# Extraction of Cytochrome c and Alcohol Dehydrogenase from Yeasts by Cationic Surface Active Agent

# Extraction of Cytochrome c and Alcohol Dehydrogenase from Yeasts by Cationic Surface Active Agent

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## Introduction

A method for the extraction of cytochrome c of yeast has been proposed by Hagihara et al[1]. In their method cytochrome c is extracted with 10 % ammonium sulfate solution from ethylacetate treated cells. Alcohol dehydrogenase of yeast is commonly extracted by autolysing of dried cells.

During the course of an investigation on the bactericidal action of surface active agents, it was observed that the addition of cationics to the suspension of intact cells of yeast results in the release of cytochrome c and alcohol dehydrogenase. This paper deals with a method for the extracton of these substances from yeast cells.

## Materials and Methods

Intact cells of *Candida krusei*: The organisms were grown in 60-ml medium in 300-ml Erlenmeyer flask on a shaker at 30° for 52 hours. The medium was composed of the following: glucose, 10 %; urea, 1 %; $(NH_4)H_2PO_4$, 0.1 %; $K_2HPO_4$, 0.1 %; KCl, 0.05 %; $MgSO_4 \cdot 7H_2O$, 0.05 %; $FeSO_4 \cdot 7H_2O$, 0.001 % (pH 5.8). Cells were harvested from the medium by centrifugation, and washed three times with distilled water.

Baker's yeast: Commercial pressed baker's yeast was used after washing with 10 volume of distilled water.

Surface active agent: Cetyltrimethyl ammonium bromide (CTAB) was used.

Procedures for the extraction by CTAB: The following procedures were adopted, unless otherwise stated. Washed cells were suspended in either pH 5.5 or pH 8.3, M/20-phosphate buffer. Samples of cell suspensions (4.5 ml) were mixed with 0.5 ml of CTAB solution (the amount of CTAB corresponding to 60 $\mu$g per mg dried cells). After allowing the mixtures to stand for an adequate period, the extracts were obtained by removing the cells by centrifugation.

Determination of dry-weight of yeast cells: As stated in the preceding paper[2], dry-weight of yeast cells was calculated from a previously prepared standard curve relating dry-weight to optical density.

Assay of cytochrome c: A sample of a spectrum of the extract obtained is shown in Figure 1. According to the method of Paul[3], cytochrome c was determined from the difference in light absorption between the $\alpha$-band (548 m$\mu$) and minimum (534 m$\mu$) between the $\alpha$ and $\beta$ bands of the spectrum of reduced cytochrome c after reducing with $Na_2S_2O_4$. A value of 12,000 was adopted to the molecular weight of cytochrome c[4].

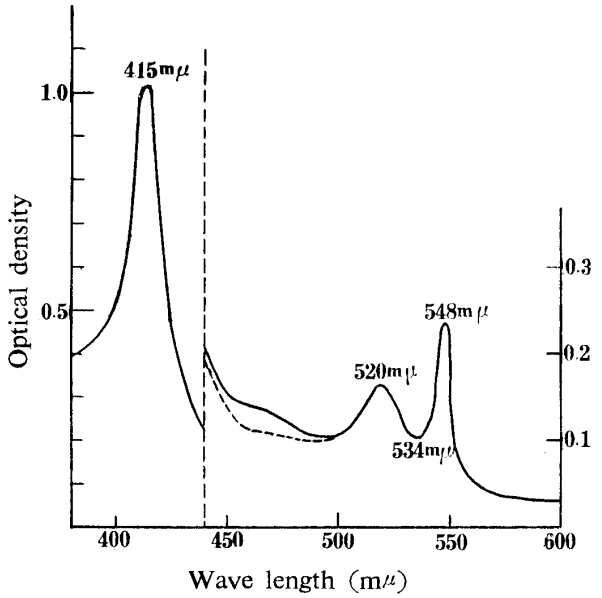


Fig. 1. Absorption spectra of the extract obtained by CTAB-treatment of *Candida krusei*.
The extract was obtained by treating with CTAB (corresponding 60μg/mg dried cells) at pH 8.3, 33° for 1 hour in the cell concentration of 88mg dried cells per ml. Broken line: addition of $Na_2S_2O_4$.

For the extinction coefficient of cytochrome c at 548 mμ and 534 mμ, $2.77\times10^7$ cm²/mole and $0.77\times10^7$ cm²/mole were adopted, respectively[3].

Assay of alcohol dehydrogenase: Activity of alcohol dehydrogenase in the extract was determined by the method of Racker[5].

Detemination of protein: Protein was determined by the biuret reaction by the method of Robison and Hogden[6].

Expression for the amount of the substrates in the extract: From the contents of these substances in the extract obtained, the contents in such extract that will be obtained when the extraction is carried out in the cell concentration of 100 mg dry weight per ml were calculated using the equation below.

(Content in the extract which will be obtained in the cell concentration of 100 mg dried cells per ml)

$$=(\text{content in the extract obtained})\times\frac{100}{A}$$

A: a value in mg of the cell concentration (dry weight) per ml in the extraction procedures.

## Results

I. Extraction of cytochrome c and alcohol dehydrogenase from *Candida krusei*.

Intact cells of *C. krusei* suspended in pH 5.5 or pH 8.3, M/20 phosphate buffer were treated with CTAB at 4 or 33° for 90 mimutes. Cytochrome c and alcohol dehydrogenase in the clear extract obtained by centrifugation were measured. The extraction from lyophilized cells was carried out in parallel by substituting lyophilized cells for intact cells. These results is shown in Table 1. By CTAB-treatment of intact cells cytochrome

c was extracted on either temperature 4 or 33°, although a more rapid extraction was observed at 33° than at 4°. Efficient extraction of alcohol dehydrogenase was only achieved at the temperature of 33°. In the case of lyophilized cells, alcohol dehydrogenase was extracted by incubating the suspension at 33° without CTAB, in contrast cytochrome c could not be extracted without CTAB.

Table 1. Extraction of cytochrome c and alcohol dehydrogenase from *Candida krusei* (1).

| Conditions of extraction | | | | Extract[a)] | |
|---|---|---|---|---|---|
| Cells used | Addition of CTAB 60μg/mg cells | Initital pH | Temp. | Cytochrome c μg/ml | Alcohol dehydrogenase unit/ml |
| Intact cell | + | 5.5 | 33° | 118 | 14,000 |
| 〃 | + | 8.3 | 〃 | 115 | 24,000 |
| 〃 | + | 5.5 | 4° | 36 | 200 |
| 〃 | + | 8.3 | 〃 | 46 | 400 |
| Dried cell | — | 5.5 | 33° | 0 | 3,000 |
| 〃 | + | 〃 | 〃 | 68 | — |
| 〃 | — | 8.3 | 〃 | 0 | 10,000 |
| 〃 | + | 〃 | 〃 | 76 | — |

Cell concentration on the extraction procedures: 56mg dried cells/ml.
Extraction period: 90minutes.
a): The contents of cytochrome c and alcohol dehydrogenase in the extracts were expressed as those contents in the extract which will be obtained on the extraction in the cell concentration of 100mg dried cells per ml.

The relative courses of extraction of cytochrome c and alcohol dehydrogenase were studied by treating the cell suspension with CTAB at 33 or 4°. As comparison, cytochrome c was extracted with 10 % ammonium sulfate solution adjusted the pH to 8.0 with $NH_4OH$ from ethylacetate treated cells (the method of Hagihara et al.[1]), and alcohol dehydrogenase was extracted with pH 8.3, M/20 phosphate buffer for one hour at 33° from the cells ground with quarz sand. As shown in Table 2, cytochrome c comparable to the amount extracted by the method of Hagihara et al. was extracted by CTAB-treatment at 4° for 5 hours or at 33° for 3 hours. The activity of alcohol dehydrogenase extracted by CTAB-treatment at 33° for 3 hours was equivalent to about 80 % of that extracted by grinding. It is interesting that (a) a marked stimulation of the extraction of cytochrome c and alcohol dehydrogenase was observed by an addition of 10 % ammonium sulfate to CTAB treating solution, (b) a relatively small amount of cytochrome c was extracted by CTAB-treatment of ethylacetate treated cells.

II. Extraction of cytochrome c and alcohol dehydrogenase from commercial baker's yeast.

Cytochrome c and alcohol dehydrogenase were extracted from baker's yeast in a manner similar to that described for *C. krusei*, although it took a longer time for their extraction (Table 3).

III. Preparation of cytochrome c from commercial baker's yeast.

Commercial pressed yeast (450 g, containing 67 % water) was suspended in 500 ml of

Table 2. Extraction of cytochrome c and alcohol dehydrogenase from *Candida krusei* (2).

| Conditions of extraction[a] | | | | Extract[e] | | |
|---|---|---|---|---|---|---|
| Cells used | Addition of CTAB 60μg/mg cells | Temp. | Time hr. | Cytochrome c μg/ml | Aldohol dehydrogenase unit/ml | Protein mg/ml |
| Intact cells | + | 33° | 1 | 85 | 10,000 | 3.0 |
| 〃 | + | 〃 | 2 | 95 | 27,000 | 7.5 |
| 〃 | + | 〃 | 3 | 111 | 37,000 | 10.0 |
| 〃 | + | 〃 | 2[b] | 109 | 39,000 | 10.6 |
| 〃 | + | 4° | 1 | 27 | — | — |
| 〃 | + | 〃 | 2 | 55 | — | — |
| 〃 | + | 〃 | 3 | 73 | — | — |
| 〃 | + | 〃 | 5 | 98 | 1,000 | 0.5 |
| 〃 | Extraction by gringing | | | — | 45,000 | 22.7 |
| Cells treated with ethylacetate | — | 4° | 15[c] | 100 | — | 2.0 |
| 〃 | + | 〃 | 15[d] | 33 | — | 1.1 |

a) : Cell concentration on the extraction procedures: 72mg dried cells per ml.
b) : Ten % of ammonium sulfate was added to the extraction.
c) : Cytochrome c was extracted according to the method of Hagihara et al. by extracting with 10% ammonium sulfate solution from the cells treated with ethylacetate.
d) : Cytochrome c was extracted by the addition of CTAB to the suspension of the cells treated with ethylacetate.
e) : See Table 1.

Table 3. Extraction of cytochrome c and alcohol dehydrogenase from commercial baker's yeast.

| Conditions of extraction[a] | | | | | Extract[e] | | |
|---|---|---|---|---|---|---|---|
| Cells used | Addition of CTAB 60μg/mg cells | Initial pH | Temp. | Time hr. | Cytochrome c μg/ml | Alcohol dehydrogenase unit/ml | Protein mg/ml |
| Intact cells | + | 8.3 | 33° | 2 | 15 | 3,000 | 0.8 |
| 〃 | + | 〃 | 〃 | 4 | 29 | 11,000 | 1.4 |
| 〃 | + | 〃 | 〃 | 2[b] | 32 | 8,000 | 3.8 |
| 〃 | + | 〃 | 〃 | 4[b] | 36 | 22,000 | 8.8 |
| 〃 | + | 〃 | 4° | 5 | 17 | — | — |
| 〃 | + | 〃 | 〃 | 15 | 34 | 1,000 | 0.8 |
| 〃 | + | 5.5 | 33° | 4 | 17 | 4,000 | 1.2 |
| 〃 | + | 〃 | 4° | 15 | 14 | 600 | — |
| Cells treated with ethylacetate | — | 8.3 | 4° | 15[c] | 34 | — | 0.5 |
| 〃 | + | 〃 | 〃 | 15[d] | 6 | — | 0.4 |

a) : Cell concentration on the extraction procedures: 120mg dried cells per ml.
b, c, d, e) : The same with Table 2.

distilled water in which was dissolved 7 g of CTAB. After adjusting the pH to 8.3 with 1 N-NaOH, the mixture was held at 4° for 20 hours. By centrifugation 630 ml of clear

extract, containing 32 $\mu$g of cyfochrome c per ml, was obtained. The extract was passed through a column (2.2×12 cm) of $NH_4$–Amberlite IRC–50 (buffered at pH 7.0). After washing the column with distilled water and then 100 ml of 0.2 M ammonium acetate, the cytochrome c was eluted with 5 % ammonium sulfate solution adjusted the pH to 8.0 with $NH_4OH$. After dialysing against distilled water, the solution was again passed through a small column, eluted and dialysed. Thus, 35 ml of the solution, containing 370 $\mu$g of cytochrome c per ml, was obtained (yield of 64 % to cytochrome c in the original extract.) and its spectrum is shown in Figure 2.

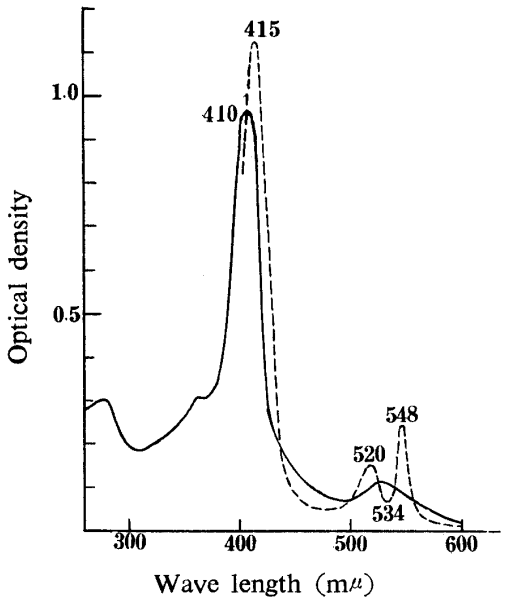


Fig. 2. Absorption spectra of purified cytochrome c.
Solid line: oxidized form with potassium ferricyanide.
Broken line: reduced form with $Na_2S_2O_4$.

IV. Stepwise extraction of nicotinamide adenine dinucleotide, cytochrome c and alcohol dehydrogenase.

In the preceding paper[2], it was reported that nicotinamide adenine dinucleotide (NAD) of *C. krusei* could be extracted by CTAB–treatment at pH 3.5, 4° for 45 minutes. As described above, cytochrome c and alcohol dehydrogenase of *C. krusei* could be extracted at pH 8.3, 4° for about 4 hours and at pH 8.3, 33° for 3 hours, respectively. From these facts, a stepwise extraction of NAD, cytochrome c and alcohol dehydrogenase was tested with the cells of *C. krusei* according to the procedures shown in Figure 3. The possibility of a stepwise extraction of these substances was observed (Table 4).

In this procedures, further addition of CTAB did not need for the extraction of cytochrome c after NAD–extraction. This phenomenon should be explained as follows; CTAB binds tightly with cells during NAD–extraction[7,8], and acts for the subsequent extraction of cytochrome c.

In the final cell-debris, from which alcohol dehydrogenase has been extracted, nucleic acids seem to remain in a form of nucleoprotein. Therefore, it may be possible to extract nucleic acids after alcohol dehydrogenase extraction.[9]

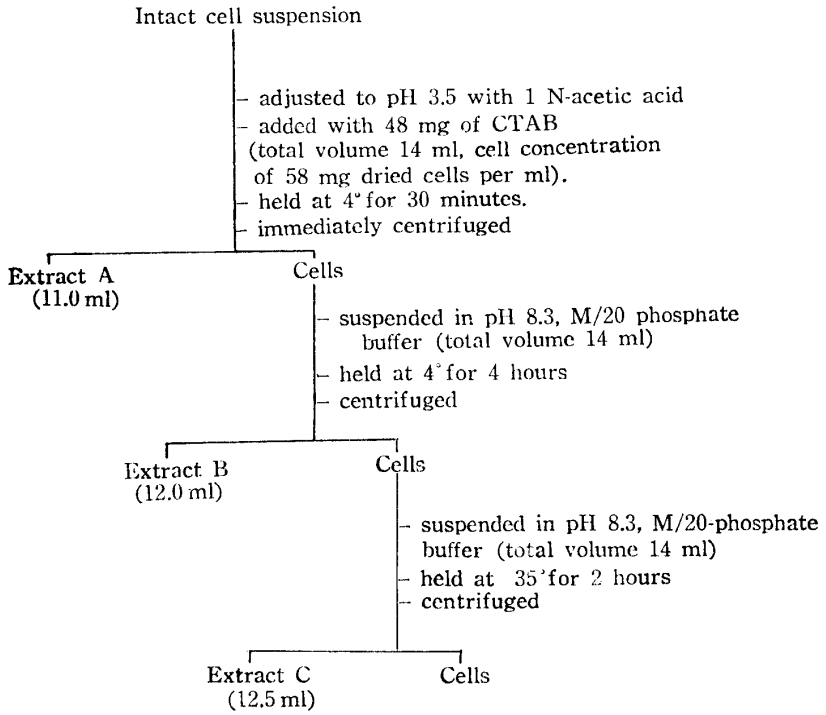


Fig. 3. Procedures for a stepwise extraction of NAD, cytochrome c, and alcohol dehydrogenase from *Candida krusei*.

Table 4. Stepwise extraction of NAD, cytochrome c and alcohol dehydrogenase from *Candida krusei*

| | Content[a] | | | |
|---|---|---|---|---|
| | NAD μg/ml | Cytochrome c μg/ml | Alcohol dehydrogenase unit/ml | Protein mg/ml |
| Extract A | 230 | 6 | — | 0.3 |
| Extract B | — | 92 | 900 | 0.3 |
| Extract C | — | 9 | 38,000 | 11.6 |
| Comparative extraction[b] | | | | |
| Conditions of extraction by CTAB | | | | |
| pH 8.3, 4°, 4hr. | — | 103 | 1,000 | 0.5 |
| pH 8.3, 33°, 2hr. | — | 108 | 38,000 | 12.4 |

a) : Contents were expressed similarly with Table 1.
b) : For the purpose of reference, cytochrome c and alcohol dehydrogenase were extracted with CTAB from the same intact cell by usual method.

## Discussion

If cytochrome c was extracted with 10 % ammonium sulfate solution from ethylacetate treated cells, it is necessary to remove ammonium sulfate from the extract obtained prior to purification of cytochrome c by resin-absorption. If alcohol dehydrogenase was

extracted by autolysing of dried cells, it is necessary to dry the cells prior to extraction; moreover, it is feared that denaturation of alcohol dehydrogenase may occur during drying of the cells. Therefore, the extraction of cytochrome c and alcohol dehydrogenase by the treatment of intact cells with CTAB should be convenicent.

That cytochrome c is binding with cell structure has been suggested[10]. Therefore, CTAB must enter the cell and have an action to separate cytochrome c from cell structure. It has been reported that cytochrome c binds easily with phospholipids[11,12], and CTAB binds also with phospholipids of the cell[13,14]. The fact that both cytochrome c and CTAB bind with phospholipids may be a reason why cytochrome c is extracted by CTAB-treatment. But the explanation of the mechanism of the extraction of cytochrome c by CTAB must await further work.

In this paper, only extraction of alcohol dehydrogenase of enzymes by CTAB was considered representatively. Negoro, Hirano and Fukumoto[15,16] reported that cationic surfactants were very effective to elute saccharase from yeast cells. Therefore, it is probable that the other enzymes are extracted by CTAB-treatment. In fact, a larger activity of catalase was observed in the extract in which large activity of alcohol dehydrogenase was observed.

## Summary

Cytochrome c and alcohol dehydrogenase were extracted from either cell suspension of *Candida krusei* or baker's yeast by the treatment with cetyltrimethyl ammonium bromide (CTAB).

Cytochrome c was extracted at pH 8.3 on either temperature of 4 or 33°. Efficient extraction of alcohol dehydrogenase by CTAB-treatment at pH 8.3 was only achieved at the temperature of 33°.

The fractional extraction was done using the cells of *Candida krusei*, according to the following procedures: cell suspension was first extracted by CTAB at pH 3.5, 4° for 30 minutes, cell debris was extracted secondly by M/20 phosphate buffer of pH 8.3 at 4° for 4 hours and the third extraction was done by the same buffer solution at 33° for 2 hours. The three extracts obtained were rich in nicotinamide adenine dinucleotide (NAD), cytochrome c, and alcohol dehydrogenase, respectively. Thus, the possibility of the fractional extraction of NAD, cytochrome c and alcohol dehydrogenase from *Candida krusei* by CTAB-treatment was suggested.

## Acknowledgement

The author is indebted to Prof. K. Kitahara, the Institute of Applied Microbiology, University of Tokyo, and Prof. M. Kanie, Kagoshima University, for their kindly guidance and suggestions. He wishes to express his thanks to Dr. K. Kodama, Laboratory of Kodama Brewing Co. Ltd. for his kindness in identifying *Candida krusei*. Thanks are also given to Mr. T. Kobayashi, student of Kagoshima University, for his assistance in the experiments.

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