# Transport kinetics of ectoine, an osmolyte produced by *Brevibacterium epidermis*

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## Abstract

*Brevibacterium epidermis* DSM 20659 is a halotolerant Gram-positive bacterium, which can synthesize the osmolyte, ectoine, but prefers to take it up from its environment. The present study revealed that *B. epidermis* is equipped with at least one transport system for ectoine, with a maximal transport velocity of 15.7 ± 4.3 nmol/g CDW/min. The transport requires energy and is completely inhibited by the proton uncoupler CCCP. The ectoine uptake system is constitutively expressed at a basal level of activity and its activity is immediately 10-fold increased by hyper-osmotic stress. Initial uptake rates are not influenced by the intensity of the hyper-osmotic shock, but the duration of the increased activity of the uptake system could be directly related to the osmotic strength of the assay solution. Competition assays indicate that betaine, but not proline, is also transported by the ectoine uptake system.

## Introduction

Microorganisms respond to external hyper-osmotic stress by accumulating -through *de novo* synthesis or uptake- osmotically active solutes, which help to restore the osmotic balance (Csonka 1989). These solutes can be inorganic ions but a more flexible mode of adaptation to high osmolarities involves the accumulation of organic solutes, called ‘compatible solutes’ or osmolytes.

Compatible solutes are best described as small, highly water-soluble organic solutes which can be accumulated to high intracellular levels without disturbing essential functions of the cell. They include sugars, polyols, amino acids and their respective derivatives, betaines and ectoines (Galinski 1995, Ventosa *et al*. 1998). They can be synthesized *de novo* by certain

producer strains, but are preferably taken up from their environment by specific transport systems. Ectoine is the most abundant *de novo* synthesized osmolyte of aerobic chemoheterotrophic eubacteria, including *Brevibacterium* species (Frings *et al*. 1993, Onraedt *et al*. 2005a, b, 2004, Severin *et al*. 1992).

Compatible solutes are naturally present in the environment, after release by primary microbial producers upon dilution stress, by decaying plant and animal cells and by mammals in the form of excretion fluids (e.g. urine). However, the concentrations of these compounds are low and variable, so osmoprotectant uptake systems usually exhibit a high affinity for their substrates with Km values in the micromolar range and a high accumulation capacity. Several uptake systems for compatible solutes have been identified. They are all active transporters, belonging to the class of the secondary transporters or the ABC transporters. Well-studied examples of such uptake systems are ProP and ProU in *Escherichia coli* and *Salmonella typhimurium* (Cairney *et al*. 1985a, b), and OpuA, C and D in *Bacillus subtilis* for betaine (Kappes *et al*. 1996).

High affinity uptake systems for ectoine have been described in non-halotolerant micro-organisms, such as EctP in *Corynebacterium glutamicum* (Peter *et al*. 1998) or in halophilic and halotolerant bacteria, such as TeaABC (*Halomonas elongate* and *Marinococcus halophilus*) (Grammann *et al*. 2002). In this paper, we present data identifying and characterising an ectoine uptake system in *Brevibacterium epidermis*. To our knowledge, this is the first report of an ectoine transport system in a *Brevibacterium* species.

## Materials and methods

*Bacterial strain and growth conditions*

*Brevibacterium epidermis* DSM 20659 was grown aerobically in 500 ml shake flasks, at 200 rpm and 30 C, filled with 100 ml complex medium (MSGYE) containing (in g/l) monosodium glutamate (50), yeast extract (2.5), $KH_2PO_4$ (3), $K_2HPO_4$ (9), $MgSO_4.7H_2O$ (0.4), $MnSO_4.4H_2O$ (0.01), $FeSO_4.7H_2O$ (0.01). The pH was adjusted to 7.0 with HCl. High osmolarity growth conditions were obtained by addition of NaCl (1 M).

The bacterial cells were grown for 18 h and then used for the uptake assay. At this point, the cells were in their mid-exponential growth phase, reaching a biomass concentration of approx. 3.5 g cell dry weight (CDW)/l.

*Ectoine uptake assay*

Uptake experiments were initiated by adding 10 ml cell suspension to 10 ml uptake buffer, containing 10 mM potassium phosphate (pH 7.0) and 1 g ectoine/l; the ectoine concentration was varied within the range of 0.1 to 0.25 g ectoine/l. For the determination of the kinetic parameters of the transport system, ectoine uptake was activated by subjecting the cells to a hyper-osmotic shock. NaCl was added to the buffer, in varying concentration depending on the intensity of the hyper-osmotic shock envisaged. Other activation assays were performed with an uptake assay solution containing sucrose (1 M) or tetracaine (1 mM), an anesthetic. As to ectoine uptake inhibition experiments, the following inhibitors were added (final concentration in uptake assay solution): carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)

(50 µM), betaine (5 g/l) or L-proline (5 g/l). The uptake assay was performed at room temperature, while the suspension was continuously stirred with a magnetic stirring bar.

*Determination of the ectoine concentration*

During the uptake assay, 1 ml samples were taken at time intervals and centrifuged (10 000 g, 5 min); the residual extracellular ectoine concentration was measured by HPLC using an Aminex HPX-87C column (Bio-Rad Laboratories), with water containing $CaCl_2$ (5mM) as mobile phase and detection at 210 nm.

**Results**

*Ectoine uptake by Brevibacterium epidermis*

The uptake by *B. epidermis* cells, grown on MSGYE medium without NaCl, of externally supplied ectoine was measured as a function of time in the presence of 1 M NaCl as well as without NaCl (Figure 1). The final NaCl concentration in the assay was then varied from 0 M (no hyper-osmotic shock) to 2 M NaCl and the initial ectoine uptake rate for the different intermediate NaCl concentrations was determined (Figure 2).

**Figure 1, Figure 2**

The uptake of ectoine was clearly stimulated by applying a hyper-osmotic shock, increasing the initial uptake rate about 10-fold. Remarkably, the initial uptake rates did not really depend on the strength of the hyper-osmotic shock once above a threshold of 0.5 M NaCl. However, the duration of the activated uptake and the final amount of ectoine taken up at that time were directly related to the osmotic strength of the ectoine uptake assay suspension (Figure 3).

**Figure 3**

*Kinetis of the ectoine uptake system*

Decreasing the ectoine concentration in the uptake assay solution to 0.1 or 0.25 g ectoine/l did not affect the initial uptake rates. This means that the ectoine transport system is saturated at these concentrations. Ectoine is thus transported by *B. epidermis* cells with a Vmax of 2.23 ± 0.06 mg ectoine/g CDW/min (15.7 ± 4.3 nmol/g CDW/min). Since concentrations of ectoine below 0.1 g/l were difficult to measure accurately by HPLC, the Km value of the transport system could not be determined.

*Induction of ectoine uptake by NaCl*

Previous ectoine uptake assays were performed with *B. epidermis* cells, grown in MSGYE medium without NaCl. In order to see the effect of salt-stress during growth on the uptake rates, a series of uptake assays was performed with *B. epidermis* cells grown on MSGYE medium with 1 M NaCl. These cells were subjected to an iso-osmotic shock (final NaCl concentration of the assay solution is 1 M NaCl), a hypo-osmotic shock (0.5 M NaCl) or a hyper-osmotic shock (2 M NaCl) (Figure 4). The initial ectoine uptake rates as a function of the NaCl concentration are given in figure 5.

**Figure 4, Figure 5**

If induction during NaCl-growth would have taken place, it could be expected that more transport proteins would be present in the cells. Then follows that the basal and activated ectoine uptake rate would be significantly higher in these assays, than when the uptake assays were performed with cells grown in MSGYE medium without NaCl. A comparison of the data in figure 5 and figure 2 shows that this is not the case.

As the cells were previously grown under salt-stress, their ectoine uptake system apparently was only activated in the presence of added ectoine and the observed ectoine uptake rates were thus maximal. However, it is remarkable that the transport system remains in its activated state for some time, even when the cells are subjected to a hypo- or iso-osmotic shock. Particularly in the case of a hypo-osmotic shock, one would expect an immediate de-activation of the ectoine uptake system, bringing the uptake rate quickly back to its basal level. However, it seems that the response of the cells to a hypo-osmotic shock, by decreasing the uptake rate, was postponed.

*Regulation of the activity of the ectoine uptake system*

From the previous results it is clear that a hyper-osmotic shock in a NaCl containing uptake solution 'activates' the uptake system: it increases the uptake rate 10-fold with respect to its 'basal' level. When the osmotic stress was applied with sucrose (1 M) or a combination of sucrose and NaCl (0.5 M each), the initial ectoine uptake rates were significantly lower (Figure 6). Sucrose is rapidly accumulated by the bacterial cells and as such reduces the osmotic gradient across the membrane.

**Figure 6**

The activity of some osmolyte transporters, such as the betaine BetP from *Corynebacterium glutamicum*, has been shown to be activated by the addition of the local anesthetic tetracaine (Ruebenhagen *et al*. 2000). Tetracaine is an amphipath that partitions into the cytoplasmic membrane and alters the physical state of the membrane, through which it can activate membrane proteins. We tried to find out whether tetracaine also had a stimulating effect on ectoine transport in *Brevibacterium epidermis* cells. Tetracaine was added to an ectoine uptake assay (1 mM final conc), performed with cells grown without NaCl and suspended in

an uptake buffer with no NaCl added (no hyper-osmotic shock). Tetracaine addition did not lead to a significant increase in the ectoine uptake rate (data not shown).

*Inhibition of ectoine uptake by Brevibacterium cells*

As ectoine is taken up against a concentration gradient, it needs an energy source as a driving force. The initial uptake was completely blocked by the $H^+$ uncoupler (CCCP) (50 µM final conc.). Only after 60 min, a gradual but low uptake could be observed (Figure 7).

**Figure 7**

Ectoine uptake rates were also determined in the presence of a 10-fold excess of betaine or L-proline., As described for the EctP uptake system of *Corynebacterium glutamicum*, these two other osmolytes can be transported by the same transport system as that for ectoine (Peter *et al.* 1998).

An excess of betaine in the uptake assay solution significantly reduced the initial uptake rate, which suggests that betaine competes with ectoine for uptake. L-Proline had no significant inhibitory effect on the initial ectoine uptake rate, but it decreased the duration of the activated uptake (Figure 8).

**Figure 8**

**Discussion**

Several high affinity uptake systems for ectoine have been described in non-halotolerant micro-organisms, such as EctP in *Corynebacterium glutamicum* (Peter *et al*. 1998) or in halophilic and halotolerant bacteria, such as TeaABC (*Halomonas elongata*) (Grammann *et al.* 2002).

*Brevibacterium epidermis* is a halotolerant Gram-positive bacterium that synthesizes ectoine *de novo* when grown on MSGYE medium. Its synthesis is inhibited by the presence of added osmolytes, including ectoine, since uptake is preferred to *de novo* synthesis. *B. epidermis* has also been shown to grow on ectoine when it is supplied as only source of carbon. These data indicate that *B. epidermis* is able to take up ectoine from its environment.

In this study, we have shown that *Brevibacterium epidermis* is equipped with at least one uptake system for ectoine with a Vmax of 15.7 ± 4.3 nmol/g CDW/min. Its transport requires energy and is completely inhibited by the proton uncoupler CCCP. The transport system is constitutively expressed at a basal level of activity and this activity can be suddenly increased 10-fold by applying a hyper-osmotic stress to the cells. Although the activity increase was not significantly influenced by the intensity of the hyper-osmotic shock, the duration of the increased uptake activity could be directly related to the osmotic strength of the uptake assay solution.

Once a certain amount of ectoine, related to the intensity of the hyper-osmotic shock, was accumulated, the uptake rate fell down again to its basal level. Unlike Gram-negative bacteria, which only accumulate compatible solutes under stress, Gram-positive bacteria need to accumulate them even under normal growth conditions, to assist in maintaining their high turgor (Glaasker *et al*. 1996, Whatmore & Reed 1990).

When *Brevibacterium* cells were grown under salt-stress, in MSGYE medium with 1M NaCl, the initial ectoine uptake rates were maximal and similar to those of cells grown without salt-stress and then exposed to a hyper-osmotic shock. Also, the basal ectoine uptake rate was similar for cells grown with or without salt-stress. These findings exclude the induction of

expression of the genes involved in the uptake of ectoine to be an important regulatory mechanism.

In order to respond to osmotic stress and to (in)activate the transport systems for osmolyte uptake, bacteria can sense one or more different physicochemical parameters, which can be a change in external osmotic pressure, turgor pressure, membrane strain, internal osmolarity or the concentration of specific cytoplasmic signal molecules (Glaasker *et al*. 1998, Poolman & Glaasker 1998). We suggest the ectoine uptake by *Brevibacterium* cells described here to be a case of turgor-regulated activity, since uptake rates are significantly lower in the presence of sucrose. Rapid intake of sucrose, possibly through facilitated diffusion, restores the osmotic equilibrium and the cellular turgor, after which the ectoine uptake rate falls down to its basal level (Glaasker *et al.* 1998). Ectoine uptake could not be activated by an alteration of the membrane strain by addition of the amphipath tetracaine, as was the case for other compatible solute transporters, such as BetP in *Corynebacterium glutamicum* (Ruebenhagen *et al*. 2000).

With *Brevibacterium* cells grown under salt-stress, it was observed that the activity of the ectoine uptake system was at its maximal level, even without subjecting the cells to a hyper-osmotic shock. If induction of gene expression has no significant influence on the transporter activity, this could indicate that the response to a hypo-osmotic shock is rather slow while the response of the increased uptake activity to a hyper-osmotic shock is immediate. For *B. epidermis*, the response to a decrease in turgor is rapid and the ectoine uptake protein is immediately activated. However, increase in turgor can be imposed up to a certain maximum, since Gram-positive bacteria have a rigid cell wall, that can withstand very high internal osmotic turgor pressures of 15 to 25 atm (Csonka & Hanson1991).

An excess amount of betaine had a decreasing effect on the initial maximal ectoine uptake rate and on the basal uptake rate as well. We suggest that the ectoine uptake system also has a strong affinity for betaine. L-Proline had no significant lowering effect on the ectoine uptake rate, both activated or basal, but it reduced the duration of activated uptake period and thus the amount of ectoine taken up before its uptake rate falls back to its basal level. L-Proline must be taken up by (an) other uptake system(s) and helps to restore the turgor-pressure, after which the ectoine uptake system is ‘deactivated’ and its uptake rate is reduced to its basal level.

In conclusion, we have described here an ectoine uptake system, which is constitutively expressed at a basal level of activity, and which is (in)activated by a change in turgor pressure. The transport of ectoine is energy requiring and the uptake system also displays a high affinity for betaine. To our knowledge, this is the first report on an ectoine uptake system in *Brevibacterium* species.

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**Figures:**

**Figure 1: Uptake of ectoine by *B. epidermis* cells, grown on MSGYE medium without NaCl and subjected to a hyper-osmotic shock (final NaCl concentration 1 M) or not (0 M NaCl).** Data points are averages of 3 experiments using the same starter cell solution.

**Figure 2: Initial ectoine uptake rates as a function of the final NaCl concentration of the assay solution.** *B. epidermis* cells grown in MSGYE medium without NaCl, data points are averages of at least 3 independent experiments; there is a maximal variation of 25% of the average

**Figure 3: Duration of activated uptake (A) and final ectoine level accumulated (B) as a function of the final NaCl concentration in the assay solution.** Data points were calculated from uptake experiments performed with the same starter cell solution. Variation on this value was max. 15%

**Figure 4: Uptake of ectoine by *B. epidermis* cells, grown on MSGYE medium with 1 M NaCl and subjected to a hypo- (0.5 M), iso- (1 M) or hyper-osmotic shock (2 M) (final NaCl concentration of the ectoine uptake assay solution)**

**Figure 5: Initial ectoine uptake rates as a function of the final NaCl concentration of the ectoine assay solution.** *B. epidermis* cells grown in MSGYE medium with 1 M NaCl. Data points are averages of at least 3 independent experiments

**Figure 6: Ectoine uptake profile for *B. epidermis* cells grown in MSGYE medium without NaCl and suspended in uptake buffer with (final conc.) 1 M NaCl (▲), 1 M sucrose (◇) or 0.5M sucrose and 0.5 M NaCl (□)**

**Figure 7: Inhibition of the initial ectoine uptake by the addition of CCCP (50 µM final conc.) to the assay solution.** *B. epidermis* cells were grown in MSGYE medium with 1 M NaCl, final NaCl concentration in assay solution is 2 M (hyper-osmotic shock)

**Figure 8: Uptake of ectoine in the presence of a 10-fold excess of betaine or L-proline.** *B. epidermis* cells were grown in MSGYE medium without NaCl, final NaCl concentration in the assay solution is 2 M (hyper-osmotic shock)

**Figure 1:**

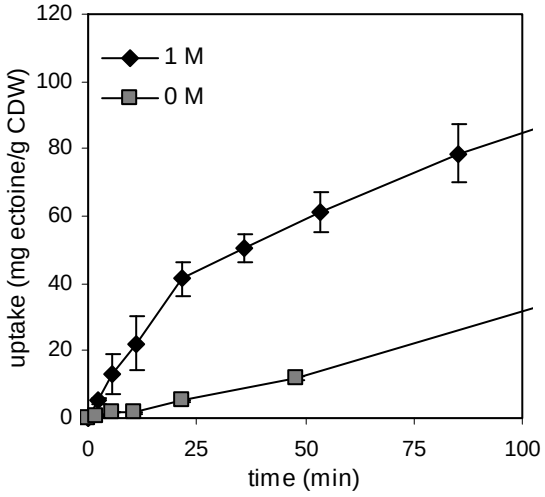



**Figure 2:**

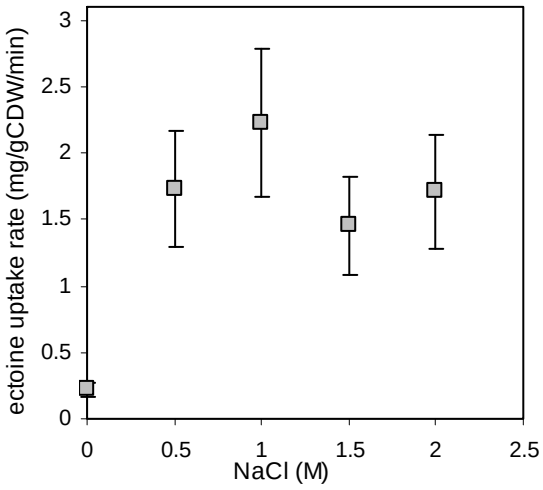
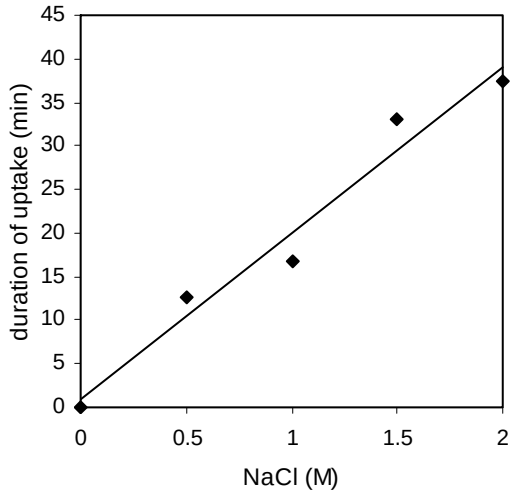


**Figure 3:**

A.

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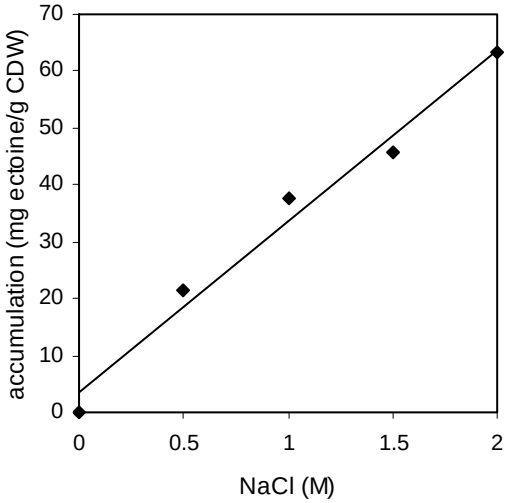


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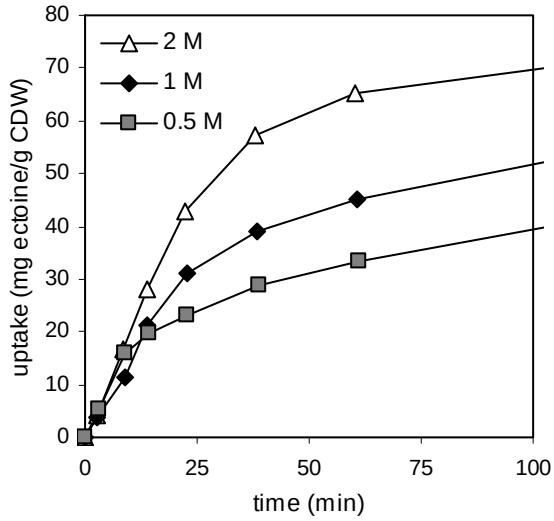

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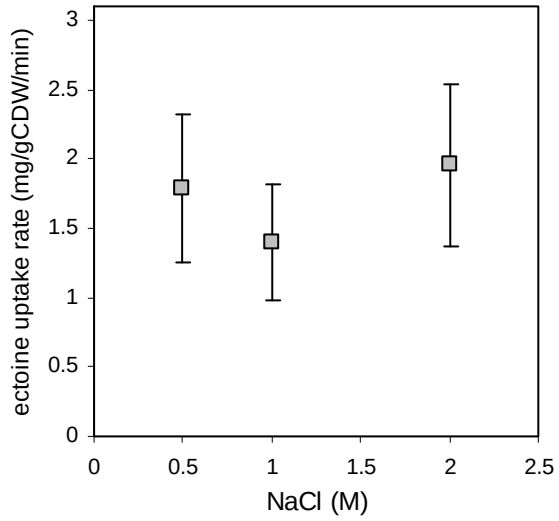


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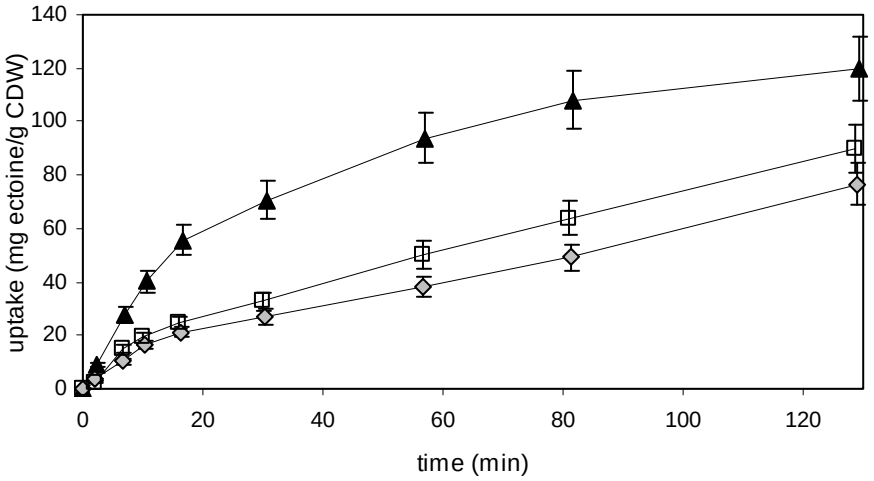

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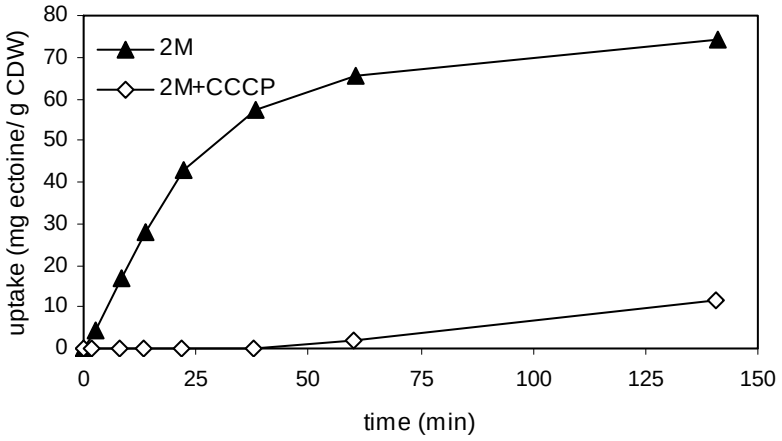


**Figure 8:**