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# Application of Alcohol-Salt Aqueous Biphasic System for the Recovery of Ectoine

**Yin Hui Chow [1,2], Chin Tee Poh [1], Phei Er Kee [3], Hui Suan Ng [3,*], John (Chi-Wei) Lan [4,5,*] and Li Wan Yoon [1]**

[1] School of Engineering, Faculty of Innovation and Technology, Taylor's University, Lakeside Campus, No. 1, Jalan Taylor's, Subang Jaya 47500, Selangor, Malaysia; yinhui.chow@taylors.edu.my (Y.H.C.); chintee.poh@sd.taylors.edu.my (C.T.P.); liwan.yoon@taylors.edu.my (L.W.Y.)
[2] Food Security and Nutrition Impact Lab, Taylor's University, Lakeside Campus, No. 1, Jalan Taylor's, Subang Jaya 47500, Selangor, Malaysia
[3] Centre for Research and Graduate Studies, University of Cyberjaya, Persiaran Bestari, Cyberjaya 63000, Selangor, Malaysia; kee@cyberjaya.edu.my
[4] Biorefinery and Bioprocess Engineering Laboratory, Department of Chemical Engineering and Materials Science, Yuan Ze University, Chungli, Taoyuan 320, Taiwan
[5] Graduate School of Biotechnology and Bioengineering, Yuan Ze University, Chungli, Taoyuan 320, Taiwan
* Correspondence: grraceng@cyberjaya.edu.my (H.S.N.); lanchiwei@saturn.yzu.edu.tw (J.L.)

**Abstract:** Ectoine is an osmoregulatory compound synthesized by halophiles which has attracted attention in the biotechnology, pharmaceuticals, and cosmetics industries due to its stabilizing and protective properties. Conventional methods for ectoine recovery are complex, costly, and often result in low yields. Therefore, there is a growing interest in exploring simple and cost-effective strategies for ectoine recovery. The aqueous biphasic system (ABS) has been employed for the recovery and purification of numerous biocompounds, but the study of low-molecular weight compounds partitioning in ABS remains limited. This study aimed to investigate the feasibility of alcohol–salt ABS for ectoine recovery from *Halomonas salina* DSM5928$^T$ cells. The influences of types and compositions of phase-forming components, crude load concentration, pH, and adjuvants on ectoine recovery were evaluated. Results revealed that ectoine favoured partitioning into the salt-rich bottom phase of alcohol–salt ABS owing to its inherent hydrophilic characteristic. ABS consisting of 16% (*w*/*w*) 1-propanol, 20% (*w*/*w*) sulphate at pH 6.0, 30% (*w*/*w*) crude load, and 1% (*w*/*w*) sodium chloride resulted in a partition coefficient ($K_E$) of 9.61 ± 0.05 and a yield ($Y_B$) of 97.50% ± 0.21. A purity ($P_E$) of 86.73% was achieved with the 1-propanol-sulphate ABS. Alcohol–salt ABS proved to be an effective approach for ectoine recovery, meeting the raising market demand for industrial applications.

**Keywords:** aqueous biphasic system; alcohol; salt; ectoine; recovery



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## 1. Introduction

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) is a hydrophilic compound produced by halophilic bacteria as a means of adapting to high osmotic pressure in highly saline environments [1]. Its primary function is to serve as an osmoregulatory compound, protecting cells from dehydration under extreme conditions. Additionally, ectoine offers protection against various stressors, such as UV radiation, heat, freezing, and inflammation [2,3]. Moreover, ectoine acts as a stabilizer for intracellular macromolecules, such as proteins, enzymes, and nucleic acids, thereby enhancing their functionality within cells. These unique properties have attracted significant interest from researchers to explore ectoine's potential application in diverse sectors, including biotechnology, pharmaceuticals, and cosmetics [4]. For instance, ectoine has been incorporated into anti-inflammation creams for the treatment of dermatitis. Clinical trials have revealed that the inclusion of 2% ectoine in skincare products can effectively slow down skin aging, as well as improve skin hydration, skin elasticity, and the overall skin surface structure [5]. Thus, there is a need for

the development of efficient production and downstream processing strategies to meet the increasing demand for this valuable compatible solute.

Ectoine can be chemically synthesized, but the large-scale production of highly purified and stereo-specific ectoine is challenging and costly due to the high cost of precursors [5,6]. The biotechnological approaches have been developed as an alternative for the production of ectoine using halophilic bacteria. Various species of *Halomonas*, including *Halomonas elongata*, *Halomonas salina*, and *Halomonas boliviensis*, are typically employed in the fermentation process to produce ectoine. The production process involves the fermentation of these halophiles, followed by microfiltration, osmotic downshock, and purification. Downstream processing techniques, including electrodialysis, chromatography, and crystallization, are commonly used for ectoine recovery [7]. However, the multiple steps in downstream processing are often associated with increased production costs and reduced overall yield of ectoine, presenting a significant challenge. Furthermore, it is important to promptly recover ectoine from the fermentation broth to prevent depletion, as the compound also serves as a nutrient source for the bacteria [8].

Aqueous biphasic system (ABS) is an effective alternative to conventional purification and separation methods that involves the formation of two immiscible liquids. It is typically comprised of two different polymers: a polymer with water-soluble salt, alcohol with water-soluble salt, or an ionic liquid with water-soluble salt [9,10]. ABS offers several advantages over conventional liquid–liquid extraction methods by reducing the use of toxic, volatile, and hazardous chemicals that can be harmful to human health. It generally utilizes biocompatible components, mainly consisting of water, minimizing the risk of toxicity and potential protein denaturation that are often associated with organic solvents [11,12]. Moreover, ABS is easy to scale up and can be coupled with other purification technologies, such as precipitation, chromatography, magnetic particle adsorption, and packed bed column, to enhance the efficiency of separation processes [7,13]. Thus, ABS offers a cost-effective approach by combining clarification, concentration, and purification into a single stage. This reduces the number of downstream processing unit operations and, ultimately, lowers the overall production cost [14].

ABS has been extensively employed for the recovery and purification of various bio-products, such as proteins, cells, viruses, organelles, genetic materials, and secondary metabolites. However, there is still lack of research on the partitioning behaviour of low-molecular weight compounds in ABS. Previous studies have explored the use of poly(propylene) glycol (PPG) 425-sulphate ABS and ionic liquid-based ABS for the intracellular ectoine recovery, achieving recovery yields of 94.7% and 96.32%, respectively [8,15]. Although ionic liquids are considered green solvents and PPG is highly biodegradable, their high costs and viscosities may present challenges in large-scale processes. An alternative approach, utilizing alcohol-salt ABS, offers a more cost-effective solution. The lower viscosity of alcohol–salt ABS can facilitate a faster phase formation and improve the mass transfer of solute, thereby enhancing the efficiency of the recovery process [16]. Additionally, the alcohol component can undergo recycling through evaporation, and the salt component can be recycled via dilution crystallization methods following the ABS recovery process. This approach could reduce the cost associated with phase-forming components and alleviate the generation of chemical waste [17]. In view of this, the current study aims to explore the efficacy of alcohol–salt ABS for the recovery of intracellular *Halomonas salina* ectoine. Various parameters of alcohol–salt ABS, including the types and compositions of phase-forming alcohol and salt, crude load concentration, pH, as well as the types and concentrations of adjuvants, were examined.

## 2. Materials and Methods

### 2.1. Materials

The bacterial strain, *Halomonas salina* DSM 5928$^{T}$ was obtained from Taiwan's Food Industry Research and Development Institute (FIRDI). Difco Marine Broth 2216, glycerol (≥99%), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium

sulphate, ectoine standard, monosodium glutamate, glucose, magnesium sulphate heptahydrate, manganese (II) sulphate tetrahydrate, calcium chloride, sodium chloride (NaCl), potassium chloride (KCl), 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF4), 1-ethyl-3-methylimidazolium tetrafluoroborate ([Emim]BF4), 1-butyl-3-methylimidazolium bromide ([Bmim]Br), and 1-ethyl-3-methylimidazodium bromide ([Emim]Br) were supplied by Sigma-Aldrich (St. Loius, MO, USA). Absolute ethanol (≥99.8%), 1-propanol, and 2-propanol were purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical grade, unless otherwise stated.

### 2.2. Bacterial Cultivation and Fermentation

For *H. salina* cell cultivation, 5 mL of cells-containing glycerol stock was added to 45 mL of Difco Marine Broth 2216 with a concentration of 37.4 g/L in a 250 mL Erlenmeyer flask [8]. The flask was placed in a shake flask incubator for 24 h at 30 °C and 200 rpm. Subsequently, the inoculum (10% (*v*/*v*)) was transferred into the cultivation medium comprised of 220 g/L glucose, 100 g/L monosodium glutamate, 30 g/L sodium chloride, 0.4 g/L magnesium sulphate heptahydrate, 0.01 g/L manganese (II) sulphate tetrahydrate, 2.2 g/L dipotassium hydrogen phosphate, and 0.8 g/L potassium dihydrogen phosphate. The cells were grown at 30 °C and 200 rpm for 60 h.

### 2.3. Intracellular Ectoine Extraction

Intracellular *H. salina* ectoine was retrieved using the osmotic downshock method [8]. Following the centrifugation of the cell-containing fermentation medium (10,000 rpm, 10 min) and the subsequent removal of the supernatant, the resulting cell pellets were resuspended in ultrapure water at a ratio of 1:1. To disrupt the cells and facilitate the release of intracellular ectoine, the cell suspension was subjected to sonication using an ultrasonic waterbath (Elmasonic P60H, Elma Schmidbauer GmbH, Singen, Germany) for 30 min, followed by another round of high-performance centrifugation (10,000 rpm, 10 min). The crude ectoine-containing supernatant was then harvested and kept at 4 °C for further investigations.

### 2.4. Ectoine Recovery in ABS

To prepare the alcohol–salt ABS, the binodal curves were adopted from a previous study [18]. An appropriate weight of alcohol, 40% (*w*/*w*) salt stock solution, and 20% (*w*/*w*) of crude ectoine were mixed. The mixture was then supplemented with ultrapure water until it reached a final weight of 5 g, and it was vortexed to ensure complete homogenization of the components. Subsequently, the mixture was centrifuged to induce phase separation (4000 rpm, 10 min). The top and bottom phases volumes were determined, and samples were collected separately for quantification of the ectoine concentration.

### 2.5. Ectoine Quantification

High-Performance Liquid Chromatography (HPLC) (Agilent Technology, Santa Clara, CA, USA) was employed to quantify ectoine concentration using the Eclipse Plus C18 column (150 × 4.6 mm, 5 µm particle size). The elution of the phase sample solution was carried out isocratically using ultrapure water, added with 5 mM calcium chloride, as the mobile phase. The entire process was conducted at a temperature of 30 °C, with a constant flow rate of 0.3 mL/min for the delivery of eluent into the system. The ectoine concentration in the samples was detected and quantified using the UV-Visible HPLC detector at 210 nm. A sample injection volume of 10 µL was used for the analysis [19].

### 2.6. Calculations

The partition coefficient of ectoine, $K_E$ was determined using Equation (1):

$$K_E = \frac{[Ectoine]_B}{[Ectoine]_T} \quad (1)$$

where $[Ectoine]_B$ and $[Ectoine]_T$ represent the ectoine concentration (mg/L) in the salt-rich bottom phase and alcohol-rich top phase, respectively.

The recovery yield, $Y_B$ of ectoine was quantified using Equation (2):

$$Y_B = \frac{M_B}{M_I} \times 100\% \quad (2)$$

where $M_B$ and $M_I$ are the ectoine content (mg) in the salt-rich bottom phase and the crude feedstock, respectively.

The purity, $P_E$, of ectoine was determined using Equation (3):

$$P_E = \frac{A_B}{A_C} \times 100\% \quad (3)$$

where $A_B$ and $A_C$ denote the area shown by a sample of the salt-rich bottom phase and crude feedstock, respectively.

### 2.7. Statistical Analysis

The results were presented as mean ± standard deviation of duplicate measurements. Additionally, one-way ANOVA was performed using IBM SPSS Statistics V22.0 software.

## 3. Results and Discussion

### 3.1. Effect of Types of Phase-Forming Alcohol and Salt on Ectoine Recovery

The influence of different types of alcohols and salts on ectoine recovery in ABS was examined, and the results are presented in Table 1. The compositions of alcohol and salt were maintained within a similar range for 1-propanol-based ABS and 2-propanol-based ABS, except for ethanol-based ABS. Due to its higher polarity, ethanol needed a higher concentration of salt to achieve the formation of ABS. However, the recovery of ectoine by using ABS formed with higher concentrations of propanol and salt was not feasible as it resulted in the precipitation of the target compound [18].

**Table 1.** Effect of types of phase-forming alcohols and salts on ectoine recovery.

| Alcohol-Salt ABS | | Phase Composition, % (*w*/*w*) | | Partition Coefficient, $K_E$ | Yield, $Y_B$ (%) |
|---|---|---|---|---|---|
| **Alcohol** | **Salt** | **Alcohol** | **Salt** | | |
| Ethanol | Phosphate | 22 | 18 | 0.09 ± 0.03 $^{a}$ | 5.72 ± 2.01 $^{a}$ |
| | | 24 | 19 | 0.10 ± 0.00 $^{a}$ | 5.89 ± 0.27 $^{a}$ |
| | | 26 | 20 | 0.11 ± 0.01 $^{a}$ | 6.78 ± 0.55 $^{a}$ |
| | Sulphate | 22 | 18 | 1.16 ± 0.04 $^{a,b}$ | 72.94 ± 0.94 $^{d,e,f}$ |
| Ethanol | | 22 | 20 | 1.40 ± 0.12 $^{a,b,c}$ | 68.44 ± 8.20 $^{c,d,e}$ |
| | | 24 | 22 | 1.35 ± 0.00 $^{a,b,c}$ | 59.47 ± 2.76 $^{b,c,d}$ |
| 1-propanol | Phosphate | 18 | 14 | 3.24 ± 0.85 $^{b,c,d}$ | 89.72 ± 1.72 $^{g}$ |
| | | 20 | 16 | 2.90 ± 1.51 $^{b,c,d}$ | 82.57 ± 8.80 $^{e,f,g}$ |
| | | 22 | 18 | 1.68 ± 0.98 $^{a,b,c}$ | 73.04 ± 9.33 $^{d,e,f}$ |
| | Sulphate | 18 | 14 | 4.70 ± 0.91 $^{d}$ | 94.86 ± 0.94 $^{g}$ |
| 1-propanol | | 20 | 16 | 3.57 ± 0.12 $^{b,c,d}$ | 89.29 ± 0.32 $^{f,g}$ |
| | | 22 | 18 | 3.72 ± 0.32 $^{c,d}$ | 88.74 ± 0.85 $^{f,g}$ |
| 2-propanol | Phosphate | 18 | 14 | 1.06 ± 0.04 $^{a,b}$ | 47.41 ± 0.97 $^{b}$ |
| | | 20 | 16 | 1.12 ± 0.12 $^{a,b}$ | 52.62 ± 1.26 $^{b,c}$ |
| | | 22 | 18 | 1.15 ± 0.05 $^{a,b}$ | 56.71 ± 1.09 $^{b,c,d}$ |
| | Sulphate | 18 | 14 | 1.94 ± 0.03 $^{a,b,c}$ | 89.72 ± 1.41 $^{g}$ |
| 2-propanol | | 20 | 16 | 2.68 ± 0.73 $^{b,c,d}$ | 85.82 ± 3.31 $^{f,g}$ |
| | | 22 | 18 | 3.30 ± 1.24 $^{b,c,d}$ | 84.42 ± 6.49 $^{e,f,g}$ |

Data were expressed as mean ± standard deviation, $n$ = 2. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

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The efficiency of ectoine recovery was significantly influenced by the type of phase-forming alcohol used, as reflected by $K_E$ and $Y_B$. In all the studied ABS, ectoine was found to be predominantly partitioned into the salt-rich bottom phase with $K_E$ greater than 1 recorded, except for the ethanol-based ABS ($K_E < 1$), which promoted the partitioning of ectoine into the alcohol-rich top phase. Among the alcohols investigated, 1-propanol demonstrated the highest $K_E$ and $Y_B$, followed by 2-propanol and ethanol. When comparing ABSs with the same concentration of 22% ($w/w$) alcohol and 18% ($w/w$) phosphate or sulphate salt, it showed that 1-propanol-based ABS exhibited greater $K_E$ and $Y_B$ of ectoine than that of 2-propanol-based ABS and ethanol-based ABS. This is owing to the longer chain length and higher hydrophobicity of propanol compared to ethanol, with 1-propanol exhibiting better hydrophobicity than 2-propanol [20]. The increased hydrophobicity of 1-propanol increases the movement of ectoine towards the salt-rich bottom phase, resulting in improved $K_E$ and $Y_B$.

Ectoine with hydrophilic surface properties displays a greater affinity for the hydrophilic bottom phase enriched with salt in the sulphate-based ABS [21]. This attributes to the presence of hydrophilic functional groups, including amino and hydroxyl groups, which facilitate the interactions with water molecules. The efficiency of ABS for the recovery of ectoine was also notably influenced by the type of phase-forming salt used. As shown in Table 1, ectoine demonstrated higher $K_E$ and $Y_B$ in sulphate-based ABS compared to phosphate-based ABS. For instance, at the same composition of 18% ($w/w$) 1-propanol and 14% ($w/w$) salt, 1-propanol-sulphate ABS achieved the greatest $K_E$ of 4.70 ± 0.91 and $Y_B$ of 94.86% ± 0.94, whereas 1-propanol-phosphate ABS yielded a lower $K_E$ of 3.24 ± 0.85 and $Y_B$ of 89.72% ± 1.72. A similar trend was observed in 2-propanol-based ABS with the same phase composition, as 2-propanol-sulphate ABS ($K_E$ = 1.94 ± 0.03, $Y_B$ = 89.72% ± 1.41) exhibited significantly higher recovery efficiency than 2-propanol-phosphate ABS ($K_E$ = 1.06 ± 0.04, $Y_B$ = 47.41% ± 0.97). This partitioning behaviour can be attributed to the phase-forming salts' salting-out ability. Sulphate salts with greater salting-out ability and ionic strength, compared to phosphate salts, increase the hydrophilicity of salt-rich bottom phase because of their stronger molecular interactions with water molecules [22]. Consequently, sulphate-based ABS exhibits a larger hydrophobicity difference between the two aqueous phases, facilitating a higher amount of ectoine transfer into the salt-rich bottom phase. Thus, 1-propanol-sulphate ABS was constructed in the subsequent experiments for ectoine recovery due to its highest recovery efficiency among the investigated alcohol-salt ABS.

### *3.2. Effect of Concentration of Phase-Forming Alcohol and Salt on Ectoine Recovery*

The impact of varying the composition of 1-propanol (16–22% ($w/w$)) and sulphate salt (14–20% ($w/w$)) on ectoine recovery was investigated (Table 2). Overall, there was a decreasing trend observed in the efficiency of ABS, for ectoine recovery, with an increase in the 1-propanol concentration while keeping the sulphate salt concentration constant. This effect was most pronounced when the concentration of 1-propanol was raised from 16% ($w/w$) to 22% ($w/w$) at a constant salt concentration of 18% ($w/w$) and 20% ($w/w$), respectively. Specifically, $K_E$ was decreased by about one-fold from 6.99 ± 1.28 to 3.72 ± 0.32 and 7.20 ± 0.57 to 4.22 ± 1.03. Moreover, an increase in the volume ratio was observed as the 1-propanol concentration was elevated from 16% ($w/w$) to 20% ($w/w$). This phenomenon occurs because the salt-rich bottom phase undergoes gradual dehydration as the 1-propanol concentration in ABS increases [18,23]. As the salt-rich bottom phase volume decreases, there are fewer available water molecules in that phase, reducing its overall hydrophilicity and resulting in a lower recovery efficiency of ectoine. Similar partitioning behaviour was reported in the extraction of *Lilium davidiivar var* polysaccharides using alcohol/salt ABS [22].

**Table 2.** The effect of the concentration of phase-forming alcohol and salt on ectoine recovery.

| Phase Composition, % (*w*/*w*) | | Partition Coefficient, $K_E$ | Yield, $Y_B$ (%) |
|---|---|---|---|
| **Ammonium Sulphate** | **1-Propanol** | | |
| 14 | 16 | 5.28 ± 1.69 [a] | 95.26 ± 1.45 [a,b,c] |
| | 18 | 4.70 ± 0.91 [a] | 94.86 ± 0.94 [a,b,c] |
| | 20 | 4.01 ± 1.80 [a] | 91.20 ± 3.63 [a,b,c] |
| | 22 | 6.03 ± 0.83 [a] | 93.31 ± 0.86 [a,b,c] |
| 16 | 16 | 5.66 ± 1.63 [a] | 95.07 ± 1.36 [a,b,c] |
| | 18 | 3.81 ± 1.13 [a] | 92.84 ± 1.98 [a,b,c] |
| | 20 | 3.57 ± 0.12 [a] | 89.29 ± 0.32 [a,b] |
| | 22 | 4.41 ± 0.32 [a] | 89.95 ± 0.13 [a,b,c] |
| 18 | 16 | 6.99 ± 1.28 [a] | 96.05 ± 1.08 [b,c] |
| | 18 | 5.40 ± 1.47 [a] | 91.80 ± 2.00 [a,b,c] |
| | 20 | 4.21 ± 1.05 [a] | 90.51 ± 2.14 [a,b,c] |
| | 22 | 3.72 ± 0.32 [a] | 88.74 ± 0.85 [a] |
| 20 | 16 | 7.20 ± 0.57 [a] | 96.76 ± 0.15 [b,c] |
| | 18 | 5.33 ± 0.53 [a] | 92.52 ± 0.68 [a,b,c] |
| | 20 | 5.24 ± 1.50 [a] | 90.74 ± 2.41 [a,b,c] |
| | 22 | 4.22 ± 1.03 [a] | 90.08 ± 2.76 [a,b,c] |

Data were expressed as mean ± standard deviation, $n$ = 2. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

Data were expressed as mean ± standard deviation, $n$ = 2. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

The concentration of the phase-forming salt also had a notable impact on the efficiency of ABS for ectoine recovery. Increasing the sulphate salt concentration from 14% (*w*/*w*) to 20% (*w*/*w*) enhanced both the $K_E$ and $Y_B$ of ectoine in ABS with 16% (*w*/*w*) 1-propanol. At 14% (*w*/*w*) sulphate salt, ABS achieved a $K_E$ of 5.28 ± 1.69 and $Y_B$ of 95.26% ± 1.45, and the values were further increased to a $K_E$ of 7.20 ± 0.57 and $Y_B$ of 96.76% ± 0.15 with the increase in sulphate concentration to 20% (*w*/*w*). The higher sulphate salt concentration facilitates the transfer of the hydrophilic ectoine to the salt-rich bottom phase because of the enhanced solubility of ectoine in the salt-rich bottom phase, which has a higher ionic strength [24]. Nevertheless, it was observed that, at higher 1-propanol concentrations (22% (*w*/*w*), the increase in sulphate salt concentration from 14% (*w*/*w*) to 20% (*w*/*w*) had an adverse effect on the recovery efficiency of ectoine, resulting in lower $K_E$ and $Y_B$. Additionally, precipitation at the interphase was also observed when the sulphate concentration exceeded 20% (*w*/*w*). Hence, the subsequent experiment proceeded with ABS consisting of 16% (*w*/*w*) 1-propanol and 20% (*w*/*w*) sulphate, which yielded the highest $K_E$ and $Y_B$.

### *3.3. Effect of Crude Load Concentration on Ectoine Recovery*

The impacts of crude load concentration, differing from 5% (*w*/*w*) to 30% (*w*/*w*), on ectoine recovery in 1-propanol-sulphate ABS are displayed in Figure 1. The $K_E$ and $Y_B$ improved as the crude load concentration increased in the ABS. For instance, $K_E$ increased from 4.78 ± 0.45 to a maximum of 9.46 ± 0.08, and $Y_B$ increased from 94.76% ± 0.47 to a maximum of 97.29% ± 0.02 at 30% (*w*/*w*) as the crude load concentration was increased from 5% (*w*/*w*) to 30% (*w*/*w*). A higher concentration of crude load in ABS results in a greater amount of the target product being recovered [25].

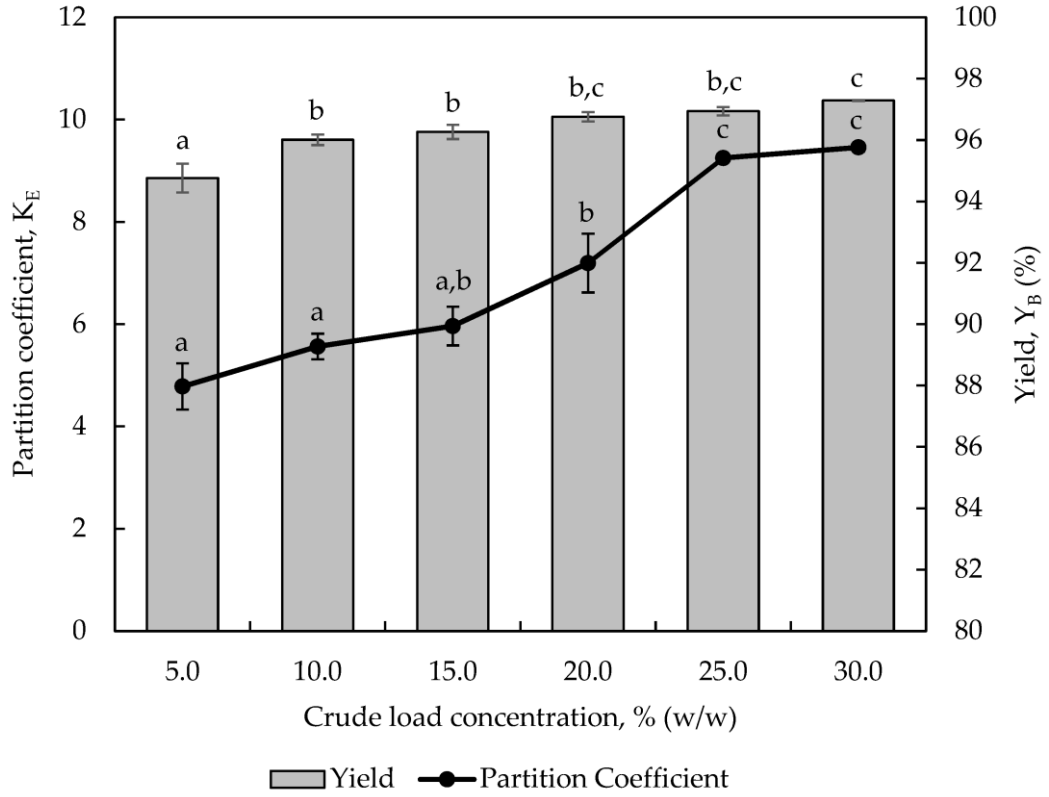


**Figure 1.** The effect of crude load concentration on ectoine recovery. The partition coefficient, $K_E$, and recovery yield, $Y_B$, of ectoine in the salt-rich bottom phase were evaluated in ABS, comprised of 16% ($w/w$) 1-propanol and 20% ($w/w$) sulphate, with a crude load concentration ranging from 5% ($w/w$) to 30% ($w/w$). Data were expressed as mean ± standard deviation, $n$ = 2. Values with different superscript letters within the same legend are significantly different ($p < 0.05$).

Additionally, increasing the crude load concentration can modify the phase volume ratio of the ABS [26]. In the partitioning experiment, it was observed that ABS with a 30% ($w/w$) crude load exhibited a 30% lower volume ratio compared to ABS with a 5% ($w/w$) crude load. As a result, there is an increase in the available free volume in the salt-rich bottom phase, allowing for the accumulation of ectoine and leading to the maximum $K_E$ and $Y_B$. Nevertheless, as the crude load concentration was above 30% ($w/w$), the loss of ectoine at the interphase was observed. Higher crude load concentration often introduces a higher amount of impurities into the system, which can affect the phase composition of ABS and lead to phase saturation [25]. Therefore, a maximum crude load concentration of 30% ($w/w$) was determined to be optimal for the ectoine recovery.

### *3.4. Effect of pH on Ectoine Recovery*

The net charges on the surface of biomolecules can be manipulated by adjusting the pH of an ABS, which, in turn, facilitates their partitioning into the desired phase of the ABS [5,27]. To examine the influence of pH on ectoine recovery, ABS consisting of 16% ($w/w$) 1-propanol and 20% ($w/w$) sulphate, loaded with 30% ($w/w$) crude, was constructed. The system pH varied from pH 5.0 to 8.0, and the recorded $K_E$ and $Y_E$ of ectoine are presented in Figure 2.

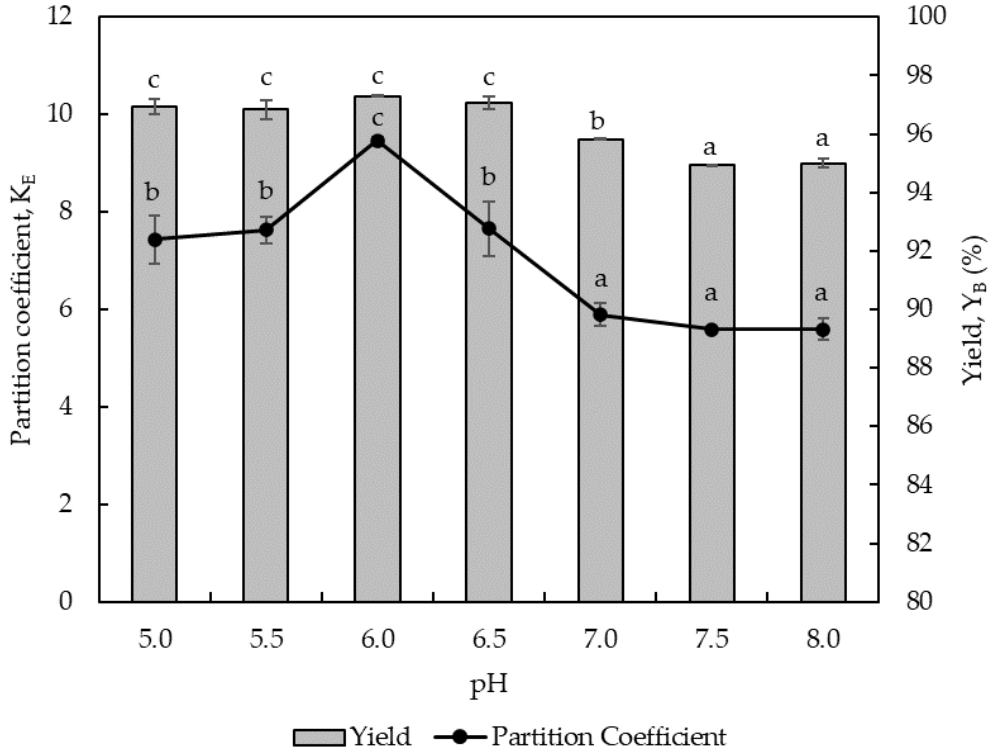


**Figure 2.** The effect of pH on ectoine recovery. The partition coefficient, $K_E$, and recovery yield, $Y_B$, of ectoine in the salt-rich bottom phase were evaluated in an ABS, comprised of 16% ($w/w$) 1-propanol, 20% ($w/w$) sulphate, and 30% crude, at system pH ranging from pH 5.0 to pH 9.0. Data were expressed as mean ± standard deviation, $n$ = 2. Values with different superscript letters within the same legend are significantly different ($p < 0.05$).

The results indicated that increase in pH from pH 5.0 to 6.0 improved the $K_E$ of ectoine from 6.93 ± 0.81 to a maximum of 9.46 ± 0.08. However, the $Y_B$ remained relatively constant above 96%, as the pH was increased from pH 5.0 to pH 6.5. Ectoine only exhibits cationic characteristics at low pH levels (pH < 2) due to the protonation of carboxylate anions (-$COO^-$) into carboxyl groups (-COOH) [28,29]. At near-neutral pH, ectoine exists as a zwitterionic molecule with zero net charge, and its partitioning in the ABS is mainly governed by hydrophobic interactions with the 1-propanol molecules rather than electrostatic interactions [8,30].

However, the recovery efficiency of ectoine gradually decreased as the system pH increased, and the lowest $K_E$ of 5.59 ± 0.23 and $Y_B$ of 94.99% ± 0.16 were recorded at pH 8.0. This reduction can be due to the acquisition of a net negative surface charge by ectoine, through the deprotonation of $NH_3^+$ ions, to form amine groups (-$NH_2$) [15,30]. The negatively charged ectoine molecules repel sulphate ions, hindering the movement of ectoine towards the salt-rich bottom phase and causing a decrease in recovery efficiency. Hence, the highest $Y_B$ of 97.29% ± 0.02 was attained in the 1-propanol-sulphate ABS with a pH of 6.0.

### *3.5. Effect of Type and Concentration of Adjuvants on Ectoine Recovery*

The incorporation of adjuvants, such as ionic liquids and neutral salts, in ABS have been explored to improve the separation of target biomolecules by increasing the electrical potential difference between the two aqueous phases. The influence of different types of ionic liquids ([Bmim]$BF_4$, [Emim]$BF_4$, [Bmim]Br, [Emim]Br) and neutral salts (NaCl, KCl) on ectoine recovery in the alcohol–salt ABS was determined. The imidazolium-based ionic liquids were chosen due to the stability of imidazolium rings under oxidative and reductive conditions, high solubility, low viscosity, and ease of synthesis [31]. Each adjuvant of 1%

(*w*/*w*) was added into the ABS, and the corresponding values of $K_E$ and $Y_B$ are depicted in Figure 3.

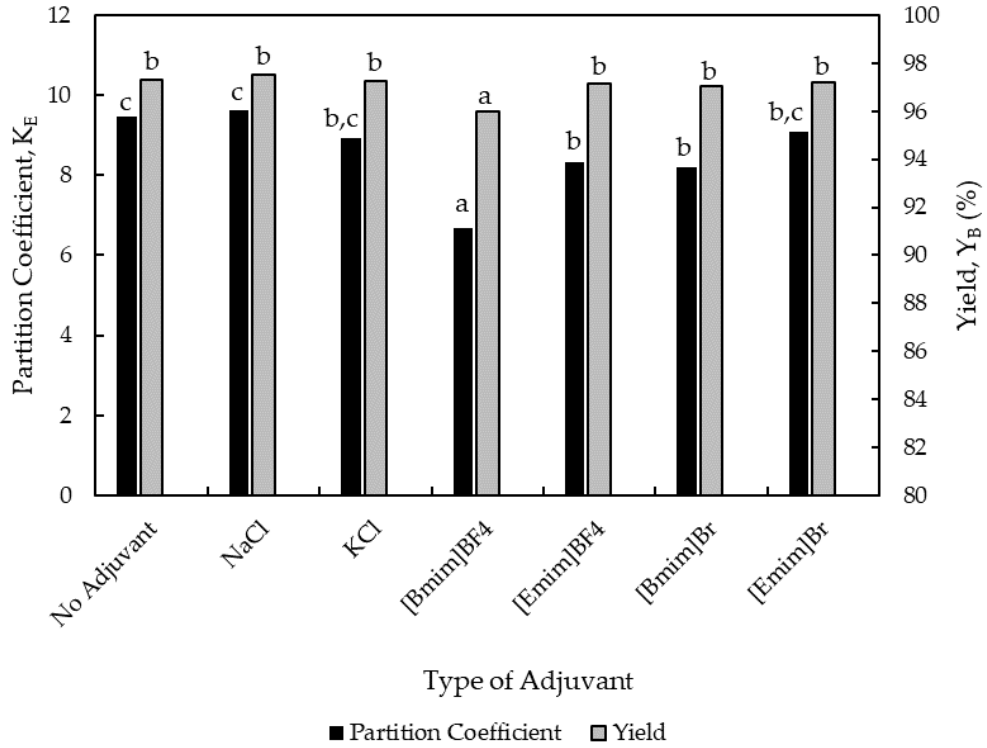


**Figure 3.** The effect of types of adjuvants on ectoine recovery. The partition coefficient, $K_E$, and recovery yield, $Y_B$, of ectoine in the salt-rich bottom phase were evaluated in an ABS of pH 6.0 comprised of 16% (*w*/*w*) 1-propanol, 20% (*w*/*w*) sulphate, and 30% crude, with the addition of 1% (*w*/*w*) of different types of adjuvants. Data were expressed as mean ± standard deviation, $n = 2$. Values with different superscript letters within the same legend are significantly different ($p < 0.05$).

The addition of adjuvants did not significantly improve the recovery efficiency of ectoine compared to ABS without adjuvants. In fact, the addition of ionic liquids had a negative impact on the recovery efficiency, resulting in lower $K_E$ and $Y_B$ of ectoine in ABS containing these additives. Particularly, [Bmim]$BF_4$ exhibited a notable decrease in the $K_E$ (6.69 ± 0.40) and $Y_B$ (95.97% ± 0.13) of ectoine. The partitioning of biomolecules in ABS is governed by ionic liquids through two mechanisms, namely hydrophobic and electrostatic interaction. The decrease in recovery efficiency can be explained by the preferential partitioning of ionic liquids towards the top phase, which leads to the modification of physical and chemical properties of the alcohol-rich top phase [32,33]. The enhanced capability of the alcohol-rich top phase for biomolecule recovery mainly attributes to the presence of benzyl groups or double bonds in the IL cation, forming a strong interaction between ectoine and the ionic liquids [34]. Additionally, ionic liquids with longer alkyl chain length exhibit greater hydrophobicity, thereby causing the transportation of more ectoine, along with the ionic liquid molecules, towards the hydrophobic alcohol-rich top phase [35].

Among the neutral salts investigated, NaCl performed better than KCl in ABS by enhancing the recovery of ectoine in the salt-rich bottom phase compared to ABS without adjuvants. The addition of NaCl resulted in the highest $K_E$ and $Y_B$ of ectoine, with $K_E$ of 9.61 ± 0.05 and $Y_B$ of 97.50% ± 0.21 recorded. This can be attributed to the more favourable electrical interactions and repulsions between NaCl and ectoine in the ABS, facilitating the migration of ectoine to the salt-rich bottom phase. The dissociation NaCl in water leads to the formation of $Na^+$ and $Cl^-$ ions, which are unevenly distributed and interact with water molecules in the ABS. This alteration of water structure enhances the solubility of the

hydrophilic ectoine in the salt-rich bottom phase and strengthens the hydrogen bonding interactions [24].

To investigate the impact of the concentration of NaCl addition on ectoine recovery, different concentrations of NaCl, differing from 0% (*w*/*w*) to 3% (*w*/*w*), were added to the 1-propanol-sulphate ABS (Figure 4). In the presence of 1% (*w*/*w*) NaCl, a slight increase in both the $K_E$ (9.61 ± 0.05) and $Y_B$ (97.50% ± 0.21) was observed compared to the ABS without adjuvant ($K_E$ = 9.46 ± 0.08 and $Y_B$ = 97.29% ± 0.02). The hydrophobic difference between the two aqueous phases improves with the NaCl addition, thereby promoting the repulsion of ectoine towards the salt-rich bottom phase [35,36]. However, a further increase in NaCl concentration, from 1.5% (*w*/*w*) to 3% (*w*/*w*), gradually reduced the recovery efficiency. An ABS with 3% (*w*/*w*) NaCl exhibited the lowest $K_E$ of 8.59 ± 0.12 and $Y_B$ of 95.09% ± 0.37. This decline in recovery efficiency can be attributed to the precipitation that occurred in ABS due to the high neutral salt concentration.

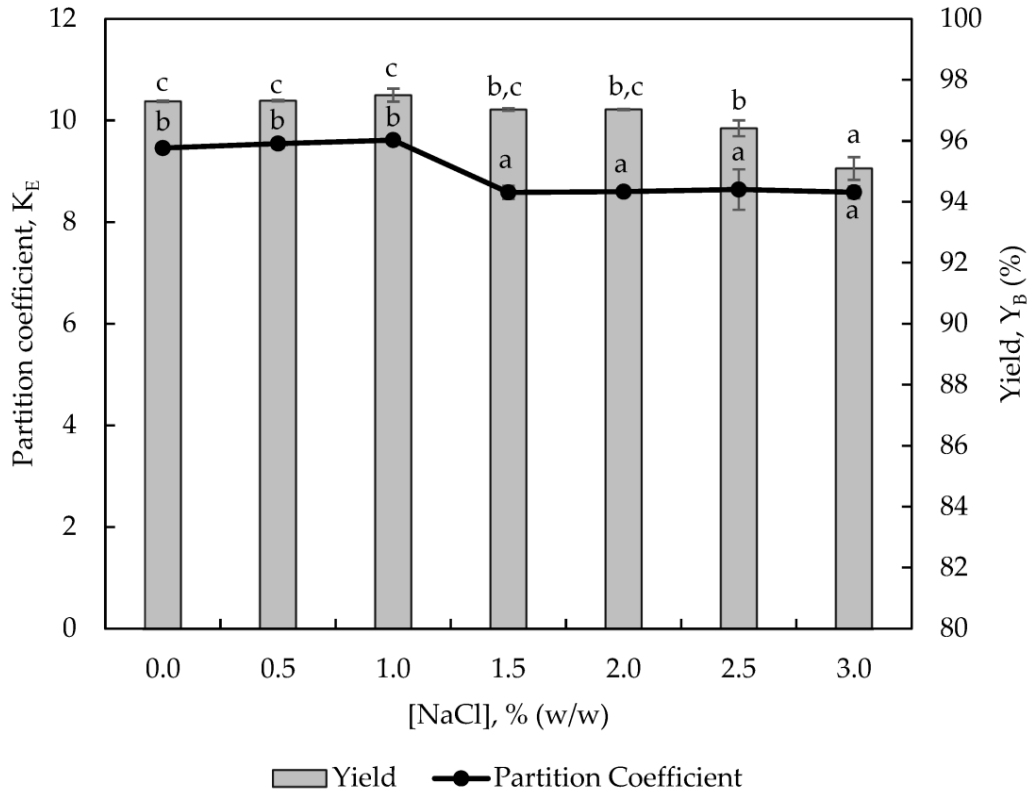


**Figure 4.** The effect of NaCl concentration on ectoine recovery. The partition coefficient, $K_E$, and recovery yield, $Y_B$, of ectoine in the salt-rich bottom phase were evaluated in an ABS of pH 6.0, comprised of 16% (*w*/*w*) 1-propanol, 20% (*w*/*w*) sulphate, and 30% crude, with the addition of NaCl at different concentrations ranging from 0% (*w*/*w*) to 3% (*w*/*w*). Data were expressed as mean ± standard deviation, $n$ = 2. Values with different superscript letters within the same legend are significantly different ($p < 0.05$).

## 4. Conclusions

The recovery of intracellular *H. salina* ectoine, utilizing an alcohol–salt ABS, was successfully accomplished. The highest $K_E$ of 9.61 ± 0.05 and $Y_B$ of 97.50% ± 0.21 were demonstrated in the ABS comprised of 16% (*w*/*w*) 1-propanol and 20% (*w*/*w*) sulphate at pH 6.0, with a crude load concentration of 30% (*w*/*w*) and the addition of 1% (*w*/*w*) NaCl. The 1-propanol-sulphate ABS resulted in the production of ectoine with $P_E$ of 86.73%. Furthermore, it was determined that the addition of ionic liquids had an adverse effect on the efficiency of the 1-propanol-sulphate ABS for ectoine recovery. Alcohol–salt ABS shows great potential for the recovery of ectoine from bacterial cell supernatant, offering a cost-effective approach for industrial-scale production of ectoine. These findings highlight

the practicability and effectiveness of alcohol–salt ABS as a strategy for the recovery of low-molecular weight molecules.

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