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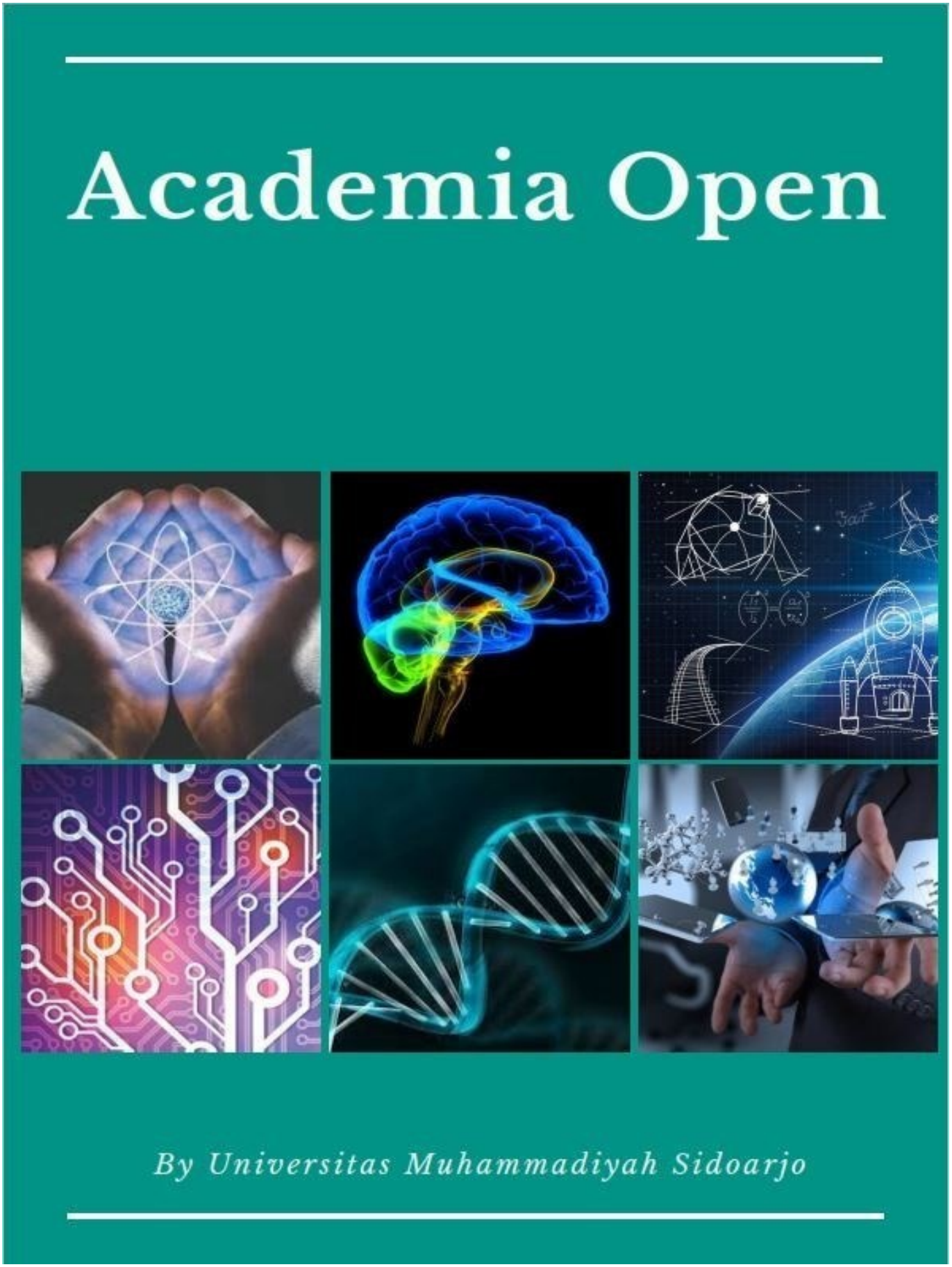

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Exploiting Klebsiella pneumoniae Arginine Deiminase: Purification, Characterization, and Selective Anticancer Activity

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Abstract

General Background: Arginine deiminase (ADI) represents a promising therapeutic enzyme for treating arginine-auxotrophic cancers by catalyzing L-arginine conversion to citrulline and ammonia, exploiting the metabolic vulnerability of cancer cells deficient in arginosuccinate synthetase (ASS). **Specific Background:** Colorectal cancer, ranking second in mortality and third in global incidence, demonstrates heterogeneous arginine metabolism with certain tumor subtypes exhibiting impaired ASS expression, rendering them susceptible to arginine deprivation therapy. **Knowledge Gap:** Despite ADI's therapeutic potential, comprehensive characterization of *Klebsiella pneumoniae*-derived ADI and its selective cytotoxicity against colorectal cancer remains insufficiently explored. **Aims:** This study isolated, purified, and characterized ADI from clinically isolated *K. pneumoniae* strains and evaluated its selective anticancer activity against HCT-116 colorectal cancer cells versus NCM460 normal colon cells. **Results:** Isolate No. 5 demonstrated optimal ADI production (12 U/mg), achieving 11-fold purification through three-step chromatographic procedures yielding 32.9 U/mg specific activity with 40.9% recovery; optimal enzymatic performance occurred at pH 7 and 37°C, with Mn^{2+} and Fe^{3+} enhancing activity (120% and 105% respectively) while EDTA inhibited it (73%), confirming metalloenzyme properties; cytotoxicity assays revealed selective anticancer activity with IC_{50} values of 390 µg/mL against HCT-116 cells and >800 µg/mL against NCM460 cells, demonstrating 2.3-fold selectivity. **Novelty:** This investigation provides the first comprehensive biochemical characterization of *K. pneumoniae*-derived ADI with demonstrated selective cytotoxicity parameters specifically targeting colorectal cancer. **Implications:** These findings establish *K. pneumoniae* ADI as a promising selective therapeutic candidate for colorectal cancer treatment, warranting further development including PEGylation strategies and combination therapy investigations.

Keywords : Arginine Deiminase, Klebsiella Pneumoniae, Enzyme Purification, Colorectal Cancer, Cytotoxicity

Highlight :

- Enzyme achieved 11-fold purification yielding 32.9 U/mg through three-step protocol.
- Optimal activity occurred at pH 7 and 37°C with metalloenzyme characteristics.
- HCT-116 cancer cells showed 2.3-fold greater sensitivity than normal colon cells.

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Introduction

Catabolic enzyme is arginine deiminase (ADI), a deaminase of L-arginine that catalyzes the deamination of L-arginine to citrulline and ammonia which is playful in the metabolism of arginine in a broad variety of organisms. (1). This enzyme has been of interest to many ideas over the past few years due to its exceptional therapeutic application as an anticancer (2).The effect of ADI is in contrast to that of arginase, which synthesizes ornithine, which can be used to grow tumors by the formation of polyamine; ADI synthesizes citrulline, which is only effective in depleting arginine in the tumor microenvironment and does not stimulate the proliferation of cancer cells. (3).The antimalignant effect of ADI also takes advantage of an inherent metabolic weakness in cancer cells: they are arginine auxotrophic due to the down-regulation or silencing of the expression of arginosuccinate synthetase (ASS), the rate-limiting enzyme that produces endogenous arginine (4).Colorectal cancer has been listed as one of the most common and dangerous types of cancer in the world as its rank is number two in death and number three in terms of incidence among the other types of cancers in the world. (5). The prognosis of patients with advanced or metastatic colorectal cancer has not improved; thus, new treatment methods are to be developed (6). Recent works on metabolic profiling have established that there is a high heterogeneity of arginine metabolism in colorectal cancer, and that some of the tumor subtypes are severely impaired in ASS expression, which makes them highly sensitive to the arginine deprivation therapy (7,8). Enzyme-based anticancer therapy has been found to be a potential approach to conventional chemotherapy, providing higher selectivity to cancerous cells without harming healthy tissue (9). Arginine-depleting enzymes such as ADI and Arginase have been found to have proven anticancer effects in preclinical and clinical settings for several cancers such as melanosoma, hepatocellular carcinoma, and colon cancer (10, 11).

K. pneumoniae is a Gram-negative bacterium, which has also become a valuable source of new therapeutic enzymes like ADI owing to its complex arginine metabolic system (12). This research focuses on the isolation, purification, bioevaluation, and characterization of ADI from K. pneumoniae isolated from cancer patients and its subsequent selective cytotoxic effects on HCT-116 colorectal cancer cells rather than on NCM460 normal colon epithelial cells, thus ascertaining its importance for targeted treatment against colorectal cancer.

Materials and Methods

Sample Collection 36 clinical samples were sampled prospectively in different hospitals in the Wasit Governorate, Iraq. Sampling was carried out during a given time with various clinical sources of patients of different ages and genders so that the representation of the local bacterial population can be facilitated.

Isolation and Phenotypic Identification The initial isolation of Klebsiella pneumoniae was done through inoculating the samples on MacConkey agar,Blood agar, and Eosin Methylene Blue(EMB) agar. After the (24 hours) incubation in aerobic conditions at 37 oC, the plates were checked using characteristic growth. The isolates with the characteristic mucoid and pink-colored colonies on the MacConkey agar were chosen as lactose fermenters to be further confirmed. The standard microbiological tests, such as Gram staining to visualize the typical Gram-negative bacilli morphology and biochemical analysis using IMViC tests, were used to offer a preliminary identity of the isolates.

Molecular Confirmation and Growth Induction The conclusive identification of K. pneumoniae was done by Polymerase Chain Reaction (PCR) was performed with the 16S rRNA gene and gel electrophoresis done to confirm the size of the amplicon. Isolates that were confirmed were then grown in a Minimal Media supplemented with 10-20 mM L-arginine as a sole nitrogen source. To maximize the induction of the Arginine Deiminase (ADI) enzyme the cultures were incubated at 37oC with orbital shaking at 150 rpm until the late exponential stage was achieved (13).

Extraction of arginine deiminase (ADI) from bacterial cells

The bacterial cell weight was harvested by centrifugation (6000 rpm, 30 min, 4 oC) to collect the bacterial cells and their wet mass. The pellet was resuspended in a buffer of extraction solution with the addition of 1 mM B-mercaptoethanol. The cell wall was disrupted by three different methods to release the intracellular enzyme: Enzymatic Lysis, cell suspension was incubated with 200 µg /mL lysozyme and left at 37°C to disrupt the cell wall and release the intracellular enzyme. The suspension was vortexed with mechanical Disruption with sterile glass beads in 20 minutes under chilled conditions. Chemical Permeabilization, cell was incubated at 30 min at 25 oC in a solution of 12.5% K2HPO4 and 2% Triton X-100. The lysates were centrifuged immediately after treatment with 8000 rpm for 15 minutes at 4 oC. The resulting pellets were thrown away and the supernatants were measured in order to determine the enzyme activity, protein concentration, and specific activity to choose the most effective extraction method(14).

Enzyme activity

The enzyme activity of crude Arginine Deiminase is analyzed on the basis of L-citrulline formed. For this, the mixture containing the crude enzyme extract and L-arginine as the substance is reacted with phosphate buffer, where it was kept at 37°C for 30 minutes. After that, an acid colorimetric agent was added, stopping the reaction. This forms a complex, whose intensity was detected spectrophotometrically at 490 nm. The ammonia formed is measured using Nesser's reagent, where the strength of the orange-brown complex developed is directly proportional to the efficiency of ADI catalytic activity, making it react with specific activity measured as the micromole amount of substrates consumed per minute per milligram of proteins (15).

Purification of Arginine Deiminase

Partial Purification by Ammonium Sulfate Fractionation

The cold solution (4oC) of the crude enzyme extract (100mL) was then treated by fractional precipitation using solid ammonium sulfate. A range of ammonium sulfate saturation (20%-80%) was systematically tested to determine the optimal salt concentration for desorption of the enzymes. To saturate the ammonium sulfate, a slow addition to the solution was made at 45 minutes with a slight stir to dissolve the solution but not to cause the denaturation of the proteins. The aggregated proteins were subsequently centrifuged at 8,000 rpm in 20 minutes at 4oC and a fraction of the overnight centrifugates was taken out.The proteinaceous fraction (pellet)was carefully obtained and redissolved in a small amount of potassium phosphate buffer(pH 7.4) for subsequent dialysis analysis(16).

Ion exchange chromatography

A NaCl concentration gradient from 0.1 to 1.0 M was employed to elute bound proteins after loading the partially purified enzyme on to an ion

exchange column consisting of DEAE-cellulose that was pre-equilibrated using 0.05 M Tris-HCl buffer at pH 8. Concentration of the protein was followed by monitoring the UV-Vis absorbencies at280 nm using a UV-Vis spectrophotometer. Fractions of 3 mL flow rate were collected. The arginine deiminase activity of the fractions was determined, and the fractions showing the highest activity of the enzyme were pooled for subsequent purification steps (17).

Gel filtration chromatography

It was further purified by loading the partially purified enzyme from the ion-exchange step onto Sephadex G-150 gel filtration chromatography (20×2 cm) equilibrated with potassium phosphate buffer at a concentration of 50 mM. Protein components were eluted in the same equilibration buffer at a constant flow rate of 3mL per fraction. The elution profile was monitored by measuring the optical density at 280 nm for protein estimation, with each fraction being assayed simultaneously for arginine deiminase activity. Fractions containing peak activity were pooled and concentrated for further studies.

Arginine deiminase characterization

Effect of pH on Purified (ADI) Activity

The optimal pH for the purified arginine deiminase (ADI) was evaluated by the enzyme activity assay between a pH range 5 to 9. The assay mixture is prepared with a concentration of 10 mM/mL for the purified enzyme and a substrate concentration of 20 mM/mL. The following buffer solution was employed: Acetate buffer (pH 4.5), potassium phosphate buffer solution (pH 6.5), and Tris HCl buffer solution (pH 8, 8.5, 9. The pH with the highest level of activities for the given enzyme would be determined.

pH Stability of Purified (ADI)Activity

The pH stability of purified arginine deiminase(ADI) was assessed by exposing the enzyme to different concentrations of buffers, ranging from (pH 5 to 9), at a temperature of 37 °C for a period of 30 minutes.The samples were then immediately chilled in a chilled bath to stop the biological effect caused by the high temperatures. The remaining activity of each sample was then determined, and the optimal pH at which the enzyme is stable was calculated based on the remaining percentages of active enzyme.

Effect of Temperature on Purified (ADI)Activity

Optimal Temperature of purified enzyme allowed for reaction with the substrate at varying temperatures from 20 to 80° C. The catalytic activity measured and used to determine the optimal temperature.Thermal Stability: The purified enzyme pre-treated by exposing it to temperatures from 27 to 50° C for 30 minutes. The samples immediately subjected to an ice bath, and the remaining activity measured to determine the stability of the enzyme.

Temperature stability of purified(ADI)Activity

Prior to the assay, the enzyme was also pre-incubated for 30 minutes at temperatures between 30 and 80°C. Subsequent to the thermal assay, the samples were quickly cooled using an ice bath to keep the enzyme fresh. The percentage activity was graphed using the temperatures to determine the heat resistance and critical concentration of the enzyme .

Determination of various ions and inhibitors effect on (ADI) activity

In order to test the effects of different chemical compounds on the (ADI) activity,the enzyme was incubated with various metal ions such as $FeCl_3$, NaCl, $ZnSO_4$, and $MnCl_2$, as well as the chelating agent EDTA,at a final concentration of 5mM at 37 °C for 30 minutes. As control, the same amount of the enzyme without any treatment was also incubated under similar conditions to measure 100% activity. On the basis of residual activity, the activators and inhibitors were checked accordingly (18).

In vitro Anticancer and Cytotoxicity Assay

The cell line, HCT-116, NCM460, was cultured using DMEM growth medium supplemented with 10% fetal bovine serum, antibiotics, and incubated at 37°C with 5% CO_2. The cytotoxicity test involved culturing cells to a density of 1×104 cells/well into a 96-well plate. After cell adhesion, the purified ADI concentration range of 12.5 to 800 µg/mL was prepared in arginine-free media, and the cells were incubated for 72 hours. Viability testing was done using the MTT test, where the formed formazan crystals were dissolved using DMSO.The resultant absorbance at 570nm was measured, and results were converted to percentage viability, which was determined by comparing treated cells to untreated controls (19)

Result and Discussion

A. Isolation and identification of *K. pneumoniae*

These isolates came from clinical and environment specimens, isolated by the use of selective differential agar media such as MacConkey agar, Eosin Methylene Blue agar, as well as Blood agar. After they were aerobically incubated at 37°C,lactose-fermenting isolates were detected based on their characteristic metallic sheen/pink color on EMB and later differentiated using MacConkey agar. The isolates from the genus Klebsiella were later detected based on their bright pink and mucoid colony characteristic on MacConkey agar and pale and gamma-hemolytic (non-hemolytic) colonies characteristic on Blood Agar media. Molecular identification was conducted using PCR with species-specific primers. Gel electrophoresis results confirmed the presence of 16 isolates of *K. pneumoniae*, as evidenced by identifying the target diagnostic bands. Recent studies verify that *K. pneumoniae* has a unique mucoid characteristic on a selective medium, and molecular PCR targeting 16S rRNA serves as a definitive tool for species identification (20).

B. Extraction of (ADI) from bacterial cells

Quantitative screening was performed to assess the arginine deiminase production potential of each K. pneumoniae isolated strain using their specific enzyme activity. Though all the isolates were found to be ADI producers, considerable variation was observed in their production levels, with specific activities in the range of 0.8-12 U/mg in the culture filtrate. Out of the tested isolates, the maximum production was found in isolate No. 5,with an optimum specific activity of 12 U/mg.Therefore, owing to higher enzymatic production, strain No. 5 was considered as the candidate strain for further research work.

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The degree of variability in arginine deiminase (ADI) specific activity observed among Klebsiella pneumoniae strains is likely linked with strain-regulated expression and metabolic processes impacting ADI production, as experienced in various microbial models demonstrating differing levels of enzyme production based on genetic and environmental sources. Optimization approaches have recently verified the influence of culturing and strain capabilities on the production of ADI, leading to optimal selection of strains maximizing enzyme production for various applications. This is in current agreement with reports on microbial variability in ADI production and its optimization for therapeutic enzyme production (21).

C. Purification of ADI Steps

3.1 Ammonium Sulfate Precipitation

Ammonium sulphate precipitation at 70% saturation caused a significant purification and enrichment of arginine deiminase activity. Following this step, the volume of the enzyme preparation was reduced from 100 to 20 ml with a corresponding increase in enzyme activity from 0.9 U/ml to 1.9 U/ml, while the level of protein dropped from 0.30 to 0.25 mg/ml, causing a significant increases in specific activity from 1.7 U/mg to 5.2 U/mg protein. The fold purification was therefore realized at 3.5 with a total activity of 39.5 U and a yield of 61.5%. Purification by ammonium sulphate precipitation is thus quite effective as a first step in concentrating arginine deiminase and removing as much as possible of the unwanted proteins Table 1.

The ammonium sulfate precipitation method proved efficient in enriching ADI activity and specific activity, as supported by recent studies which conclude that this method is an effective primary purification for microbial ADI production (22).

3.2 Ion-exchange chromatography

The chromatographic method using DEAE-cellulose led to a prominent separation of arginine deiminase from Klebsiella pneumoniae. It can be inferred from Figure 1 that contaminating proteins are separated in early fractions, marked by an early peak of protein absorbance at 280 nm and very low enzymatic activity. However, it can be seen in Figure 1 that enzymatic activity is primarily focused in a single sharp peak between fractions 46 and 57, marked by moderate protein absorbance. It can be inferred from the table of purification that this step raises the specific activity of arginine deiminase from 5.2 to 12 U/mg, representing 5.3-fold purification based on the initial crude extract, with an overall activity 25.2 U and 40.5% recovery. These observations clearly indicate that DEAE-cellulose chromatographic technique is an effective second-step method in protein purification, based on the differing negative charge of arginine deiminase and non-target protein molecules due to the nature of proven buffer requirements.

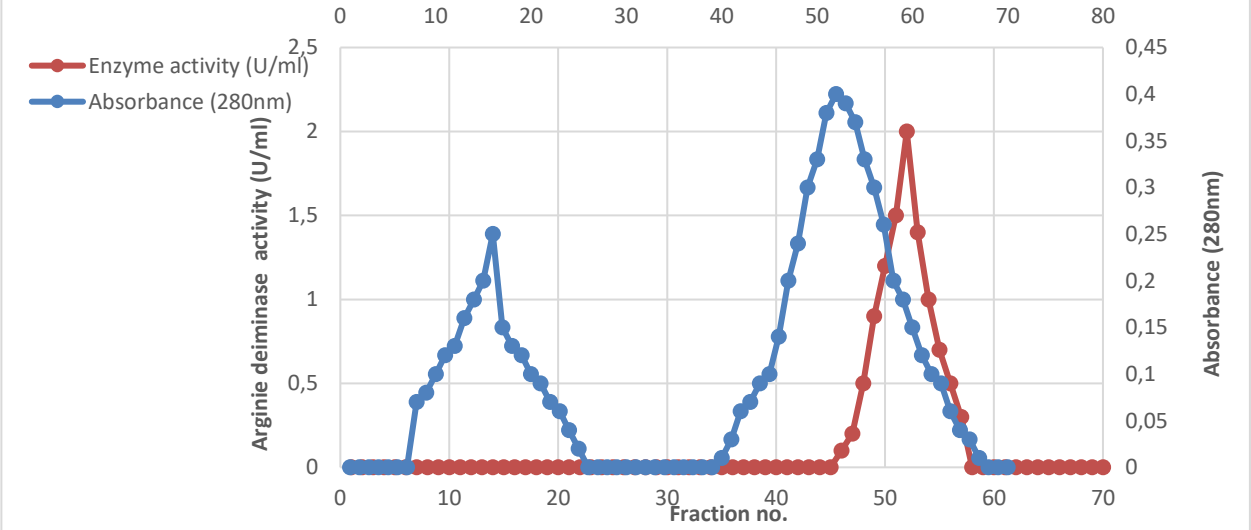

Figure1: Ion exchange chromatography of (ADI) produced by *K. pneumoniae* DEAE-cellulose column (2.5x20 Cm) with a flow rate of 30ml/hrs.

Ion exchange chromatography on DEAE cellulose proved efficient in the separation of arginine deiminase activity from the prominent contaminating proteins, taking advantage of the differential net charge of the molecules at the operative pH, resulting in a clear separation peak distinct from the early eluting samples. Consequently, there was a marked enhancement of the specific activity and purification fold, as would be expected in the current trends of ADI purification in microorganisms, where anion exchange chromatography is a pivotal step in the elevation of the purity of the enzyme before gel filtration (23). DEAE cellulose has been effectively incorporated into multifaceted purification techniques of ADI in fungi.

3.3 Gel filtration chromatography

Another round of purification of arginine deiminase from *Klebsiella pneumoniae* was done through Sephadex G-150 gel filtration chromatography Figure 2. Additionally, this method allowed for the separation of the desired enzyme and contaminants of lower and higher molecular weights as indicated by the elution profile, in which the major peak of enzyme activity correlated very closely with that of protein absorbance at 280 nm. The procedure led to an overall purification of 8.5-fold, with the specific activity of arginine deiminase increasing to 16.5 U/mg, with an overall activity of 24.4 U and an enzyme yield of 38.3%. The overall homogeneity achieved can be demonstrated by the fact that the elution peak of arginine deiminase activity became sharper, while absorption in other fractions became lower. This process implies that this method had high efficiency in removing contaminants, and following this treatment, high purity with moderate enzyme yield can be achieved. The method of gel filtration chromatography allows for protein separation based on their molecular weights without influencing enzymatic activities, and therefore, this method can be considered effective in handling arginine deiminase samples in this experiment by removing possible contaminants. This method has, in fact, high efficiency in removing contaminants, and therefore, this procedure can be considered an effective final purification step in arginine deiminase. In this case, it can be seen that this method allowed for high purification with an acceptable level of enzyme yield.

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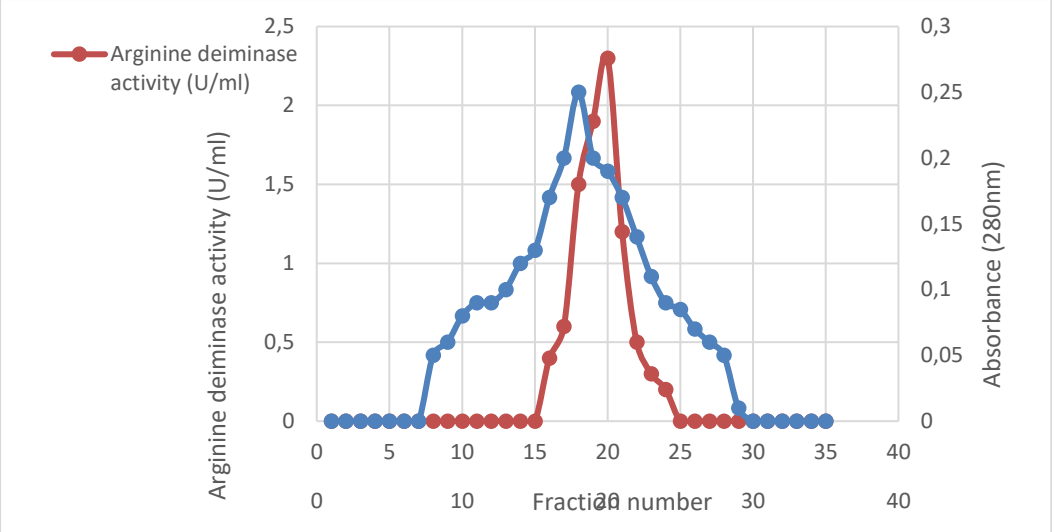

Figure2: Gel filtration chromatography of (ADI) produced by *K. pneumoniae* On Sephadex G150 column (1.5cmx35cm) with a flow rate of 30ml/hrs**.**

The position of elution peaks recorded in this study is consistent with a study carried out recently by that emphasized the superiority of Sephadex G-150 over others for achieving enzymatic homogeneity (24). Also reported recently was that size-exclusion chromatography is an important polishing step that greatly increases the specific activity of bacterial arginine deiminase. Also consistent with this study is the high recovery yield and purification fold recorded (25), which confirms that this technique is effective for maintaining the natural conformation of this enzyme and effectively separating high-molecular-weight bacterial contaminants.

Table 1: Purification steps for arginine deiminase produced by *K. pneumoniae*

Purification Steps	Volume (ml)	Enzyme Activity (U/ml)	Protein Concentration (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification (folds)	Yield (%)
Crude Enzyme	100	0.9	0.30	3.0	90	1	100
Ammonium Sulphate Precipitation 70%	20	1.9	0.25	7.6	38	2.5	42.2
DEAE-cellulose	18	1.2	0.10	12.0	21.6	4.0	24.0
Sephadex G150	16	2.3	0.07	32.9	36.8	11.0	40.9

Characterization of arginine deiminase(ADI)

Effect of pH on purified arginine deiminase(ADI) activity

The pH dependence of activity was determined for purified arginine deiminase within a pH range of 5–9 (Figure. 3). The purified enzyme was seen to have a significant pH dependence in activity, with maximum specific activity (1.8U/mg protein) at pH 7, implying that optimal activity for this enzyme takes place at near-neutral pH values. There was a progressive decrease in activity at both low and high pH values, however, at pH 6 and pH 8, there was a corresponding decrease in specific activity to 1.2 U/mg and 1.1 U/mg, respectively, with a marked decrease at pH 5 and pH 9 (8 U/mg). From these observations, it can thus be concluded that optimal biochemical and biomedical applications for purified arginine deiminase shall require near-neutral pH values and constitute firm evidence for understanding optimal intracellular bacterial pH values for potential applications in future research.

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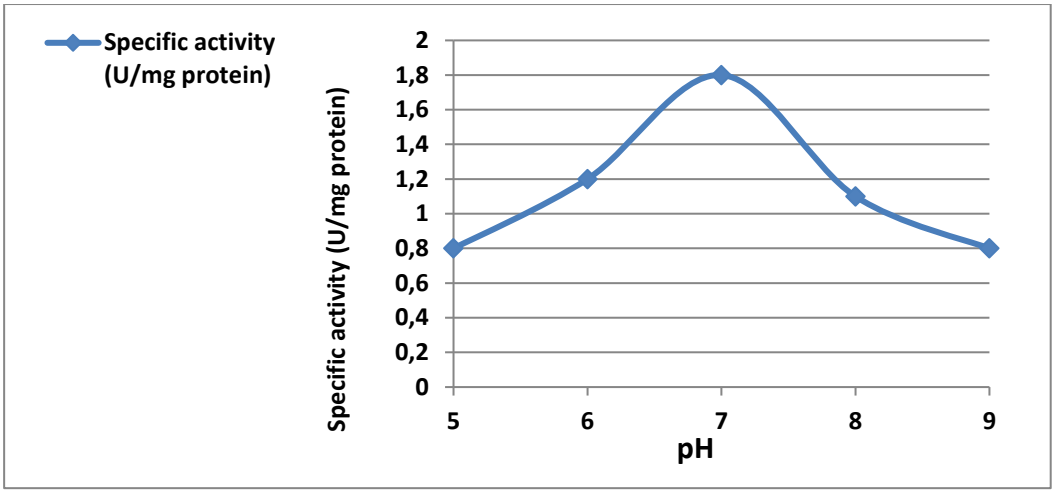

Figure 3: Effect of pH on the activity of purified arginine deiminase(ADI) produced by *K. pneumoniae*

The pH 7 was optimum for the purified enzyme corresponds well to the previous observations that the highest enzymatic activities for bacterial ADI occur around pH 7, based on the preference for the catalytic amino acids (26).

Optimum pH for arginine deiminase(ADI) stability

The enzyme with maximum stability had a pH 7 Figure 4, with 100% activity remaining relative to initial activity. The stability of the enzyme at pH 6 and 8 remained relatively high, with 95% and 80% activity, respectively. However, for higher and lower pH levels, higher drops in stability were recorded, with only 75% and 55% activity retention at pH 5 and 9, respectively. The above experiment, therefore, shows that a neutral environment is ideal for keeping this purified enzyme.

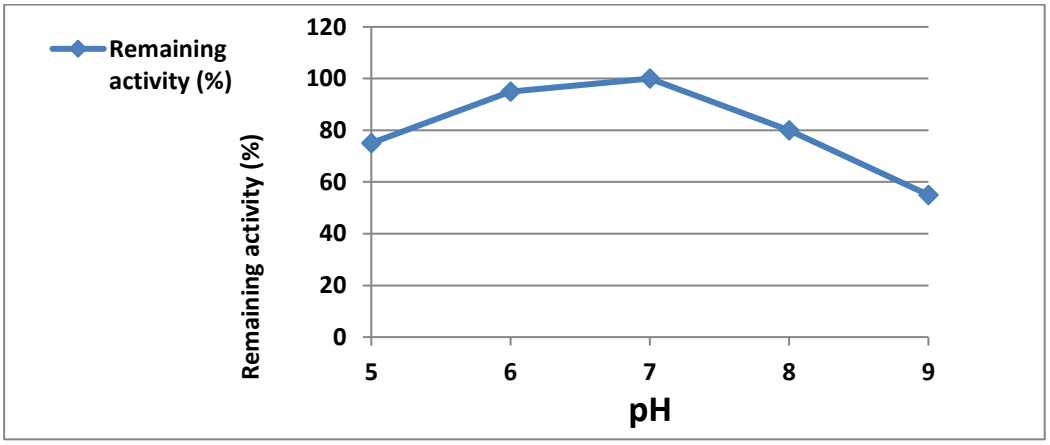

Figure 4: Effect of pH on stability of purified arginine deiminase(ADI) produced by *K. pneumoniae*

The optimal pH for arginine deiminase from a bacterial source and with regards to maximal stability is also consistent with previous research, which indicated that bacterial arginine deiminase remains optimally intact at pH 7 (27).

Effect of temperature on arginine deiminase activity

As indicated in figure 5, arginine deiminase activity gradually increased with a rise in temperature, attaining a plateau at 37 °C, which was determined to be the optimal temperature for catalytic activity. At temperatures below the optimal, the activities of the enzyme decreased, possibly because of lower molecular kinetics. At temperatures above 37°C, a slow decrease in enzyme activity was noticed, with a sharp decrease between 47 and 50°C, possibly because of the enzyme being denatured at higher temperatures. The findings indicate that the purified arginine deiminase reaches optimal activity at a physiological temperature and has moderate thermostability.

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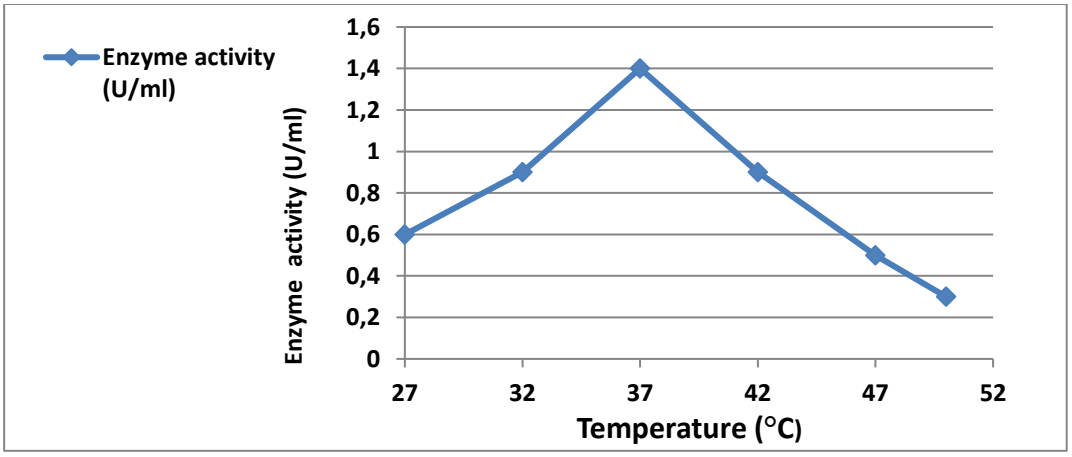

Figure 5: Effect of temperature on the activity of purified arginine deiminase(ADI) produced by *K. pneumoniae*

Optimum temperature at 37°C for arginine deiminase activity is in line with results from previous literature on bacterial ADI regarding optimal catalytic activity at biologic temperatures. This may be attributed to the adaptability of the catalyst at a cellular level and optimal turnover rates for substrates. Decreased enzyme activity at temperatures above 40°C may result from denaturation at high temperatures due to high turnover and changes in active-site structure, as seen with mesophilic bacterial enzymes (28).

Effect of temperature on(ADI) stability

On analysis of the heat stability pattern of Arginine Deiminase (ADI), it has been revealed that the enzyme maintains its shape and reaches its highest catalytic efficiency from 25°C to 40°C Figure 6. However, upon crossing the critical temperature threshold of 50°C, it begins to decrease steadily. Beyond the critical threshold temperature of 60°C, the enzyme becomes unstable rapidly; this could be because of the denaturation of its polypeptide molecules at higher temperatures, thus altering its active sites. Rising temperatures could be causing denaturation of its molecules, resulting in loss of activity.

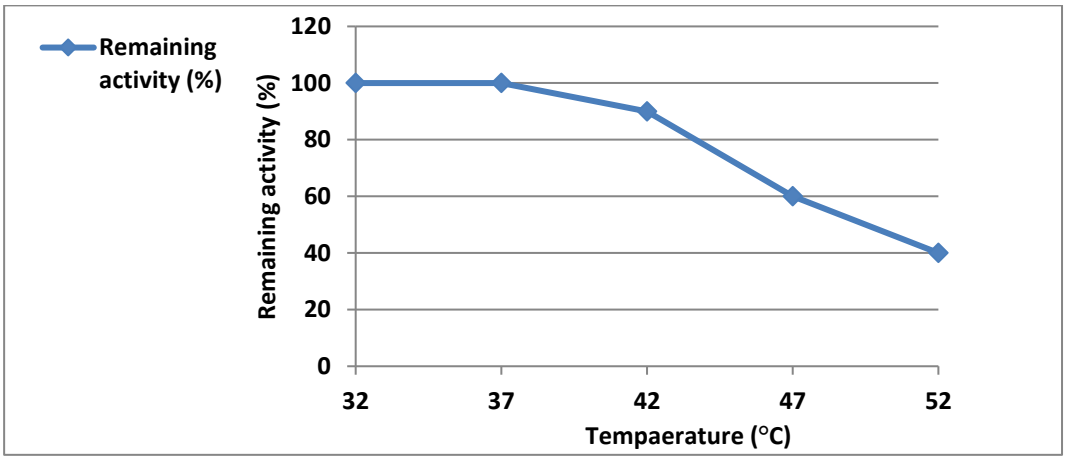

Figure 6: Temperature stability of arginine deiminase produced by *K. pneumoniae*

The heat stability profile indicates that ADI has retained its form and function until a maximum of 40°C but begins to dissociate well beyond the ranges of either 50-60°C. This corresponds perfectly to what mesophilic enzymes would behave like, where the polypeptide structure would denature at a relatively higher temperature. The extent of branching of activities of both bacteria and fungi ADIs corresponds to this trend, where both begin to shed a substantial level of their activities at temperatures beyond what would normally constitute body temperatures (29).

Effect of metal ions and inhibitors on arginine deiminase(ADI) activity

The experiment tested the effect of various 5mM chemicals on the enzymatic activity of ADI from *K. pneumoniae*. $MnCl_2$ and $FeCl_3$ were observed to activate the enzyme, increasing it by 120% and 105%, respectively, from the original baseline. EDTA, however, substantially decreased the enzyme's activity, retaining only 73% of it, which points towards ADI being a metalloenzyme. $ZnSO_4$ and NaCl, however, showed nominal effects, retaining almost all their original catalytic activities, around 92–95%. Show the in Table 2.

Table 2: Metal ions and inhibitors effect on arginine deiminase activity produce by *K.pneumoniae*

Reagent	Concentration (mM)	Remaining activity (%)
Control (Enzyme)		100
EDTA	5	73
$ZnSO_4$	5	92
NaCl	5	95

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$MnCl_2$	5	120
$FeCl_3$	5	105

The presence of Mn^{2+} and Fe^{3+} indicating an increase in ADI activity suggests the involvement of these ions as cofactors or allosteric activators in the enzyme, facilitating the enzyme to assume its active conformation. The inhibitory effect, especially by the strong chelator EDTA, suggests the binding of these ions to the enzyme and their involvement in stabilizing the enzyme structure. Similar effects in the presence and absence of these ions on the activity in the ADI enzyme in bacteria had been previously shown, emphasizing the important roles the ions play in the activity and stability.

Cytotoxic Activity of ADA Using MTT Assay

ADI demonstrated a dose-dependent cytotoxicity against HCT-116 colorectal cancer cells. Cell viability was successively lowered with an increment in the concentration of enzyme from 12.5 to 800 µg/mL (Table 3). In contrast, there was a gradual decline in cell viability, but NCM460 normal colon epithelial cells remained relatively viable in the same range, indicating that they are more resistant to ADI. Therefore, ADI induced selective cytotoxicity against the cancer cells. Viability is reported as mean % ± SD.

Table 3: Cytotoxicity effect of arginine deiminase on HCT-116 and NCM460 cell line

Concentration µg/mL-1	**Mean of cell viability (%) ± SD**	
	ADI on HCT-116 cell line	ADI on NCM460 cell line
800	40.20 ± 1.95	70.50 ± 1.40
400	49.60 ± 1.80	78.30 ± 1.55
200	58.40 ± 1.65	85.90 ± 1.60
100	70.80 ± 1.90	92.40 ± 1.45
50	82.90 ± 1.75	95.20 ± 1.30
25	93.60 ± 1.50	97.80 ± 1.10
12.5	96.40 ± 1.30	98.10 ± 0.95

The higher the concentration, the stronger the cytotoxic effect produced by ADI on HCT-116 cancer cells, whereas normal NCM460 cells experience fewer effects. The results highlight the selective anti-cancer use of ADI (31). The cancer cells sourcing more arginine from their environments and their reduced expression levels of arginosuccinate synthetase make them more susceptible to ADI (32). The selective killing effect was the same for microbial ADI in colorectal and other solid tumors. Being normal cells, they were more resistant to the drug (33). Collectively, the above findings confirm the therapeutic value and applicability of ADI.

The half-maximal inhibitory concentration (IC_{50}) values for the arginine deiminase (ADI) enzyme against the HCT-116 human colorectal carcinoma cells are around 390 µg/mL, and the values are higher than 800 µg/mL against the NCM460 human normal colon epithelial cells, indicating that the sensitivity to ADI treatment is lower in the normal cells.

Figure 7 illustrates the effects of arginine deiminase (ADI) on cell viability for two different cell lines: HCT-116 colon carcinoma cells and NCM460 cells that represent normal colon epithelium cells. Various concentrations (12.5 to 800 µg/mL) of ADI were used for these experiments, and all results are presented as means with standard deviations in % ± SD. The IC_{50} value was determined for HCT-116 cells; however, for NCM460 cells, 50% inhibition was not reached within that range.

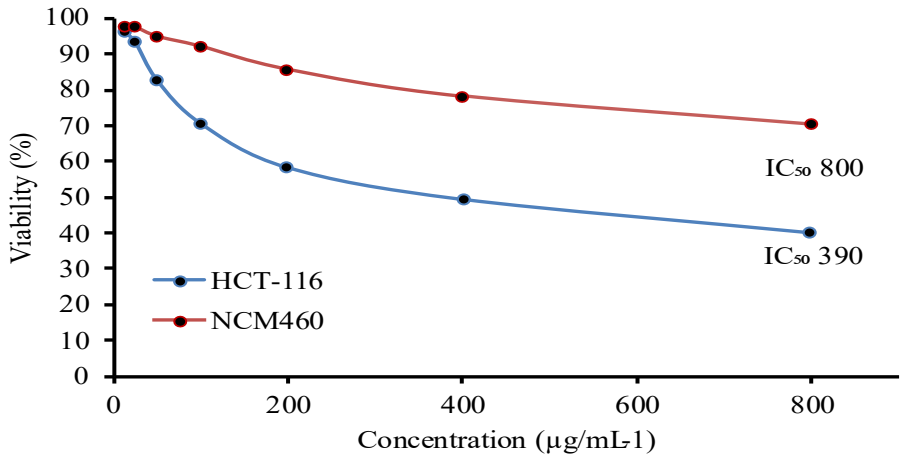

Figure 7. IC_{50} Effect of arginine deiminase on NCM460 and HCT-116 cell lines

Our data resonate with existing evidence that HCT-116 is still susceptible to ADI despite the presence of arginosuccinate synthetase (34). The most impressive part is that normal NCM460 cells are much more resistant to ADI, and their IC_{50} is above 800 µg/mL. This verifies the hypothesis that cells with ASS can still produce arginine from citrulline and survive ADI treatment, contrary to cancer cells that slumps into apoptosis (35). Instead, this therapeutic index reveals the promising capability of ADI to target cancer cells without harming normal cells (36), paving the way for its application in colorectal cancer treatment.

Conclusion

This study successfully to described the isolation, purification, and evaluation of the selective anticancer activity of arginine deiminase derived

from Klebsiella pneumoniae. Among the strains, the superior producer of this enzyme was isolate No. 5, having an activity of 12 U/mg and 11-fold purification achieved through three steps: ammonium sulfate fractionation,DEAE-cellulose chromatography,and Sephadex G-150 filtration, producing 40.9% purity activity with an activity of 32.9 U/mg. Biochemical analysis showed optimal activity at pH 7 and 37°C. Arginine deiminase activity was increased by Mn^{2+} and Fe^{3+} ions (by 120% and 105%, respectively) and inhibited by EDTA (73%), establishing it as metallo-protease enzymes.

Most significance to this topic, however, is that the ADI from this Klebsiella pneumoniae showed targeted cytotoxicity against HCT-116 colon cancer cells with anIC50 value of approximately 390 μg/mL,whereas normal colon cells (NCM460) were found to be largely resistant with an IC50 value above 800 μg/mL . This translates into a selective index of more than 2.3-fold. It is clear from these findings that cancer cells, which do not have ASS, are highly susceptible to arginine deprivation, while normal cells, which do produce ASS, are able to produce arginine and resist the drug. Overall, these findings make Klebsiella pneumoniae a potent candidate for a high-quality drug source with potent anticancer properties. The future directions for this drug, therefore, include PEGylation for increased half-life, exploring the pathways related to apoptosis, and exploring the possibility of combination therapies, which would help advance this arginine deprivation approach towards the treatment of colorectal cancer.

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