4.6	100	18.94	125
		9.68	150	6.4	125	----	150
		12.46	175	8.53	150	----	175
				10.8	175	32.25	200
				13.25	200		
				15.6	225		
				18.4	250		
				21.7	275		

Experiment No.: 5.5-Filtration Studies

Temperature: Room Temperature

pH: 4.8

Acetone Concentration: 3.0 v/v

Special Conditions: Ball milled newsprint added as filter aid in 9:1 ratio.

Volume of Enzyme Solution:

Pressure Drop: 17 in. Hg.

25 ml		50 ml		75 ml		100 ml	
Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)
0	0	0	0	0	0	0	0
0.37	25	0.85	25	1.13	25	1.98	25
0.76	50	1.92	50	2.36	50	4.07	50
1.18	75	3.2	75	3.76	75	6.18	75
		4.72	100	5.33	100	8.36	100
		----	125	7.02	125	10.56	125
		8.17	150	8.72	150	12.84	150
				10.5	175	15.16	175
				12.46	200		
				14.38	225		

Experiment No.: 5.6-Filtration Studies

Temperature: Room Temperature

pH: 4.8

Acetone Concentration: 3.0 v/v

Special Conditions: Ball milled newsprint added as filter aid in 9:1 ratio.
Pressure Drop: 24 in. Hg.

Volume of Enzyme Solution:

25 ml		50 ml		75 ml		100 ml	
Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)	Time (minutes)	Volume (ml)
0	0	0	0	0	0	0	0
0.31	25	0.67	25	1.52	50	1.21	25
0.55	50	1.53	50	2.5	75	2.26	50
0.94	75	2.59	75	3.68	100	3.58	75
		3.85	100	5.0	125	4.97	100
		5.27	125	6.33	150	6.55	125
		6.69	150	7.76	175	8.28	150
		8.14	175	9.35	200	10.10	175
				11.12	225	12.0	200
				12.15	250		
				13.62	275		

APPENDIX II

APPENDIX II

Dielectric Constant of Acetone-Water Mixtures (2)

Acetone wt.%	Temperature 20°C	25°C	30°C	40°C	50°C
0	80.37	78.54	76.73	73.12	69.85
10	74.84	73.02	71.37	68.07	65.01
20	68.58	66.98	65.34	62.28	59.45
30	62.48	61.04	59.47	56.77	54.17
40	56.00	54.60	53.23	50.82	48.52
50	49.52	48.22	46.99	44.81	42.81
60	42.93	41.80	40.75	38.86	37.04
70	36.51	35.70	34.63	33.03	31.44
80	30.33	29.62	28.74	27.50	26.20
90	24.61	23.96	23.38	22.32	21.26
100	19.56	19.10	18.67	17.80	16.98

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