Investigation of the Effects of Deep Eutectic Solvents Based on Fructose, Sucrose, and Glycerol on the Kinetic Parameters of Uricase

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ABSTRACT

Urate oxidase (Uricase), an important enzyme in the fields of industry and pharmaceuticals, plays a significant role in the conversion of uric acid to allantoin and the reduction of serum uric acid levels. This has led to increased interest among researchers in the stabilization of this enzyme. One effective method for stabilizing uricase involves the use of deep eutectic solvents (DES). In the present study, conducted at the Graduate University of Advanced Technology in Kerman, we cultured the BL21 bacteria and expressed and purified recombinant uricase. The impact of a glycerol-based eutectic solvent combined with the sugars fructose and sucrose on the enzyme's kinetic parameters was examined. The results indicated that the eutectic solvent reduced the Michaelis constant (Km) and enhanced the catalytic efficiency of the enzyme in the presence of the eutectic compared to the free enzyme. This solvent, with its hydroxyl groups and influence on the enzyme's intramolecular hydrogen bonds, significantly improved the enzyme's kinetic stability.

Keywords: *Uricase, Deep Eutectic Solvent, Catalytic Efficiency, Kinetic Parameters.*

1. *INTRODUCTION*

Urate Oxidase (Uricase) [1] is an enzyme belonging to the oxidoreductase class and is of considerable interest in both pharmaceutical and therapeutic contexts due to its lack of cofactor requirements [2]. The specific role of uricase in the oxidation of uric acid [3], the final product of purine catabolism [4, 5], to allantoin [6] facilitates its easier and more rapid excretion, thereby attracting significant attention from researchers [7]. Approximately two-thirds of uric acid in the body is excreted through the kidneys, while the remaining third is eliminated via the intestines [8]. Reported serum uric acid concentrations are 1.5 to 6.0 mg/dL for women and 2.5 to 7.0 mg/dL for men [9]. These levels are regulated by enzymes and membrane transporters, and elevated uric acid can lead to precipitation around joints and soft tissues, resulting in complications such as renal insufficiency [3], hyperuricemia [10], gout [11], and tumor lysis syndrome [12]. Three mechanisms have been proposed to reduce uric acid levels. The first involves the use of drugs such as allopurinol [13] and febuxostat, which inhibit xanthine oxidase and consequently reduce uric acid production, although they are associated with cardiovascular diseases [14]. The second mechanism employs agents like probenecid [15]. The third and more effective approach for lowering uric acid levels is the use of enzymatic drugs such as urate oxidase [16], which serves as a diagnostic enzyme for determining urate concentration [12, 17, 18]. Rasburicase [19], a recombinant form of uricase, has proven effective in treating such conditions [20]. However, like many protein-based enzymes, it is rapidly destabilized and inactivated under physiological conditions influenced by factors such as temperature and pH, compromising its functionality [21].

The low stability of this enzyme limits its applications in industrial, commercial, and pharmaceutical fields [22]. Numerous researchers have sought to enhance the stability of uricase through various methods [23]. Proteins, including therapeutic proteins like uricase, are susceptible to stress factors such as temperature during purification, processing, and storage, leading to alterations in their three-dimensional structure and, ultimately, reduced stability. This limitation constrains their use in food, commercial, and pharmaceutical industries [23]. To address these challenges and overcome the stability limitations of proteins, researchers have adopted various strategies, including chemical modification [24], encapsulation and stabilization in protective matrices and nanoparticles [25], optimization of formulations and storage conditions [26], the use of stabilizing additives [27], and protein engineering techniques [28]. The primary objective of these approaches is to enhance enzyme activity and stability, thereby expanding their applications in medicine, pharmaceuticals, and biotechnology.

It is noteworthy that the aforementioned strategies for improving uricase stability may vary depending on the specific application or intended use of the enzyme. Deep eutectic solvents, formed by the combination of two or more specific organic and inorganic components (not necessarily ionic) [29], exhibit significantly lower melting points than their constituent components [30, 31], offering advantages such as low cost, low toxicity, non-volatility, ease of preparation, high efficiency, scalability, and biodegradability. Enzymatic kinetics refers to the study of reaction rates and their variations across different enzymatic mechanisms, allowing for the assessment of various inhibitory and stimulatory factors affecting enzyme activity [32].

This study aims to investigate the impact of deep eutectic solvents synthesized from fructose, sucrose, and glycerol on the enhancement of uricase's kinetic stability, given the enzyme's acknowledged importance.

2. *MATERIALS AND METHODS*

2.1 Materials

The materials utilized in this study included tryptone, yeast extract, agar, NaCl, Tris, HCl, K_2HPO_4, KH_2PO_4, $CaCl_2$, nickel chloride, nickel sulfate, sodium hydroxide, uric acid, boric acid, phosphoric acid, and acetic acid.

The expression strain employed was Escherichia coli BL21, harboring the recombinant uricase plasmid, with kanamycin serving as the antibiotic. Luria-Bertani (LB) broth was used as the culture medium for various E. coli strains.

2.2 Methods

2.2.1 Bacterial Culture and Protein Expression

E. coli BL21 containing the recombinant plasmid pET28a$^+$-UOX was cultured on LB agar plates supplemented with kanamycin. Suitable single colonies were selected for inoculation into liquid LB medium. Subsequently, 1 mL of this culture was transferred to 100 mL of TB medium containing salts. Once the optical density (OD) reached 0.6, protein expression was induced by adding 1 M IPTG and incubating for 5 hours at 37°C.

2.2.2 Protein Purification

To lyse the bacterial cell walls, sonication was performed for 30 seconds, repeated seven times. Following this, the sample was centrifuged at 13,000 rpm for 20 minutes at 4°C, and the supernatant was transferred to a nickel affinity column for purification. This column operates based on affinity chromatography, allowing the histidine-tagged protein to bind to nickel. After binding, the enzyme was eluted using buffers containing varying concentrations of imidazole, and the samples were collected in 1.5 mL tubes for subsequent assays.

2.2.3 Synthesis of Eutectic Solvent

For the synthesis of the eutectic solvent, a mixture of fructose, sucrose, and glycerol was prepared in a ratio of 2:2:4. The resulting mixture was heated until melted, yielding a viscous and transparent substance that transformed into a gel-like and sticky consistency after 20 minutes of stirring.

2.2.4 Optimization of Eutectic Concentration and Determination of Optimal Concentration

*Initially, the optical absorption of the eutectic solvent was assessed in the wavelength range of 200 to 700 nm at concentrations of 1%, 5%, 10%, 15%, 20%, 30%, and 50% in Tris buffer. The results were compared with those obtained from burax buffer containing uric acid, allowing for the selection of concentrations below 10%. Subsequently, using Formula (**1**), the enzymatic activity at eutectic concentrations of 1%, 5%, and 10% was measured and compared to the activity of the free enzyme, which had been diluted in Tris buffer at the same ratios.*

$$Volume\ activity\ \left(\frac{u}{ml}\right) = \frac{\Delta(A_0 - A_{60})\,,V,D}{12{,}6,d,v} \qquad (1)$$

In this formula A_0 is the absorbance without substrate, A_{60} is the absorbance in the initial minute, V is the total reaction mixture volume (0.730 ml), D represents enzyme extinction coefficient, 12.6 represents molar absorptivity of uric acid ($mM^{-1}.cm^{-1}$), d is the path length (1 cm), and v refers to the enzyme sample volume (0.02 ml).

2.2.5 Determination of Enzyme Concentration

*The enzyme concentration was determined using the Bradford assay. This method is based on the formation of a complex between positively charged amino acids in the enzyme and the Bradford reagent. The reagent, which has a brick-red color, changes to blue upon interaction with protein, indicating an increase in concentration. To prepare the Bradford solution, 0.01 g of Coomassie Blue G-250 was weighed using an analytical balance, and 5 ml of 20% ethanol was added. In a dark environment, 10 ml of phosphoric acid and 85 ml of distilled water were subsequently incorporated (***Table 1***). The mixture was then filtered using filter paper and stored at 4°C. Different concentrations of bovine serum albumin (BSA) were prepared in seven 1.5 ml vials, including 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/ml. Using 750 µl of the Bradford solution, the spectrophotometer was calibrated at a wavelength of 595 nm. In new vials, 25 µl of each BSA concentration was mixed with 750 µl of the Bradford solution, followed by two cycles of pipetting and incubation in the dark on ice for 5 minutes. Subsequently, 750 µl of each new solution was transferred to a cuvette, and absorbance was measured three times under the same conditions. Using the average absorbance values, a calibration curve was plotted, and the line equation was calculated using Excel software. After constructing the graph and determining the line equation, 750 µl of the Bradford solution was mixed with 25 µl of uricase enzyme in a 1.5 ml vial. This mixture was then subjected to two cycles of pipetting and incubated in the dark on ice for 5 minutes. Again, 750 µl of the resulting mixture was placed in a cuvette, and absorbance was measured three times at 595 nm. The enzyme concentration was subsequently calculated in mg/ml using the average absorbance and the previously established line equation.*

Table 1. *Preparation of Bradford solution*

Materials	Amount	Storage conditions
Coomassie Blue G-250	0.01 gr	4°C
Ethanol20%	5 ml	
Phosphoric Acid	10 ml	
Distilled Water	85 ml	

2.2.6 Investigation of Kinetic Parameters

*In the context of conducting enzyme kinetics tests, a burax buffer containing substrate concentrations of 0.184, 0.161, 0.138, 0.115, 0.092, 0.069, 0.046, and 0.023 millimolar of uric acid was prepared. The activity of the enzyme, uricase, was measured in the presence of these varying concentrations. To control experimental conditions, enzyme dilution was also performed in a Tris buffer, and absorbance readings were taken for all substrate concentrations following the same methodology. Subsequently, a Michaelis-Menten plot was generated using Excel, and the corresponding parameters were calculated (**2**). After inverting the Michaelis-Menten equation, the Lineweaver-Burk equation was derived (**3**). The y-intercept of this equation was determined to be the inverse of the maximum velocity (V_{max}). Following the calculation of V_{max}, the Michaelis*

constant (K_m) was derived using the slope of the Lineweaver-Burk plot. It is noteworthy that this parameter is equivalent to the reciprocal of the x-intercept of the plot.

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (3)$$

V_0 is the reaction rate ($mM.min^{-1}$), V_{max} is the maximum velocity ($mM.min^{-1}$), S is the substrate concentration (mM), and K_m is the Michaelis constant (mM).

*Additionally, the catalytic rate constant (K_{cat}) of the enzyme, which represents its catalytic efficiency, was calculated (**4**). Finally, the catalytic efficiency constant (CE) was determined by dividing K_{cat} by K_m (**5**).*

$$K_{cat} = \frac{V_{max}}{[E]} \quad (4)$$

$$CE = \frac{K_{cat}}{K_m} \quad (5)$$

K_{cat} is the catalytic rate constant (s^{-1}), V_{max} is the maximum velocity ($mM.s^{-1}$), E is the enzyme concentration (mM), and CE is the catalytic efficiency constant ($s^{-1}. mM^{-1}$).

3. *RESULTS AND DISCUSSION*

3.1 Bacterial Cultivation, Protein Expression, and Purification

*The cultivation of BL21 bacteria was performed in a medium containing antibiotics, utilizing the resulting single colonies for bacterial culture, protein expression, and purification (**Fig 1**). This strain is employed as an expression host in laboratory tests [33], containing the laci gene, which acts as an inhibitor of the lacUV5 promoter, thus preventing expression under normal conditions. In the presence of IPTG, this gene is inhibited, leading to the activation of the aforementioned promoter and subsequent expression [34]. The expression vector pET28a$^+$ (Novagen, Cat. No. 96864-3) [35] consists of 5369 base pairs, a T7 polymerase promoter, and a kanamycin resistance marker, which facilitates the selection of bacteria harboring the plasmid. The protein produced by this expression system features a polyhistidine tag at either its carboxyl or amino terminus [36]. The histidine tag of uricase, with its high affinity for nickel, justifies the use of nickel-affinity chromatography for protein purification.*

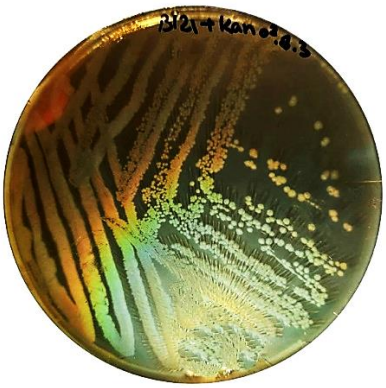

Fig 1. *Cultivation of BL21 in Kanamycin-Containing Medium.*

3.2 The synthesis of DES

Changes in the solvent and the surrounding environment of the enzyme can affect stability by altering surface tension or impacting the structure and compactness of the protein. Natural deep eutectic solvents may enhance enzyme stability by influencing intra- and intermolecular interactions. Glycerol, due to its viscous nature, low volatility, and non-toxicity, plays a significant role. This compound also mitigates the aggregation of inactive enzyme structures by interacting with hydrophobic surface residues of the protein [37]. Sucrose and fructose, which possess properties such as non-toxicity and natural origins, enhance protein stability through their abundant hydroxyl groups, contributing to protein hydration [38].

3.3 Determination of Optimal Eutectic Concentration

*Following a comparison of the absorbance spectra of the eutectic with a burate buffer containing uric acid, concentrations below 10% were selected (***Fig 2***) due to their lack of interference in the absorbance spectrum, with their spectra lying beneath that of burate. Higher concentrations interfered with absorbance measurements, as their corresponding spectra were positioned above that of uric acid. Enzymatic activity was subsequently assessed for both free enzyme and eutectic-containing enzyme, leading to the selection of a 5% concentration (***Fig 3***). The final results indicated that the presence of the eutectic resulted in a slight decrease in enzymatic activity, which may be attributed to the viscosity of the eutectic and the formation of a coating around the enzyme, thereby reducing its mobility.*

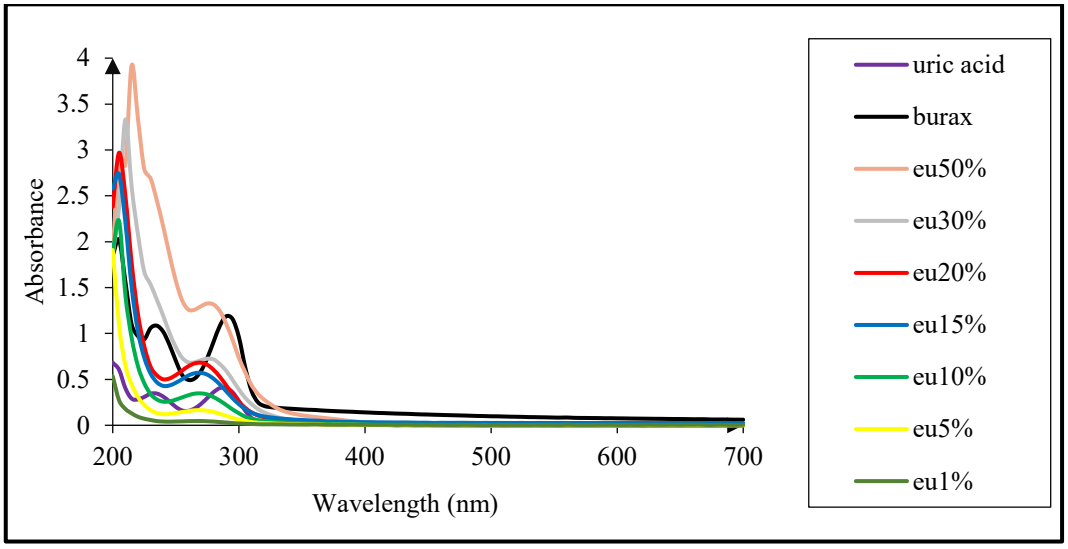

Fig 2. *Graph of Eutectic Absorbance Intensity at Concentrations of 1% to 50% Compared to Burate Buffer Containing Uric Acid (200 to 700 nm)*

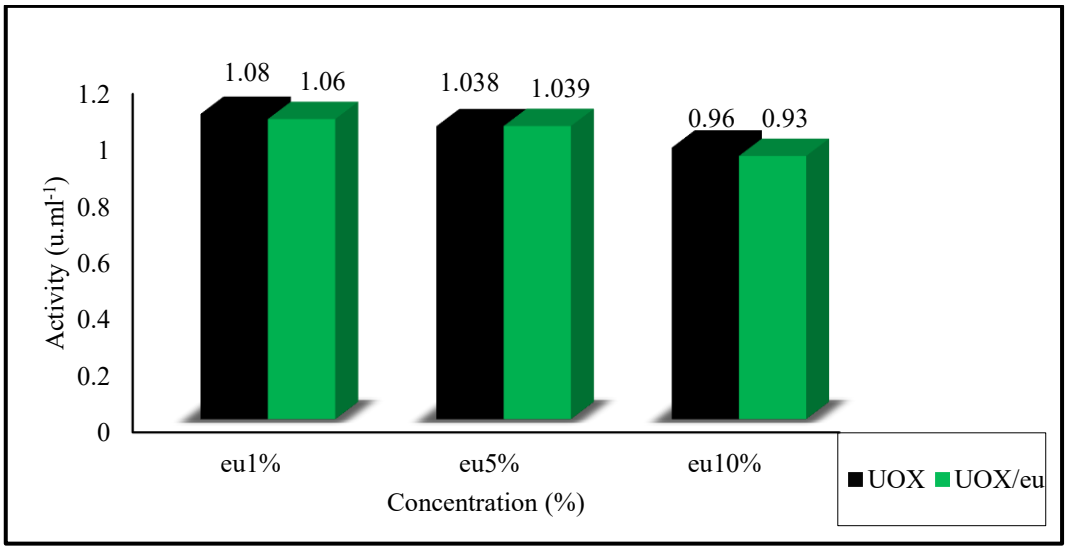

Fig 3. *Determination of Activity for Free Enzyme and Eutectic-Containing Enzyme at Three Different Concentrations*

3.4 Determination of Enzyme Concentration

*A Bradford assay was conducted (***Table** ***2****), and following the placement of enzymatic activity on the resulting graph (***Fig** ***4****), the enzyme concentration was determined to be 0.959 mg/ml.*

Table 2. *The absorbance of the BSA solution was measured at a wavelength of 595 nm.*

BSA concentration ($mg.ml^{-1}$)	Absorbance
0.05	0.475
0.1	0.514
0.2	0.725
0.4	0.953
0.6	1.155
0.8	1.428
1	1.562

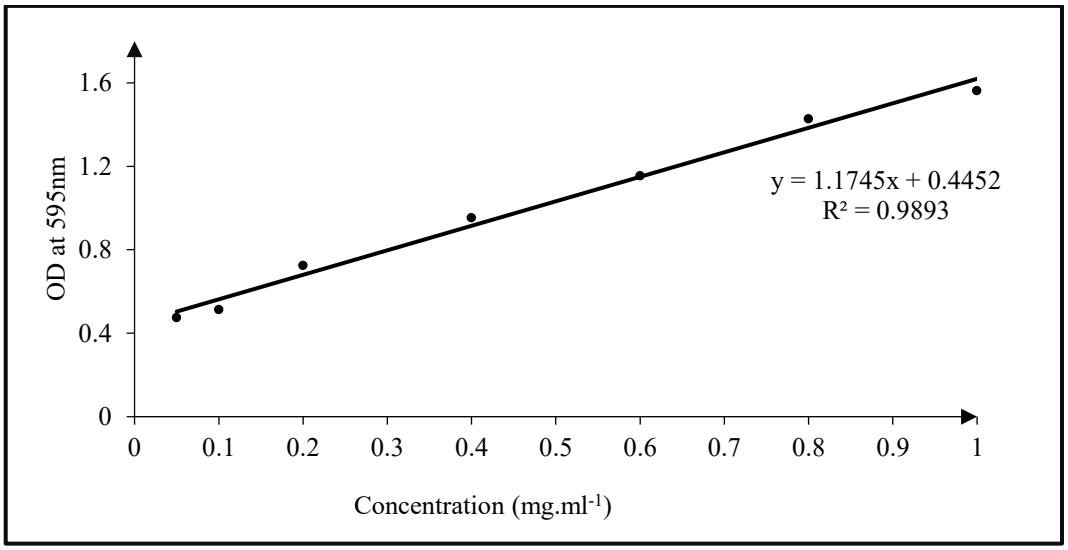

Fig 4. *The Bradford assay graph was plotted to calculate enzyme concentration, with the x-axis representing absorbance and the y-axis indicating concentration.*

3.5 Determination of Kinetic Parameters

*To study enzymatic kinetics, the Michaelis-Menten (***Fig 5***) and Lineweaver-Burk (***Fig 6***) plots were generated for both free enzyme and eutectic enzyme, facilitating the calculation of kinetic parameters. V_{max} represents the maximum enzymatic velocity at which all enzyme molecules are saturated with substrate. It was observed that this parameter decreased in the presence of the eutectic, likely due to the viscous nature of the solvent, which may reduce the likelihood of enzyme-substrate interaction.*

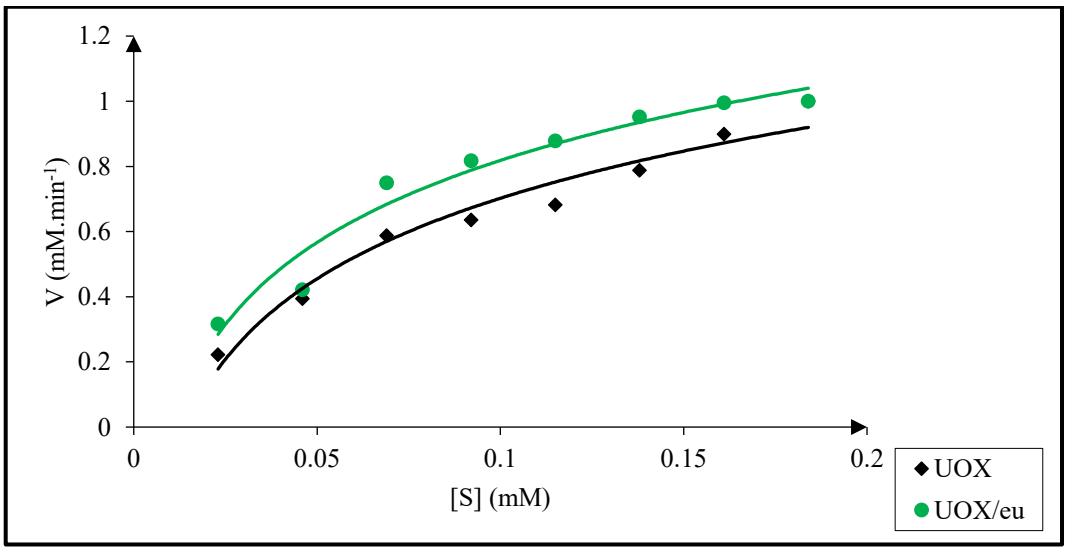

Fig 5. *Michaelis-Menten Plot in the Presence(green) and Absence(black) of Eutectic.*

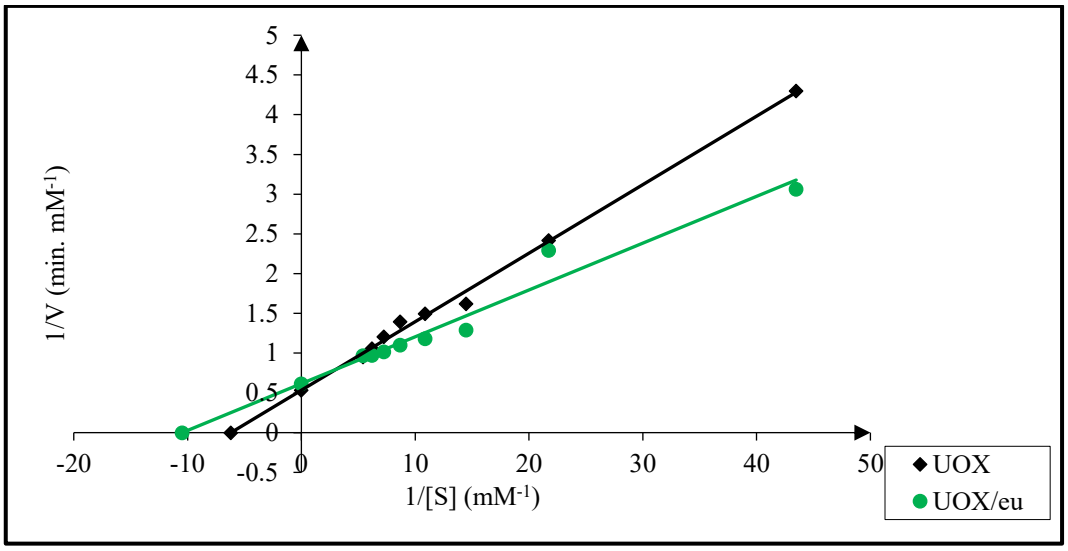

Fig 6. *Lineweaver-Burk Plot and Analysis of Enzymatic Kinetic Parameters.*

*The K_m parameter is defined as the substrate concentration at which the enzymatic reaction rate is half of V_{max}. In other words, K_m serves as an inverse measure of the affinity of the enzyme for its specific substrate, facilitating the formation of the enzyme-substrate complex. A reduction in this parameter indicates an increased affinity of the enzyme for its substrate. This effect may be attributed to the influence of the eutectic on hydrogen bonds within the enzyme. Notably, the eutectic does not appear to occupy the active site. Similar findings were reported by Taheri Mehr et al., where an increased affinity of the enzyme for the substrate was observed with a decreased Km in treated versus untreated enzymes [39]. K_{cat} reflects the catalytic power of the enzyme and serves as a measure of the conversion rate of substrate to final product. It indicates the maximum number of substrate molecules converted to product per second. An observed 46% increase in the ratio of K_{cat} to K_m suggests enhanced specificity of uricase for its specific substrate (***Table 3***).*

Table 3. *Kinetic Parameters of Uricase in the Presence and Absence of Eutectic.*

	V_{max} (mM.min^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/k_m (mM^{-1}.s^{-1})
UOX	1.87	0.16	1.95	12.08
UOX/eu	1.62	0.09	1.68	17.68

4. *CONCLUSION*

Uricase is an enzyme that plays a crucial role in the conversion of uric acid to allantoin, leading to a reduction in uric acid levels and, consequently, the prevention of diseases such as gout. To investigate the effects of natural deep eutectic solvents containing sucrose, fructose, and glycerol on the kinetic stability of uricase, bacterial culture, protein expression, and purification were performed. The enzyme concentration was determined to be 0. 959 mg/ml, with an optimal eutectic concentration of 5%. Kinetic results and parameter analysis indicated a decrease in the K_m value, which suggests an increased affinity of the enzyme for its substrate. Additionally, the increase in the ratio of K_{cat} to K_m reflects an enhancement in the specificity and catalytic efficiency of the enzyme for its substrate in the presence of the eutectic solvent compared to the free enzyme.

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