ENZYME RELEASE KINETICS IN A CELL DISRUPTION CHAMBER OF A BEAD MILL

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The kinetics of enzyme release from yeast using a bead mill was investigated. The enzymes investigated were invertase, acid phosphatase, alcohol dehydrogenase and alkaline phosphatase. A two-step consecutive process model was proposed to describe the behavior of enzyme release. The model described well the release of both membrane-bound and non membrane-bound enzymes, such as alkaline phosphatase and other enzymes, respectively.

Introduction

A large proportion of potentially useful microbial products are not secreted in the surrounding medium but are retained within the cells. The cells must be disrupted to liberate their intracellular products. Cell disruption is a process used in the initial stage of product recovery. Use of a bead mill for the release of enzymes and other bioproducts has gained wider acceptance due to its fast and efficient performance²⁾. Although many researchers have examined the release of several enzymes using a bead mill^{1, 10, 14)}, research has been concentrated on the investigation of operating parameters on the rate of release.

The enzyme release process was considered to follow a first-order process; i.e., the rate of release of a particular enzyme is in proportion to the concentration of the remaining enzyme in the cell⁸⁾. Marfy and Kula¹⁰⁾ determined the rate of release of several enzymes from *S. carlbergensis* and examined the effects of agitation speed, flow rate, cell concentration and bead diameter on the process. Woodrow and Quirk¹³⁾ examined the effect of bead mill operating conditions on the release of enzymes from *E. coli*. The rate of release of an enzyme may depend on its location within the cell, according to the study made by Heterington *et al.*⁸⁾ Schute *et al.*¹³⁾ showed that bead diameter, bead loading and location of the desired enzyme were the main factors affecting the release of the enzyme. Recently, a newly designed bead mill, the Co Ball Mill, has been developed and the operating parameters were studied for the release of biological products of unstable character⁵⁾.

This paper concerns a systematic analysis of the kinetics of enzyme release from the disruption of yeast using a bead mill. The release of four enzymes located in different parts of cells was investigated. A model

describing the enzyme release is proposed, which enables the prediction of the rate of enzyme release.

1. Materials and Method

1.1 Microorganism

Cells of *Saccharomyces cerevisiae* used in all experiments were commercial bakers' yeast obtained from Universal Food Co., Milwaukee, Wisconsin, USA. The cell suspension was prepared by suspending the cells in a medium containing 50 mM phosphate buffer solution to give a 30% dry weight/volume of cells. The prepared cell suspension was stored at 277 K until used.

1.2 The Bead mill

Disruption of cells was performed using a Dyno Mill KDL type with a disruption chamber volume of 150 cm³, depicted schematically in **Fig. 1**. The agitator of the bead mill consists of a single rotating polyurethane disc in a horizontally jacketed chamber. Rotor speed was set at 33.3 revolutions per second. Lead-free glass beads were used as grinding elements. The mean sizes of the beads were 0.375, 0.625 and 0.875 mm.

1.3 Cell Disruption process

The disruption chamber was filled with glass beads at bead loading α between 0.3-0.85. Bead loading α is defined as the volume ratio of the settled beads' volume to the chamber volume. Cell suspension was cooled to 278 K before being added to the disruption chamber. During operation, the outer jacket of the disruption chamber was cooled with brine solution at an inlet temperature of around 271 K. The temperature of the disrupted cell suspension was recorded as well as the cooling medium. The temperature of the suspension in the disruption chamber was between 286-291 K even after 5 minutes of disruption.

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Fig. 1 Schematic diagram of bead mill chamber

1.4 Enzyme assay

The enzymes assayed were invertase, acid phosphatase, alcohol dehydrogenase and alkaline phosphatase, which are considered to be located mostly in the cell wall, periplasm, cytoplasm and cell membrane, respectively. The disrupted yeast suspension was centrifuged at $2400\times g$ at 277 K for 30 minutes. The supernatant obtained was analyzed for enzyme activity. Invertase and acid phosphatase activity were measured in a sodium acetate-acetic acid buffer (pH4.5) at wavelengths of 505 nm and 340 nm respectively. Alcohol dehydrogenase and alkaline phosphatase activity were measured in a glycine-sodium pyrophosphate buffer (pH9) at a wavelength of 405 nm and Tris-HCl buffer (pH8) at a wavelength of 340 nm. The activity units of the enzymes were defined as follows: for invertase, μ mol glucose formed from sucrose per minute; for alcohol dehydrogenase, μ mol NADH produced per minute; for acid phosphatase and alkaline phosphatase, μ mol of *p*-NPP decomposed per minute.

1.5 Description of cell disruption

The number of disrupted cells was determined by counting them with a hemocytometer, where disrupted cells were distinguished from undisrupted viable cells using the methylene-blue dye exclusion method. The concentrations of disrupted cells were used in evaluating the physical disruption kinetics.

2. Results and Discussion

2.1 The enzyme release process

Figure 2 shows a comparison of the rate of release of enzymes and the rate of cell disruption. Cell disruption is faster than enzyme release. Release of the enzymes is considered to occur after the cell is disintegrated as shown below.

Fig. 2 Comparison of rates of cell disruption and enzyme release

Fig. 3 Degree of release of the four enzymes investigated

This implies a consecutive two-step process, i.e., cell disruption and enzyme release. The first process involves disruption of cells where the rate is described by the first-order rate constant k_1 as reported before¹¹⁾. The second step is the release of the enzymes themselves, the rate of which is governed by the rate constant k_2 .

Examples of the relative release of the four enzymes are shown in Fig. 3. The results show different rates of releases for each enzyme. Invertase showed the fastest rate of release, followed by acid phosphatase, alcohol dehydrogenase and alkaline phosphatase. It can also be observed from the graph that invertase, acid phosphatase and alcohol dehydrogenase were released very rapidly, such that the release shows exponential behavior.

Since the cell wall would be the part that is broken first, soluble materials trapped in it such as invertase were released the fastest. The cell wall is mostly composed of cellulose microfibrils located within a matrix

Fig. 4 Release of alkaline phosphatase as compared to the theoretical curve of Eq. (7)

made up mostly of hemicellulose such as glucans and mannans. As a soluble protein located within the cell wall matrix in association with mannans⁴⁾, invertase was observed to have a fast rate of release which is comparable with the rate of physical disruption of cells. Acid phosphatase and alcohol dehydrogenase, which are located in the periplasm and cytoplasm respectively, are soluble enzymes and thus followed a pattern of kinetics similar to that of invertase.

On the contrary, the release of alkaline phosphatase deviated from the behavior of the other three enzymes. As a membrane-bound enzyme, its release appeared to be more complex than the release of enzymes located in the cell wall, periplasm or cytoplasm of the cell. Enzymes associated with the membrane interact extensively with the hydrocarbon chains of membrane lipids³⁾. This hydrophobic interaction may have been responsible for the slow release of alkaline phosphatase.

2.2 First-order cell disruption and enzyme release process

At peripheral velocity higher than the critical velocity¹²⁾, disruption of cells is described by the first-order process as follows:

$$\frac{dC}{dt} = k_1 (C_o - C) \quad (2)$$

$$C = C_o (1 - e^{-k_1 t}) \quad (3)$$

where C_o is the initial number of cells and C is the number of disrupted cells at any time t .

The enzymes inside the cell diffuse into the surrounding liquid after the cell is disintegrated. Due to vigorous mixing of the cell suspension in the disruption chamber, the released products immediately mix with the surrounding liquid.

Let the rate of release of the enzyme be proportional to the amount of remaining enzyme in the disrupted cell:

$$\frac{dA}{dt} = k_2 (A_D - A) \quad (4)$$

where A is the amount of enzyme release at any time t

Fig. 5-a. Release of alcohol dehydrogenase after disruption of cells

Fig. 5-b. Release of alkaline phosphatase after disruption of cells

and A_D the maximum enzyme release from the disrupted cells whose value is related to the fraction of disrupted cells C/C_o as follows:

$$A_D = A_m \left(\frac{C}{C_o} \right) \quad (5)$$

where A_m is the maximum value of releasable enzyme. Substituting Eq. (3) and Eq. (5) into Eq. (4) yields the following equation:

$$\frac{dA}{dt} = k_2 [A_m (1 - e^{-k_1 t}) - A] \quad (6)$$

whose solution is given by Eq. (7), with the condition that at $t = 0$, $A = 0$.

$$A = A_m \left[\frac{k_2 e^{-k_1 t} - k_1 e^{-k_2 t}}{k_2 - k_1} \right] \quad (7)$$

For the disruption condition shown in Fig. 3, where the value of k_1 is 0.022 s^{-1} , the values of k_2 obtained for the release of invertase, acid phosphatase, alcohol dehydrogenase and alkaline phosphatase were, 3.10, 2.61, 2.22 and 0.032 s^{-1} respectively. Cell breakage will proceed in the order of cell wall, cell membrane and

Fig. 6 Release of alkaline phosphatase at different bead loadings

organelles from the most fragile to the stiffest. Therefore, the evaluated rate constants will reflect the locations of enzymes; i.e., invertase in the cell wall was the fastest, while periplasmic enzyme acid phosphatase and cytoplasmic enzyme alcohol dehydrogenase were slower. The slowest releasing rate of alkaline phosphatase might be due to its association with the cell membrane, as will be discussed later.

The values of k_2 for the first three enzymes were so large that the kinetics appeared to be first-order as shown in Fig. 3. If the release of enzyme itself is very rapid, i.e., $k_2 \gg k_1$, Eq. (7) may be reduced to Eq. (8), thus explaining the results previously reported^{8,10}.

$$A = A_m (1 - e^{-k_1 t}) \quad (8)$$

Plots of Eq. (7) as compared to the experimental values at different bead diameters are shown in Fig. 4. The values of k_1 at 0.375 mm, 0.625 mm, and 0.875 mm were 0.022, 0.0125 and 0.0076 s⁻¹, while the values of k_2 obtained were 0.032, 0.018 and 0.009 s⁻¹ respectively.

For further examination of the enzyme release process, enzyme assay was conducted after allowing the disrupted cells to stand for several minutes. After the disruption operation for 60 to 180 seconds, the mixture of glass beads, cell debris and its supernatant were separated by using 0.2 mm filter paper. The supernatant thus obtained every 120 seconds was subjected to enzyme assay. Alcohol dehydrogenase and alkaline phosphatase activities are shown in Figs. 5-a and 5-b respectively. No change in activity of alcohol dehydrogenase was observed, indicating that the release of alcohol dehydrogenase during cell disruption was instantaneous. A different trend for alkaline phosphatase was observed, with the activity increasing linearly with time. This phenomenon indicates that during cell disruption, only a part of the enzyme was released right after the cell was broken. The remaining part may have been slowly liberated from the interactions within the membrane as indicated in 2.1.

Evaluation of k_2 for alkaline phosphatase may be obtained from the experimental data presented in Fig. 5-

Fig. 7 Relation between cell disruption rate constant k_1 and k_2 of alkaline phosphatase described by Eq. (7)

b using the integrated form of Eq. (4):

$$A = A_D (1 - e^{-k'_2 t}) \quad (9)$$

where k'_2 is the rate constant of enzyme release into stagnant medium. The value of A_D can be calculated from the fraction of cell disrupted using Eq. (5). If the rate of release is very slow, i.e., $k'_2 \approx 0$, Eq. (9) reduces to Eq. (10):

$$A = A_D k'_2 t \quad (10)$$

Eq. (10) was used to evaluate k'_2 in Fig. 5-b. The average value of k'_2 obtained for 60, 120 and 180 seconds of disruption was 0.0016 s⁻¹, which is about 20 times smaller than k_2 obtained from the enzyme release during cell disruption. The discrepancy in the values obtained suggests that the release of enzyme after cell disruption was accelerated by agitation in the bead mill chamber.

2.3 Effect of bead loading and bead diameter on enzyme release process

From the above discussion, the effect of bead mill parameters such as bead loading and bead diameters on the enzyme release may be evaluated in terms of cell disruption rate constant k_1 . Since the amount of releasable enzyme is related to the fraction of disrupted cells, as shown in Eq. (5), the rate of release of the enzymes would increase by increasing the cell disruption rate (by increasing the bead loading or decreasing the bead diameters). Thus, the release of alkaline phosphatase was observed to increase with bead loading and with smaller diameters as shown in Figs. 4 and 6 respectively.

Plots of the values of k_2 against k_1 at different bead loadings and bead diameters are shown in Fig. 7, where the enzyme release rate constant k_2 of alkaline phosphatase increased linearly with cell disruption rate constant k_1 as

$$k_2 = 1.44k_1 \quad (11)$$

The kinetic constant k_2 of enzyme release described by Eq. (11) implies that the release of mem-

brane-bound enzyme would have been accelerated by the agitation of disrupted membrane itself or the increase in rate of membrane cleavage, which was accompanied by cell disruption, the kinetic rate constant being k_1 . A similar relation to that of Eq. (11) is possible for the other enzymes, but in the present study it has not been obtained.

The numerical coefficient of Eq. (11) is specific for the system adopted here. The relation will be convenient for evaluating the rate of release of enzyme from the physical disruption kinetics.

Conclusion

The release of an enzyme by cell disruption in a bead mill is intimately related to its location within the cell. A kinetic model based on a consecutive two-step process was proposed which describes the overall process from the disruption of cells to release of the enzyme. The model may well be applied to explain the release of any enzymes located in different parts of the cell. The release rate of membrane-bound enzyme was found to be dependent on the rate of cell disruption.

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Nomenclature

A	= enzyme activity	[U cm ⁻³]
A_D	= releasable enzyme from disrupted cells	[U cm ⁻³]
A_m	= maximum releasable enzyme	[U cm ⁻³]
C	= disrupted cell concentration at time t	[cells m ⁻³]

C_o	= initial cell concentration	[cells m ⁻³]
d_b	= diameter of bead	[mm]
k_1	= cell disruption rate constant	[s ⁻¹]
k_2	= enzyme release rate constant	[s ⁻¹]
k'_2	= enzyme release rate constant based on Eq. (10)	[s ⁻¹]
t	= time	[s]
α	= ratio of settled beads' volume to chamber volume	[-]

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