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Primary Recovery of Carboxymethyl Cellulase from Thermophilic *Bacillus licheniformis* 2D55 Using an Aqueous Two-phase System

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ABSTRACT

This study uses an aqueous two-phase system developed from a polymer and salt to purify a thermostable carboxymethyl cellulase (CMCase) produced by thermophilic *Bacillus licheniformis* 2D55. The effects of system parameters, such as polyethene glycol (PEG) molar mass, salt concentration, crude load, NaCl concentration and pH on partitioning and recovery efficiency, are evaluated. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used to determine the purity of the CMCase. The enzyme is successfully purified, achieving a 10.9-fold purification and 86.62% yield. The maximum purification condition is achieved in ATPS comprising 20.5% PEG 8000/15% sodium citrate, with a crude load of 17% (w/w), NaCl of 1.0% (w/w) and pH at 7.0. Under these conditions, a partition co-efficient of 0.21 is observed, indicating that CMCase preferentially partitions to the bottom phase. These results demonstrate the potential of ATPS for the purification of thermostable CMCase from the fermentation broth of thermophilic *Bacillus licheniformis* 2D55.

Keywords: Aqueous two-phase system, *Bacillus licheniformis* 2D55, carboxymethyl cellulase, enzyme, partial purification, polyethene glycol

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INTRODUCTION

Cellulases are enzymes involved in the bioconversion of cellulose, which accounts for 40%–50 % of the weight of agro-waste biomass. It catalyses the cleavage of the β-1,4-glycosidic bonds linking the glucose molecule polymer of cellulose into cellodextrins, cellobiose and glucose (Co & Hug, 2020). Among cellulases, carboxymethyl cellulases (CMCase) are of special attention due to their role in initiating the process of cellulose hydrolysis. The global enzyme market receives a significant share of CMCase due to its high demand in industrial processes such as food, feed and beverage (Kumar et al., 2019), waste management (Pham et al., 2022), detergent (Abedi et al., 2022), textile (Vajpayee et al., 2022) and biofuel (Hoffman et al*.,* 2021) industries. They have also found special usefulness in aiding the decomposition of straws to improve soil fertility (Shi et al., 2019) as well as improving animal feed digestibility (Selzer et al., 2021).

A plethora of microorganisms, bacteria and fungi (mesophilic or thermophilic) produce CMCase under induction when grown on agro-waste biomass (Kazeem et al., 2021; Sadida & Manchur, 2021). Interestingly, much attention is now drawn to enzymes from thermophilic bacteria for industrial uses due to their high stability, increased substrate solubility, reduced contamination risk and reduced cooling cost. CMCase from thermophilic bacteria has been reported in some species of *Bacillus* and (Ulucay et al., 2022) *Thermoanaerobacterium* (Harnvoravongchai et al., 2020). However, the purification of this enzyme usually involves multiple laborious chromatographic steps in which high purity and large-scale production are not economically available. Such multiple steps reported for CMCase include ammonium sulphate precipitation and anion exchange diethylaminoethanol (DEAE)-Sepharose (Banerjee et al., 2020), ultrafiltration and column Q Sepharose (Bettache et al., 2021), ammonium sulphate, DEAE-cellulose, Sephadex G-100 (Shankar et al., 2021), ammonium sulphate precipitation, DEAE- Sephadex A-50, ultrafiltration, gel filtration (Sephadex G-100) (Olukunle et al., 2021). Nevertheless, each technique faces limitations, which may include high cost, low purity and low yield. Thus, improvement in downstream processing is necessary since downstream processing accounts for 80 % of the total production cost (Júnior et al., 2020).

The ATPS is widely applied to separate and purify microscopic and macroscopic biological molecules, including enzymes (González-González et al., 2022). This technique is commonly known to yield high-purity products in a single step (Singla & Sit, 2023). The ATPS, which consists of polyethene glycol (PEG) and salt, is widely accepted owing to the varied differences in hydrophobicity between the two phases and its low cost of salt (Saddique et al., 2020). ATPS are formed by mixing polymer, salt and water at appropriate concentrations, forming two immiscible liquids where proteins are separated (Moteshafi et al., 2022). It allows simultaneous extraction, purification and concentration of the target proteins. The polymer-salt system is advantageous over the polymer/polymer system due

to the low viscosity salt phase, low interfacial tension between the phases, higher density and considerable selectivity (Hamta & Dehghani, 2017).

Hence, the present study employed ATPS based on a polymer/salt system, favouring the one-sided partition of compounds due to larger differences in the physico-chemical properties between the two phases. It is valuable during the first purification step when most contaminants must be separated. The most common inorganic salt employed was potassium phosphate. However, using high salt concentrations poses a waste disposal and an environmental problem. This problem could be surmounted by recycling the polymers and salts or by choosing other salts, such as sodium citrate, which is biodegradable and non-toxic. For similar reasons, sodium citrate was selected in this study in the construction of the ATPS as it has a lower eutrophic potential and is more biodegradable compared with other salts (Wang et al., 2022).

Until now, very few studies have reported CMCase purification via ATPS (Ho et al., 2017; Liu et al., 2019). Reports on the purification of CMCase from *Bacillus licheniformis* using ATPS are lacking in the literature so far. The present study, therefore, evaluated the partitioning competence of CMCase from thermophilic *B. licheniformis* 2D55. The appropriate ATPS composition suitable for the separation of CMCase was determined using varying PEGs and salts. Furthermore, factors such as the pH of ATPS, crude enzyme load, and sodium chloride concentration (NaCl) that affect the efficiency of ATPS for the recovery of CMCase were investigated to maximise CMCase purification.

MATERIALS AND METHODS

Supplies

PEGs of different molar mass (MM) ranging from 4000 g/mol to 10,000 g/mol were supplied by Sigma-Aldrich (St. Lious, USA). Potassium dihydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), ammonium sulphate $(NH_4)_2SO_4$, magnesium sulphate ($MgSO_4$), sodium citrate ($NaH_2C_6H_5O_7$), citric acid ($C_6H_8O_7$), sodium acetate (CH_3COONa), hydrochloric acid (HCL), acetic acid (CH_3COOH) and sodium hydroxide (NaOH) were sourced from Merck (Darmstadt, Germany). The bovine serum albumin and the bicinchronic acid (Pierce™) kit for protein assay were supplied by ThermoScientific (Rockford, USA).

Bacterial Strain and Carboxymethyl Cellulase Production

A thermostable CMCase-producing *Bacillus licheniformis* 2D55 isolated from hot compost was used (Kazeem et al., 2017). The CMCase production medium contains the following in submerged fermentation (g/L): urea, 1.0; peptone, 11.0; $MgSO_4.7H_2O$, 0.40; and $CaCl_2.2H_2O$, 0.03; Tween 80, 0.2% (w/v). The carbon source was prepared from a mixture of rice husk and sugarcane bagasse, 7% (w/v), with the final pH adjusted to 3.5

while agitation was set at 180 rpm (Kazeem et al., 2016). The bacterial cells were set at OD_{600} = 1.0, and 3% (v/v) bacteria cells were transferred into the 100 mL production medium and used as an inoculum. After 18 h of shaking at 50°C, the fermentation broth was centrifuged at 2,860 × *g*, 4°C for 10 min. A clear top layer (Crude enzyme) was gently withdrawn for storage at 4°C.

Assay for Carboxymethyl Cellulase

CMCase activity was carried out as Wood and Bhat (1988) described with minor variation. Briefly, 0.5 mL of the crude enzyme was mixed with 0.5 mL of 1% (w/v) carboxymethyl cellulose (CMC) in 0.5 mM sodium phosphate buffer (pH 7.5). The concoction was allowed to stay in a water bath (30 min; 50°C). Reducing sugar liberated was analysed following the dinitro salicylic acid method (Miller, 1959). A unit of CMCase enzyme activity was defined as the amount of enzyme that reacted with the substrate to release µ1mol of glucose/ min at the stated condition of the assay

Bicinchronic Acid Assay (BCA Assay)

The BCA kit with the bovine serum albumin (BSA) standard was employed to analyse protein concentration. A 0.1 mL enzyme was added to a 2 mL BCA reagent. The mixture was then incubated in a 37°C water bath for 30 min. The intensity of the resulting colour was determined by a spectrophotometer (SECOMAN, 30100 Ales, France) at 562 nm wavelength. The protein concentration of the samples was obtained from the BSA standard curve.

Salt Selection for ATPS

The stability of CMCase with five different salts: magnesium sulphate (MgSO4), di-potassium phosphate (K_2HPO_4), ammonium sulphate $(NH_4)_2SO_4$, sodium citrate ($Na_3C_6H_5O_7$), sodium acetate (CH_3COONa) was examined. The system pH was maintained at 7.0. The pH of the phosphate salt was adjusted with potassium di-hydrogen phosphate. Magnesium sulphate and ammonium sulphate were adjusted with sodium hydroxide and hydrochloric acid. In contrast, acetic acid and citric acid were used to adjust the pH of sodium acetate and sodium citrate, respectively. Different salt concentrations were mixed with the crude enzyme for 1 h, after which residual CMCase activities were determined to select the salt preferable for ATPS.

ATPS Studies

The ATPS were studied from 50% (w/w) stock of PEG solution of different molar mass (MM) (4000 g/mol, 6000 g/mol, 8000 g/mol and 10,000 g/mol) and sodium citrate stock solution [40% (w/w)]. The ATPS was prepared in a 15 ml centrifuge tube. The amount of the PEG solution, sodium citrate solution and distilled water was weighed and mixed with

crude enzyme at 14% (w/w) to form a 10 g system of pH 7. The solution was mixed using a vortex for 10 min and centrifuged at 2860 × *g* for 10 min to achieve a phase separation. The phase separation of PEG at the top phase and sodium citrate at the bottom phase was completed by allowing the tubes to stand for 10 min. Finally, samples from both phases were collected for analysis of CMCase activity as well as the overall protein concentration.

Determination of Partition Coefficient (K), Purification Factor (P_F) and Yield (Y *%)*

The CMCase coefficient of partition (K) was calculated using Equation 1 to extract the proportion of CMCase activities from the two phases.

$$K = \frac{A_T}{A_B} \quad [1]$$

Where, A_T and A_B are the equilibrium of the CMCase activities (U/mL) at the top and bottom phases.

Specific activity (SA) of CMCase is the ratio between CMCase activities (U/ml) in the phase sample to the total protein concentration (mg) following Equation 2.

$$SA\left(\frac{U}{mg}\right) = \frac{cellulase\ activity\ \left(\frac{U}{mL}\right)}{Protein\left(\frac{mg}{mL}\right)} \quad [2]$$

Purification factor (P_F) is the ratio of the specific CMCase activity (U/mg) of the recovered CMCase activity at the bottom phase to the original specific CMCase activity in the crude feedstock, calculated according to Equation 3

$$P_F = \frac{SA\ of\ phase\ sample}{SA\ of\ crude\ feed\ stock} \quad [3]$$

The CMCase activity yield from the bottom phase in the bottom phase was determined as illustrated in Equation 4

$$Yield\ (\%) = \frac{Enzyme\ activity\ in\ bottom\ phase\ \times\ volume\ of\ the\ bottom\ phase}{Enzyme\ activity\ in\ x\ \ supernatant\ \times\ volume\ of\ \ supernatant} \times 100 \quad [4]$$

Sodium Dodecyl Sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The electrophoresis chamber (Bio Rad, USA) was used for this analysis. The SDS-PAGE investigation was executed using an electrophoresis unit, according to Abbasiliasi et al. (2014). The bottom phase sample was precipitated with trichloroacetic acid at 10 % (w/v).

A desalting column was used to remove the salt, while a protein concentrator with a 10 kilo Dalton (kDa) cut-off (Thermo Scientific, Massachusetts, US) was used to concentrate the protein. The concentrated samples were obtained after centrifugation (12,100 × *g*, 4°C, 20 min). Thereafter, 20 µl precipitate was suspended in 5 µl denaturing buffer (100mM Tris-HCL pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and bromophenol blue). The mixture was loaded onto the gel after boiling for 10 min. The SDS-PAGE was carried out according to Laemmli (1970) by loading protein samples onto solidified gel containing 12% resolving gel [2.5mL 1.5 M Tris HCL (pH 8.8), 100 µL 10% SDS, 3.0 mL 40% (w/v) Bis-acrylamide solution, 100 µL ammonium per sulphate (APS) and 5 µL tetramethylethylenediamine (TEMED), 4.3 mL dH_2O] and 5% stacking gel [0.5 mL of 1M Tris-HCL, 0.5 mL 40% Bis-acrylamide, 40 µL 10% SDS, 40 µL APS, 2.920 dH_2O, 5 µL TEMED]. The gel was run at 180 V for 40 min. Staining commenced with the Coomassie Brilliant Blue, followed by distaining, and the resulting band was viewed.

RESULTS AND DISCUSSION

The Effect of the ATPS Salt on Carboxymethyl Cellulase Activity

Results from the influence of salt concentrations (5%–20 %) on the CMCase activity were as shown in Table 1. Increasing the concentration of the salt resulted in a decreased enzyme activity among all the salts tested. However, sodium citrate at 5% and 10% concentrations yielded 28.59 U/mL and 26.28 U/mL of CMCase activity, respectively. It was followed by 5% sodium phosphate (26.55 U/mL). The CMCase activity was strongly inhibited by magnesium sulphate. This may be due to protein denaturation by aggregation (Torosyan & Shoichet, 2019). When sodium acetate was used as a separating salt, the activity of CMCase decreased. The use of high concentrations of salt in ATPS poses a disposal problem. Hence, the problem can be addressed by either recycling salt or choosing non-toxic and biodegradable salt. The stability of CMCase in sodium citrate is advantageous to this study (Ma et al., 2018; Ng et al., 2011). Hence, sodium citrate was selected as the salt of choice for subsequent studies.

Table 1
Selection of ATPS bottom salt for carboxymethyl cellulase activity

Salt	Concentration (%, w/w)	CMCase activity (U/mL)
Sodium phosphate	5	26.55 ± 0.62
	10	25.38 ± 0.56
	20	22.93 ± 0.21
Magnesium sulphate	5	21.53 ± 0.09
	10	19.15 ± 0.31
	20	15.45± 0.18
Ammonium sulphate	5	26.41 ± 0.43
	10	25.06 ± 0.46
	20	21.51 ± 0.28
Sodium citrate	5	28.59 ± 0.21
	10	26.28 ± 0.43
	20	21.49 ± 0.53
Sodium acetate	5	25.55 ± 0.51
	10	23.98 ± 0.31
	20	20.71 ± 0.21

Influence of PEG Molar Mass, Curde Load, pH, and NaCl on Carboxymethyl Cellulase Partitioning Behaviour

The current work did not use the design of experiments to maximise CMCase purification in the partitioning experiment. Variables affect the ATPS, and interactions between the combinations of variables impact the response either positively or adversely (for example, yield, PF, and K). Significant variables that are screened have a big impact on the outcome. The ATPS partitioning of CMCase employing a factorial design will provide more accurate results and potential interdependencies between the variables.

The selection of a suitable PEG molar mass is usually the first step required in ATPS for protein recovery. The PEG molar mass decides the extent of the partitioning of the target protein and impurities in the extract. It has been previously demonstrated that the effectiveness of the extraction is largely influenced by the phase-forming molecules and the number of polymer-protein interactions, which is governed by the degree of polymerisation of the polymer (Iqbal et al., 2016). The influence of the PEGs on the partitioning behaviour of the phase system was observed in Table 2. Table 2 shows that the partition coefficient (K) was less than 1 (i.e. $K < 1$) in all the PEG /citrate examined, ranging from 0.32 to 0.63, indicating that the CMCase was preferably segregated into the bottom salt-enriched phase. In a similar result, CMCase from *B. subtilis* occupied the bottom salt-enriched phase of the PEG 6000 and sodium citrate aqueous biphasic system (Ho et al., 2017). Our findings are also in accordance with Albuquerque et al. (2020), who claimed that increasing the PEG molar mass from 1000–6000 (g/mol) resulted in K reduction (all the K values were smaller than 1) for purified tannase-acryl hydrolase from *Aspergillus sydowii* using ATPS (PEG/citrate). Variation in partitioning is usually attributed to the hydrophobic and hydrophilic interface between the phase-forming PEG/salt and the external part of the protein of interest (Liu et al., 2019). It was reported that an increased hydrophilic region on the molecular surface of CMCase increased the CMCase separation into the hydrophilic bottom phase of sodium citrate (Ilari et al., 2009). Enzymes with highly hydrophilic proteins have greater protein affinity toward the salt-rich phase (González-Valdez et al., 2011; Sofijan et al., 2017). In this study, the PEG 8000/citrate ATPS resulted in a substantial maximum CMCase with a K of 0.32 and P_F of 6.18.

Additionally, the PEG yield obtained in the PEG 8000 g/mol showed the highest compared to PEG 2000 g/mol and PEG 4000 g/mol. The PEG 2000 g/mol and 4000 g/mol results in P_F at 1.63 and 3.23, respectively. Lower PEG molar mass limits the segregation of CMCase to the PEG phase, which is favourable for purifying CMCase. It was also observed that the K decreased with an increase in PEG molar mass. The system comprising PEG 4000 g/mol and PEG 6000 g/mol achieved a reduction of K from 0.63 to 0.52. This result shows that apart from hydrophobicity, the efficiency of partitioning is also affected by the phase component's molecular conformation and molar mass. A hydrophobic PEG further increases

Table 2
Influence of polyethyleneglycol molar mass on the partitioning behaviour of carboxymethyl cellulase in aqueous two-phase system

Molar mass PEG[a] (g/mol)	Partition coefficient (K)	Purification factor (P_F)	Yield (%)
4000	0.63	1.63	47.11
6000	0.52	3.32	58.89
8000	0.32	6.18	68.72
10,000	0.58	1.96	50.34

Note. [a] 20.5 % (w/w) PEG + and 15 % (w/w) sodium citrate. Results are means of triplicate readings ± SD

the hydrophobicity of the ATPS due to an increasing PEG molar mass (Ng et al., 2011). Hence, there is a space shortage for the enzyme at the PEG phase, resulting from the volume elimination effect (Nagaraja & Iyyaswami, 2015). It is favourable to CMCase partitioning due to volume elimination resulting from space insufficiency experienced at the PEG-rich phase.

This allows efficient extraction of the CMCase to the bottom citrate phase, thereby decreasing the partition coefficient (K). In addition, increasing the molar mass of PEG up to 10,000 g/mol decreased the P_F to 1.96 while K increased to 0.58. Similarly to this study, a high PEG molar mass of up to 10,000 g/mol resulted in a concomitance decrease in P_F for the purification of polygalacturonase (Remli et al., 2018). This occurrence could be elucidated through exclusion impact, whereby long chain PEG 10,000 g/mol limits free volume in the PEG phase, thereby leading to the segregation to the bottom phase, the undesirable proteins (Nagaraja & Iyyaswami, 2015). Consequently, this allows space for competition between unwanted proteins and enzymes. This phenomenon could lead to excluded volume (Nagaraja & Iyyaswami, 2015) or a repulsion effect (Ho et al., 2017). Proteins are precipitated between phases as a result of the volume exclusion, whereas the enzyme is drawn back to the top PEG phase as a result of the repulsive volume effect. This phenomenon can be explained by declining free volume or saturation of the polymer phase, which causes protein entrapment at the interphase in systems with high molecular weight polymers (Torres-Bautista et al., 2022). Nagaraja and Iyyaswami (2015) have additionally reported a comparable outcome for the recovery of fish protein from fish processing industrial effluent in a PEG-sodium sulphate system.

Additionally, the use of larger molecular weight PEG could have an impact on the phase characteristics due to increased viscosity and compact polymeric network development (Karkaş & Önal, 2012). The ATPS is also influenced by factors that interact to enhance or decrease the purification efficiency. Several studies have shown the interaction between the PEG molar mass and the citrate concentration in ATPS. Amaral et al. (2020) demonstrated that partition coefficient (K) was influenced by PEG molar mass and citrate concentration for extraction and partitioning of protease of *Aspergillus tamarii* Kita UCP1279 using factorial design. PEG molar mass and citrate concentration exert an antagonistic effect,

increasing the K value when both variables were increased to the maximum. A similar phenomenon was observed with the purification of lectin, where PEG molar mass and citrate concentration exert the most significant effect on the K value (Porto et al., 2011). In their study, synergistic interaction between the two variables was observed. Thus, a simultaneous increase in both variables will increase the yield. Based on the previous study by Aziz et al. (2017), PEG concentration and citrate concentration showed a significant interaction effect on the partition coefficient ($p<0.0004$) and purification fold ($p< 0.0001$). However, the molar mass of PEG and citrate did not ($p>0.05$).

Hence, the interaction effects in PEG and citrate have to be considered during the experiment's design. In another study, Herculano et al. (2012) explored the purification of cellulolytic complex from *Aspergillus japonicus* using PEG/citrate aqueous two-phase systems. They reported that pH and citrate concentration demonstrated a negative correlation with the response K. They observed that a low value of PEG concentration and molar mass with high citrate concentration favoured the transfer of CMCase to the top phase, which increased K to 3.30. Therefore, the most appropriate molar mass chosen for subsequent studies was PEG 8000 g/mol.

The role played by the crude load in changing the phase behaviour in ATPS of the target protein cannot be overemphasised. The crude load feedstock may change the ATPS volume ratio (Vr), affecting the desired protein's partitioning behaviour. Enzyme loading volume in ATPS is also important to enable the processing and recovery of larger feedstock, which is advantageous to industrial downstream processing (Sofijan et al., 2017). Crude loads were applied in the range of 2% to 20% (w/w), as illustrated in Figure 1. The CMCase was greatly improved when the crude load was increased to 17%, resulting in a maximum yield of 78.13%. The increase in crude load separated the target CMCase to the bottom phase, as reflected by the lowest K value at 0.24. This concentration achieves a balance of attractive and repulsive forces between the target enzyme and phase-forming component, ensuring their interactions are highly favourable.

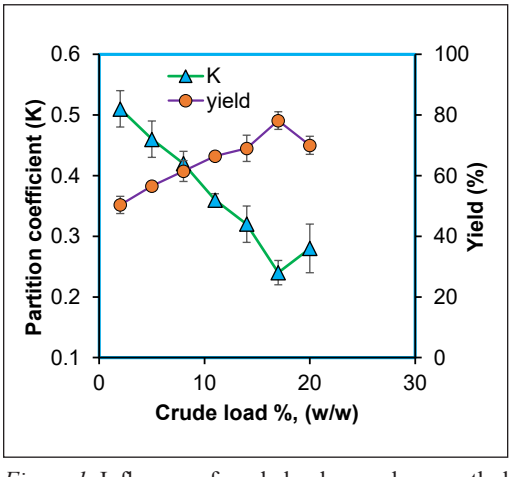

Figure 1. Influence of crude load on carboxymethyl cellulase partitioning in PEG 8000/citrate, ATPS. The crude load added to the aqueous two-phase system composition was varied from 2.5% to 20% (w/w). Results are means of 3 readings ± SD. K = Partition coefficient

However, a further increase in the crude enzyme load beyond 17% (w/w) resulted in the declination of the ATPS partitioning. Higher crude load at 20% (w/v) negatively impacted the ATPS partitioning behaviour due to a reduction in the phase volume ratio, thereby leading to a change in the

composition of the phases. This result is in accordance with previous literature (Abbasiliasi et al., 2014; Sukohidayat et al., 2018). Alteration of the system composition resulting from increasing ATPS crude load could diminish the Vr in the system (Ng et al., 2011). An increase in crude loads up to 20% resulted in a decline in yield to 70%, leading to an increase in K value to 0.28. It could be that the frontier of concentration at 17% (w/w) was exceeded by a higher crude load at 20% (w/w), therefore altering the partitioning behaviour of the target CMCase. Thus, the CMCase occupies the interface rather than being extracted from the bottom phase. In agreement with Sukohidayat et al. (2018), the precipitation of target protein at the interface resulted from a lack of space at either of the two phases. Furthermore, the precipitation of proteins at the inter-phase of ATPS resulted in the loss of enzyme activity (Chow et al., 2015). Therefore, crude enzyme at 17% (w/w) was designated the best concentration and used in further studies.

The pH affects the electrochemical characteristics of protein and ATPS. The electrochemical interaction plays a vital role in protein purification via ATPS (Iqbal et al., 2016). Thus, it will significantly affect CMCase partitioning. Hence, the pH of the system varied between the range of 5.0 to 8.5 (Figure 2). From this study, an increase in pH to 7.0 enhances the distribution of impurities at the top phase, thereby decreasing the value of K and enhancing the yield at the bottom phase. However, pH beyond 7.0 moved the contaminant into the salt-dominated phase and shifted the CMCase into the PEG-dominated phase, thereby increasing the K and leading to a decline in the yield. This phenomenon is supported by the earlier literature (Ho et al., 2017; Sofijan et al., 2017). It can be visualised that the values of K diminished (0.32 to 0.24) with pH increase (5.0 to 7.0).

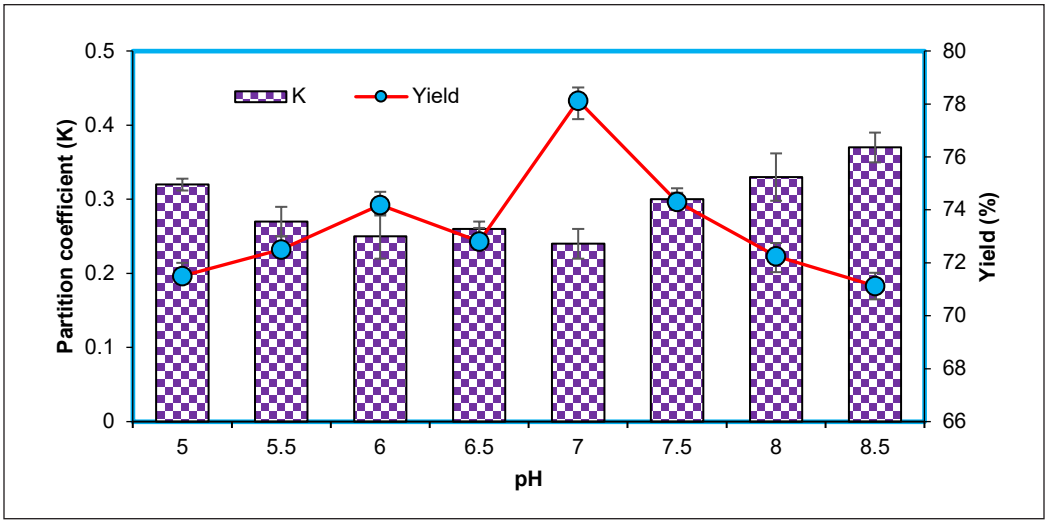

Figure 2. Influence of pH on carboxymethyl cellulase partitioning in PEG 8000/citrate ATPS.The results are means of 3 readings ± SD. K = Partition coefficient

However, pH beyond 7.0 increases the K values to 0.37 (at pH 8.0), resulting in a concomitant decrease in yield to 71%. The highest yield of 78.13% was obtained when the pH was maintained at 7.0. The pH affects the target protein net charges, altering the attractive and repulsive forces of the protein of interest and the phase composition (Amid et al., 2014). Increasing the system pH at a higher value than the pI value of the CMCase creates an enzyme with a negative charge, which develops an attraction for the PEG phase (+ charge), thereby decreasing the CMCase yield at the salt-enriched phase (Ratanapongleka, 2010). The isoelectric point (pI) value of CMCase from *Bacillus* sp ranges between 5.0 and 7.0 (Yin et al., 2010). When the pH approaches the pI value of CMCase, the net charge of the enzyme becomes constant and the total charges near zero, where the enzyme is of neutral pH; the change in yield and K was not noticeable in ATPS with pH 5.5 to 7.0 (Ho et al., 2017). Purification of CMCase from *Bacillus valezensis* A4 with ATPS composed of PEG 4000/phosphate requires a pH of 8.5 for maximum recovery (Liu et al., 2019). According to Soares et al. (2011), using a factorial design, the volume exclusion impact of PEG 8000 was stronger than the pH electrostatic effect and, combined with a low citrate concentration, favoured the partitioning of Con A protein to the bottom phase.

In another study by Herculano et al. (2016), a negative interaction effect of PEG concentration and pH was experienced. In this case, an increase in PEG concentration with a simultaneous decrease in pH increased the PF of xylanase. In this study, the PEG 8000/citrate at pH 7 with the lowest K (0.24) was selected for further studies because it was inferred that the CMCase was more stable at neutral pH.

The NaCl concentration will affect the hydrophilic proteins, which will, therefore, influence the enzyme separation in the ATPS. The NaCl concentration will affect the variation in the hydrophilic proteins, which will, therefore, influence the enzyme separation in the ATPS. The Variation in the ATPS NaCl concentration will affect the hydrophilic nature of the proteins, thereby influencing the separation of the enzyme. The NaCl concentration will affect the hydrophilic proteins, which will influence the enzyme separation. The effect of NaCl concentration on [0 to 1.25%, (w/w)] CMCase partitioning is illustrated in Figure 3. From the results, an increased NaCl to 0.75% (w/w) separated the enzyme to the bottom salt-enriched phase with a higher P_F of 9.68 compared to the ATPS without the addition of NaCl (8.94). The yield of CMCase increased from 78.12% to 83.12% with increasing NaCl concentration from 0 to 0.75%. The addition of NaCl at 1% (w/w) in PEG 8000/ citrate ATPS achieved the maximum yield at 86.62%. The P_F of 10.6 was recorded as the maximum at this concentration. It shows that the bottom phase harbours more enzymes.

The above results indicated that the NaCl concentrations influenced the enzyme partitioning. The electrical potential difference produced by adding NaCl may affect the ATPS protein partitioning. The unequal distribution of additional salt at different concentrations between the two-phase samples could improve the separation of the target

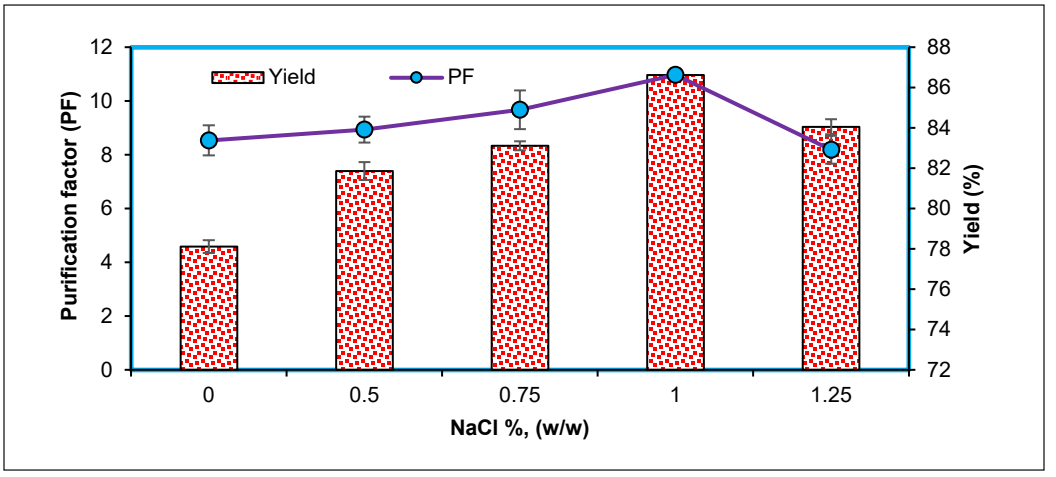

Figure 3. Influence of additional NaCl concentration on carboxymethyl cellulase partitioning in aqueous two-phase system. NaCl at concentrations between 0%–1.25% (w/w) was maximised in the ATPS. Results are means of three experiments ± SD. PF = Purification fold

and enhance the biomolecules (Anvari, 2015). A better interaction between CMCase and the NaCl could be due to the higher generated electrical potential, separating additional CMCase from the bottom phase (Ramesh & Murty, 2015). Adding sodium chloride might create a disorder in the water structure due to the higher electrical potential created amid the two phases, consequently improving the contact of the phase composition with the CMCase (Settu et al., 2015). When NaCl at 1.25% (w/w) was added to the ATPS, Y and PF decreased to 84.06 and 8.19, respectively. Excessive addition of NaCl could partition CMCase and unwanted protein to the same phase, consequently reducing the Y and the P_F.

In addition, NaCl at higher concentrations could decrease enzyme activity and modify target proteins' structural properties, thus leading to protein denaturation (Ketnawa et al., 2010). Similar to this study, Ho et al. (2017) reported that a 1% NaCl concentration was required for the purification of *B. subtilis* CMCase in ATPS consisting of PEG 6000 and sodium citrate. However, a higher NaCl concentration of up to 4% (w/w) was reported with PEG 8000/citrate ATPS partitioning of cyclodextrin glycosyltransferase (CGTase) (Ng et al., 2011). Increasing the NaCl concentration to 5% (w/w) leads to a decrease in P_F beyond the non-addition of NaCl, pulling non-target protein to the bottom phase (Ho et al., 2017). Therefore, 1% NaCl was sufficient for PEG 8000/citrate ATPS partitioning of CMCase.

The Molecular Weight of CMCase

Figure 4 shows the purified partitioned CMCase from PEG8000/citrate in ATPS. The result's evaluation shows multiple bands in Lane 2, signifying the presence of both the impurities and the target protein. However, a sample from the salt-rich citrate bottom phase (Lane 3)

shows a distinct band corresponding to 56 kDa. The result is in accordance with the CMCase produced from *Bacillus cereus* FOA-2, which was reported to have a molecular weight of 56 kDa (Olukunle et al., 2021).

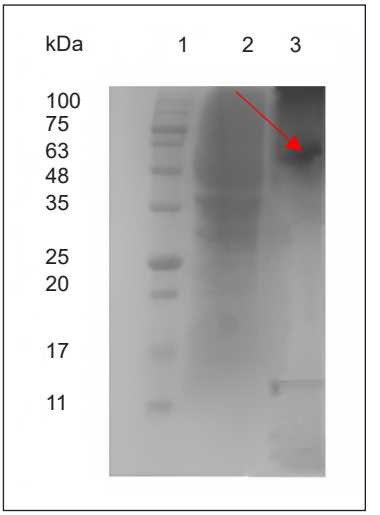

Figure 4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis for purification of carboxymethyl cellulase in polyethene glycol 8000/citrate aqueous two-phase system. The purity of the carboxymethyl cellulase was assessed with 12 % SDS-PAGE analysis. The protein maker's molecular weight ranged from 11 to 100 kDa. SDS-PAGE- Lane 1: Protein molecular marker; Lane 2: crude sample; Lane 3: purified carboxymethyl cellulase from ATPS bottom phase

Comparison of Purification Enhancement and Partitioning Behaviour of Cellulases from Different Microorganisms

The purification and partitioning behaviour of cellulases has been studied for bacteria and fungi isolated from various environments (Table 3). Different two-phase systems have been used to achieve purification enzymes. Notably, ATPS, which consists of PEG/citrate or PEG/phosphate, has been reported for cellulase purification. According to Liu et al. (2019), Cellulase from *Bacillus Valezensis* A4 was recovered at a yield of 67.8% in ATPS consisting of PEG 4000/K_2HPO_4 at pH 8.5 and 5% (w/w) NaCl concentration. Results from the current study are comparable to the previous study, as indicated in Table 3. Despite 100% purification not being achieved, partial purification of CMCase from *Bacillus licheniformis* 2D55 using ATPS has been successfully demonstrated in PEG 8000/citrate.

Table 3
Partitioning behaviour of cellulases from Bacillus species at different conditions

Isolate	Phase composition	Condition		Reference
Bacillus subtilis	PEG 6000/citrate	Vr 0.29, NaCl 1% (w/w), pH 7, crude load 20 % (w/w)	Y_B 88%, P_F 4.8, K 0.44, MW 32-46 kDa	Ho et al., 2017
Bacillus Valezensis A4	PEG 4000/K_2HPO_4	NaCl 5% (w/w), pH 8.5, crude load 2g (w/w)	Y_B 67.8%, P_F 1.14, MW 35 kDa	Liu et al., 2019
Bacillus licheniformis 2D55	PEG 8000/citrate	NaCl 1% (w/w), pH 7, crude load 17% (w/w)	Y_B 86.62%, P_F 10.9, K 0.21, MW 56 kDa	This study

Note. Vr = volume ratio

CONCLUSION

The aqueous two-phase system was a prospective method for purifying CMCase of thermophilic *Bacillus lichiniformis* 2D55. ATPS has been recognised as a potential and powerful primary separation and purification step in the overall product recovery train. This system is a viable alternative for CMCase purification because of the phase-forming polymers' low cost, making it suitable for large-scale applications. Based on the results of this study, the maximum yield (88.62%) and P_F (10.98) were successfully recovered from the salt-rich bottom phase upon incubation of the system at PEG 8000 /citrate (20.5/15%, w/w) 1%, (w/w) NaCl, at pH 7.0 and crude enzyme load at 17%, (w/w). The hydrophilic nature of the enzyme assists in separating the CMCase into the salt-rich phase obtained at the bottom layer. Hence, the ATPS provides a good degree of enzymes since it reduces the loss of enzymes and eventually improves enzyme recovery. The result shows that this technique could be advantageous to the enzyme industries since a cheaper and relatively straightforward separation and purification strategy could be achieved in a single step. It is obvious that the ATPS is an efficient recovery technique for CMCase, addressing the need for scalability and effective bioprocess technology with a high purification factor. Further investigations will be conducted using factorial design to build upon our findings and provide a complete understanding of the system.

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