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Original Article

Effects of carbamate pesticides intermediates on *Escherichia coli* membrane architecture: An *in vitro* and *in silico* approach

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Abstract

Methyl isocyanate (MIC), a low molecular weight synthetic aliphatic compound, having an isocyanate group (−NCO), has industrial application. In this study, the effects of methyl isocyanate and its mechanism on outer membrane protein of *Escherichia coli* were observed using experimental and computational methods. *In vitro* exposure of N-succinimidyl N-methylcarbamate (NSNM) a synthetic analogue of MIC on *E. coli* to a final concentration of 2 mM was found to affect the growth curve pattern and changes in cell morphology. Molecular docking studies of MIC and NSNM with *E. coli* outer membrane protein (OmpW, OmpX, OmpF OmpA), and periplasmic domain (PAL) were performed. The *in-silico* results revealed that outer membrane protein OmpF showed the highest negative binding energy, i.e. ΔG -4.11 kcal/mole and ΔG -3.19 kcal/mole by NSNM and MIC as compared to other proteins. Our study concludes that methyl isocyanate retains lethal toxicity which leads to cell death due to the membrane protein damage of *E. coli* membrane.

Keywords: Methyl isocyanate, *Escherichia coli*, Toxicity, Membrane architecture, Autodock

Introduction

Methyl isocyanate is one of the isocyanates derivatives which has many industrial uses. Methyl isocyanates are characterized by the presence of extremely reactive -N=C=O group and have application in the manufacture of adhesives materials, polyurethane foam, coating constituents, plastics, paints, and different kinds of pesticides [1,2]. The generally used isocyanates are methyl isocyanate, diphenylmethane 4, 4'-diisocyanate, hexamethylene diisocyanate, toluene diisocyanate, 1, 5-naphthalene diisocyanate, and isophorone diisocyanate, and all of these are reported for a certain degree of toxicity [3,4]. Methyl isocyanate also exerts severe chronic and acute lethal effects on different organ system [5].

Methyl isocyanate is utilized as an intermediate in the production of carbamate pesticides which were accidentally released from the Union Carbide India Limited (UCIL) plant in the Bhopal gas tragedy, the world's most horrible industrial disaster [6-8]. Thousands of people died with pulmonary oedema, and thousand survived with various diseases like cancer, pulmonary, reproductive, ophthalmic, hematologic, tuberculosis, immunologic and neurological toxicity [9-11]. Numerous theories have suggested for the interaction of MIC with different cell lines MM55.K, B/CCMA.OV, NIH.3T3 and cultured human lymphocytes which justify its toxicological effect [12]. The rigorous mechanism of cell and tissue damage by isocyanate is to induce reactive oxygen species (ROS), which interacts with a different cellular molecule like proteins and nucleic acid, shows the toxic effect, i.e. DNA damage, apoptosis and oxidative stress [12].

Methyl isocyanate binds with glutathione and makes its way into the systemic circulation, affecting the red blood cell membrane, which explains its toxic activity [13,14]. Carbamylation effect of methyl isocyanate in brain, liver, kidney, and lung proteins, were also reported [15,16]. Cellular proteins interact with methyl isocyanate and alter their membrane permeability, damage membrane structure which leads to the leakage of cell contents, and as a result, cell death occurs [17]. The effect of this methyl isocyanate has been studied to a minimal extent in microorganisms, because of its extreme volatility and toxicity. The mechanisms of toxicity and its impact on natural membrane architecture, particularly microorganism, are still in the necessity of elucidation. *Escherichia coli* was selected as a model bacterium for exploring the effect of N-succinimidyl N-methylcarbamate (NSNM) a synthetic analogue of MIC, on cell membrane structure and how it creates pores leading to morphology disruption and consequently to cell death.

In the present study, we tried to understand the effect and action mechanism of methyl isocyanate on *E. coli membrane* architecture. The lethal effect of MIC on *E. coli* membrane architecture using atomic force microscopy (AFM) enables us to explore direct microscopy imaging of cell damage. The effect and action mechanism of methyl isocyanate on *E. coli* is revealed *via* AFM imaging. To detect cell morphology, preliminary observation employing growth curve assay, colonogenic assay and cell hydrophobicity assay are also performed. Further, *in silico* study was performed using autodock to identify and provide a mechanistic study of the lethal effect of MIC and NSNM, which directly or indirectly affect *E. coli* membrane protein leading to its damage.

Materials and Methods

The strain of *E. coli* MTCC No.87 was procured from microbial type culture collection and gene bank (IMTECH, Chandigarh), N-succinimidyl N-methylcarbamate NSNM (CAS No. 18342-66-0) was purchased from Sigma Aldrich Laboratories, St. Louis, USA, and all other reagents used are of analytical grade.

Growth curve assay

E. coli was grown overnight using nutrient broth at 37 °C. After incubation 0.2 mL of 1x108 CFU/ml suspension of *E. coli* was transferred to 20 mL of fresh media containing different concentration of NSNM from 0.25 mM to 20 mM. Suspension without NSNM was used as control. Optical density was measured at different time intervals (0, 2, 4, 6, 12 and 24 h) at 600 nm. An experiment was repeated thrice.

Clonogenic assay

Clonogenic cell survival assay was used to determine the ability of a cell to proliferate and to form a large colony or a clone. *E. coli* culture was treated with different concentration of NSNM from 0.25 mM to 20 mM for 4 h, followed by spreading on nutrient agar plates. Several growing *E. coli* colonies in nutrient agar plates was counted after 24 h of incubation at 37 °C [18]. An experiment was repeated thrice.

Cell surface hydrophobicity (CSH) assay

E. coli cell-surface hydrophobicity was determined as per the described method [19]. Briefly, after 24 h incubation, treated and control cultures of *E. coli* were harvested by centrifugation (5000x rpm for 20 min at 4 °C) and washed thrice with normal saline, resuspended and absorbance was measured at 600 nm (OD1). 5 mL of *E. coli* suspension was mixed with 1 mL p-xylene and vortexed for 1min and then incubated for 30 min without disturbing to ensure that the two liquids had separated into layers. The absorbance of the aqueous layer was measured at 600 nm (OD2). Experiment was repeated thrice. *E. coli* culture surface hydrophobicity was expressed as percentage hydrophobicity index (%HI) and calculated using the following formula.

$$\%HI = [1 - (OD1/OD2)] \times 100 \quad (1)$$

Atomic force microscope

Atomic force microscope (AFM) experiments were performed by using an NT-MDT NEXT Integrated Research Atomic Force Microscope, Russia. Two to three areas of each slide were scanned using the contact mode. The cantilevers tip with force constant of 10 N/m and have a typical resonance frequency of 250 kHz was used. A 2 mL of cell suspension was centrifuged at 4000 g for 5min, the cell pellet was resuspended in normal cold saline. Finally, 50 µL portion of the cell suspension was placed on a freshly cleaved glass substrate and dried [20]. The parameters such as surface roughness include mean roughness (Ra) and root mean square roughnesses (Rq) were calculated.

In-silico analysis

E. coli membrane proteins selected for the present study are outer membrane protein OmpW, outer membrane protein OmpX, outer membrane protein OmpF porin, outer membrane protein OmpA, and periplasmic domain PAL (Protein Data Bank (PDB) ID: 2F1T, 1QJ8, 2ZFG, 2GE4 and 1OAP respectively) whose three-dimension structure were obtained for docking from protein data bank and used as a target; water molecules are removed from the PDB file. The possible binding active sites in proteins were identified with the help of the CASTp server [21].

Preparation of ligand

N-Succinimidyl-N-Methylcarbamate (NSNM) and Methyl isocyanate (MIC) (PubChem CID 4381935 and CID 12228) were used as ligands in the present study and were retrieved as a Standard Delay Format (SDF) from Pubchem Compound Database. Ligands are converted into PDB format using open babel software [22].

Molecular docking

PyRx 0.8 a plugin of Autodock software were used for molecular docking simulations studies. The Lamarckian Genetic Algorithm (LGA) and grid-based energy evaluation method were used for the docking study. Water molecules were removed, and the protein structure containing only amino acid residues are considered for the study. Using an auto grid, grid points with 0.375 Å spacing were calculated around the docking area for all ligand atoms. The parameter used for docking calculation are mutation rate, crossover rate and population size; the best-docked conformations were obtained with a population size of 150 mutation rate of 0.02 and crossover rate of 0.8. Results were evaluated based on root mean square deviation (RMSD) and orientation. Dock energy is the sum of the intermolecular and the internal energies and is represent as ΔG. Further, the best pose was selected, which possesses the lowest estimated binding free energy [23].

Statistical analysis

All the analytical experiments were carried out in triplicates (n=3). Data were presented as mean ± standard deviation (SD). Statistical analysis was performed by Graph Pad Prism 5.0 (San Diego, CA, USA).

Results and Discussion

In the current investigation, we found that N-Succinamidyl N-Methyl Carbamate (a synthetic analogue of methyl isocyanate) has an impact on cell health, and so we wanted to explore the way in which it is hazardous. MIC may enter cell membranes and then cross cell membranes to get to distant organs [5]. Study findings related to DNA damage, cell cycle arrest, and apoptosis were critical in helping us better understand the toxicological consequences of NSNM. Genotypic susceptibility to many infectious illnesses, as well as other environmental exposures, necessitates extensive discussion. The study's aim was to determine the reaction profiles of *E. coli* to NSNM, and then to investigate how different the response patterns are across other strains. In response to treatment with a synthetic counterpart of methyl isocyanate, it was found that the proliferation of *E. coli* was diminished. It illustrates that at a concentration of 2 mM, there is a substantial growth rebound after 24 hours; at a concentration of 5 mM, the growth of *E. coli* is totally blocked and there is no growth rebound (Figure 1). Additional NSNM effects, *via* modulating the growth curve of *E. coli* by either influx or extrusion, may influence a cell's ability to acquire new mutations. Toxicants entering the organism's cells and influencing their cellular activity is required for modulation to occur. The findings of the current investigation demonstrate that exposure to a final concentration of 2 mM NSNM reduced the *E. coli* growth curve pattern by entrance into it.

We evaluated the growth-inhibitory effect of NSNM in a clonogenic cell survival test as well. During this experiment, various doses (0.25 mM to 20 mM) of NSNM were used to treat *E. coli* cultures for 4 hours, followed by spreading the bacteria over nutritional agar plates. Colonies were counted after 24 hours of incubation at 37 °C on nutrient agar plates. In these experiments, it was discovered that treatment with NSNM reduced the number of viable *E. coli* cells in a dose-dependent manner (Figure 2).

The effect of oxidative stress on surface hydrophobicity was studied by quantitative surface hydrophobicity assay. The surface hydrophobicity of *E. coli* plays an important role in adherence to host cells. Surface hydrophobicity was significantly decreased when subjected to exposure with different concentration of NSNM. 5mM NSNM exhibits higher variation in cell surface hydrophobicity as compared to others (Figure 3). The above discussion supports the understanding that NSNM generates oxidative stress and plays a central role in altering the morphology, which directly affects the adherence capacity of *E. coli*. In addition, the surface properties of treated *E. coli* were found to be altered, which indicates its morphological changes. Cell surface hydrophobicity studies help us to identify morphological changes. The results indicate that the overall hydrophobicity of the cell surface was greatly influenced by the presence of NSNM. Kustos et al. 2003 showed observed similar alterations [26].

AFM was used to visualize the effect of NSNM on *E. coli* cell membrane surface. An AFM image of an untreated (control) *E. coli* shows no visible pores or ruptures (Figure 4A). Changes in membrane surface of *E. coli* treated with 0.25 mM, 0.5 mM and 1 mM were observed (Figure 4B, C and D). Further ultrastructure microscopy of treated cells was performed to justify our finding; exposure of the cells to 0.25 mM NSNM induced minor perturbations on *E. coli* in comparison to control cells. Membrane blebbing and collapse at the apical end of the bacterial envelope were observed upon treatment with 1mM NSNM [27]. Topographical parameter like surface roughness includes mean roughness (Ra), and root-mean-square roughness (Rq) was observed. The interaction of NSNM with cell membrane leads to an increase in surface roughness; which is the evidence of membrane damage (Table 1) [28].

The results obtained after docking showed strong binding affinities with MIC and its analogue NSNM (Table 2). Out of the five outer membranes protein selected, three proteins, namely 2ZFG, 2F1T and 2GE4, showed significant binding interactions with ligands (Figure 5). In outer membrane protein OmpF complex with NSNM, a strong hydrogen bonding interaction was observed with the ARG140 and SER142 amino acid residues as shown in (Figure 5A). Whereas outer membrane protein OmpF complex with MIC, a strong hydrogen bonding interaction was observed with the ARG100 and ARG132 amino acid residues as shown in (Figure 5F). In order to identify a protein involved during the membrane toxicity,

we have performed molecular docking using Autodock 4.0. The results showed that outer membrane protein OmpF shows highest negative binding energy, i.e. ΔG -4.11 kcal/mol and ΔG -3.19 kcal/mol by NSNM and MIC respectively when compared to other proteins [7]. Thus, OmpF, which is involved in the passage of solutes such as sugars, ions, and amino acids which are crucial for entry into survival mode in stress condition, was found to be inhibited. Thus, the above study infers that NSNM and MIC cause cell death due to membrane protein damage of *E. coli*.

According to the findings, the toxicity of methyl isocyanate on *E. coli* was discovered. Additionally, *E. coli* was shown to have toxic effects that restrain growth in the presence of NSNM. The *E. coli* show decreasing vitality after they have been treated with NSNM. Methyl isocyanate is toxic to cells because it increases hydrophobicity on the bacterial cell surface, and the damage it causes is thus determined by calculating the effects of *in vitro* exposure to NSNM on cellular oxidative stress, which in turn produces cell harm. *E. coli's* surface has been harmed by exposure to NSNM, according to AFM investigation, and support the significant hazardous potentials of NSNM. The presence of increased surface roughness means that the membrane has been exposed to oxidative stress, allowing a cellular component to be released from the cell envelope. According to the binding contact capacities of NSNM and MIC with membrane proteins, the findings are supported.

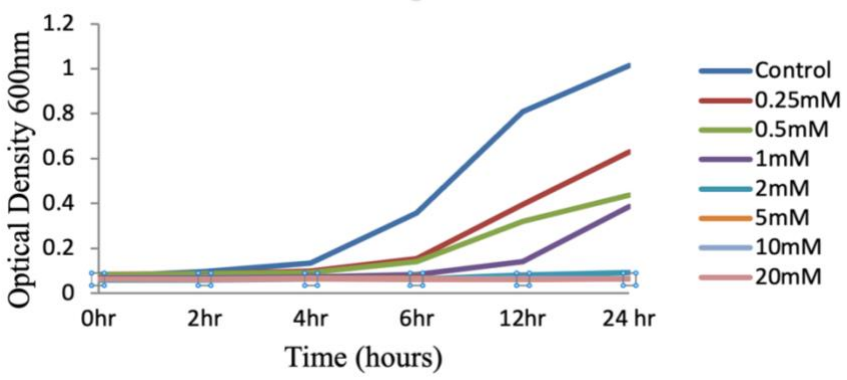

Figure 1. Growth curves assay: *E. coli* treated with 0.25mM to 20mM concentrations of NSNM along with control.

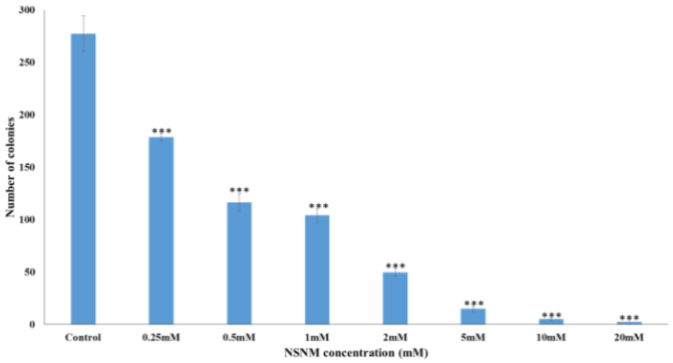

Figure 2. Clonogenic cell survival assay; number of colonies count on *E. coli* nutrient agar plate. Values are standard deviation of three independent plates (*P<0.05, **P<0.01, ***P<0.001).

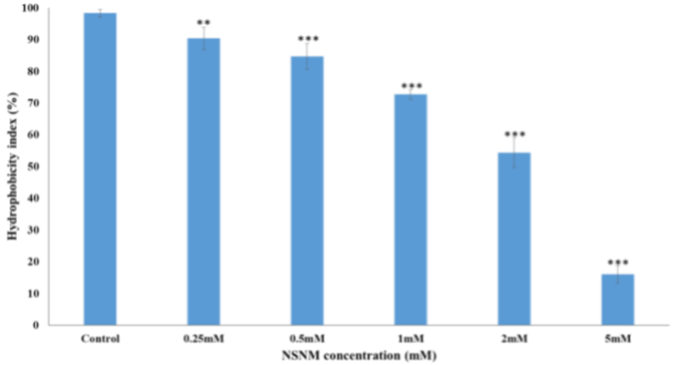

Figure 3. Cell Surface Hydrophobicity (CSH) Assay; percentages of hydrophobicity index of treated and *E. coli* culture values are standard deviation of three independent hydrophobicity indexes (*P<0.05, **P<0.01, ***P<0.001).

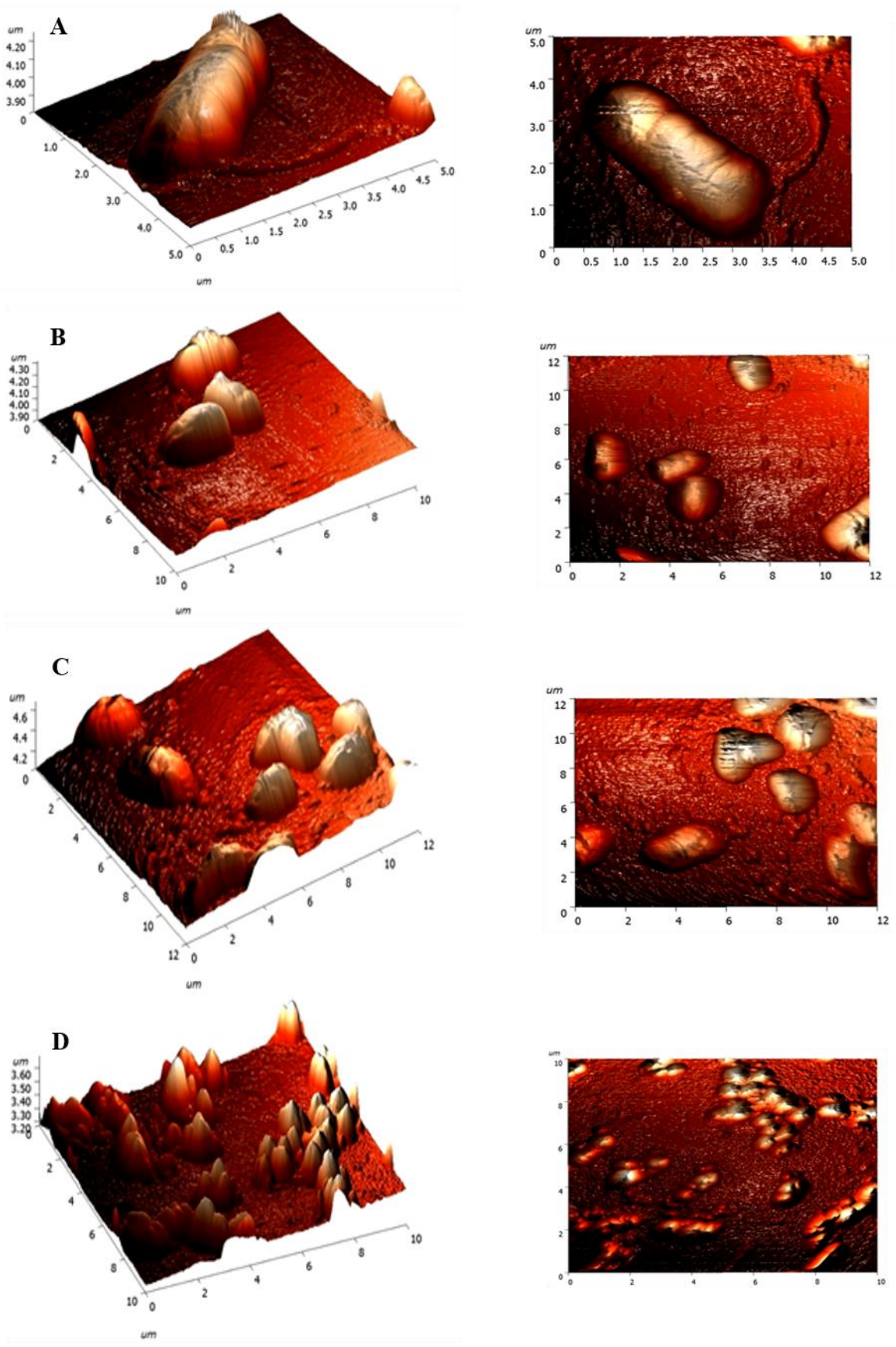

Figure 4. Atomic force microscopic images-AFM 3D images (left) and 2D images (right) of *E. coli* control culture: (A) and treated with NSNM concentration such as 0.25mM (B); 0.50mM (C); 1mM (D).

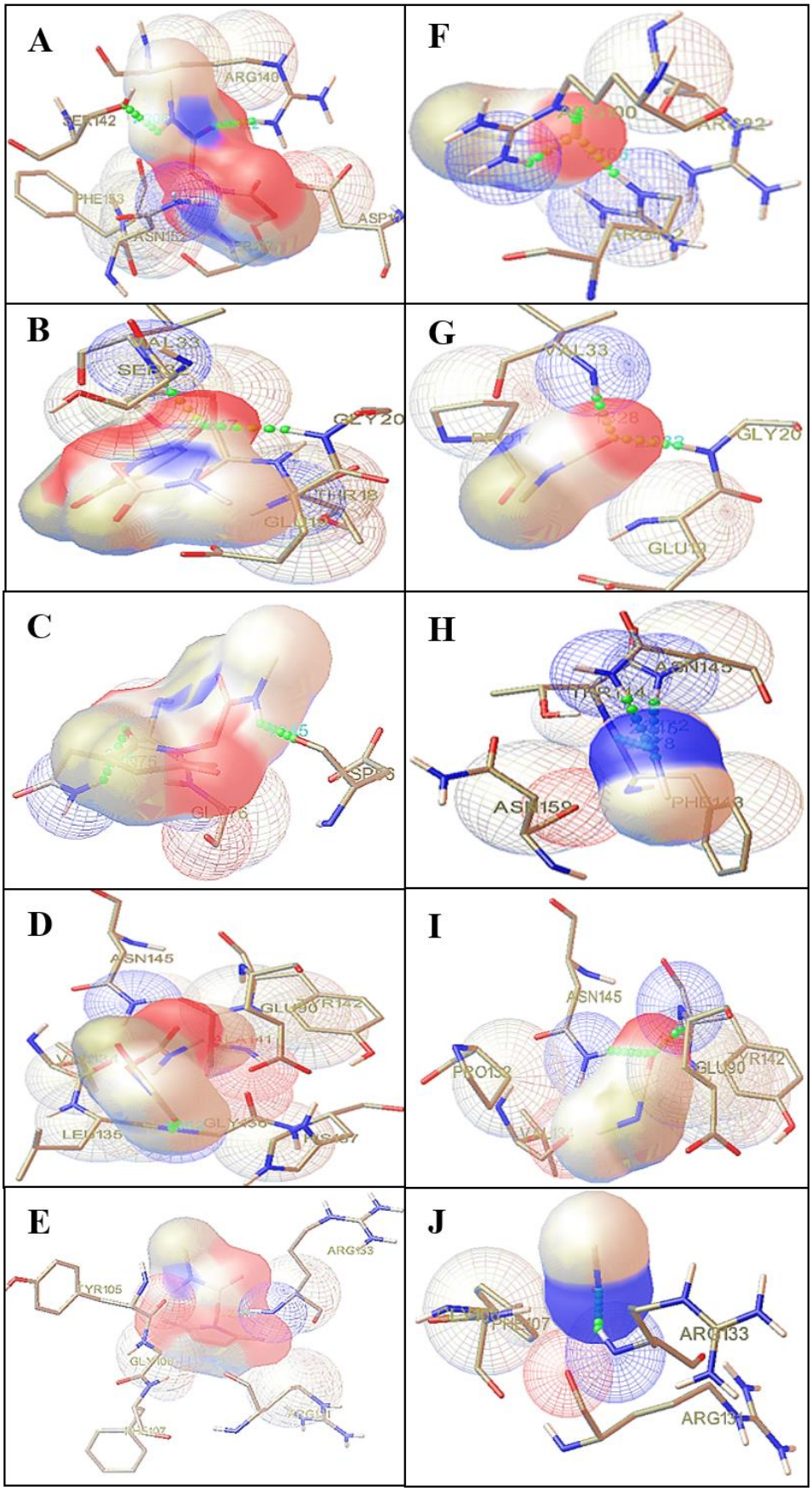

Figure 5. Protein ligand interaction: A-E indicates interaction between N-succinamidyl N-methyl carbamate (NSNM) with protein 2ZFG (A); 2F1T (B); 2GEM (C); 1OAP (D), 1QJ8 (E). F-J indicates interaction between methyl isocyanate (MIC) with 2ZFG (F); 2F1T (G); 2GEM (H); 1OAP (I); 1QJ8 (J).

Table 1. Surface roughness analysis of *E. coli* culture.

Sample	Rq (RMS profile deviation)	Ra (Arithmetic roughness)
Control	14	12
0.25mM	26	21
0.5mM	49	47
1mM	95	85

Table 2. Results of molecular docking.

Ligand	NSNM				MIC			
Protein Name & ID	BE (kcal/mol)	IC (mM)	AAR	BL (Å)	BE (kcal/mol)	IC (mM)	AAR	BL (Å)
OmpF	-4.11	0.969	ARG140	2.022	-3.19	4.59	ARG100	1.766
2ZFG			SER142	2.432			ARG132	1.953
OmpW	-3.9	1.38	GLY20	3.06	-3.13	5.12	GLY20	3.13
2F1T			VAL33	2.85			VAL33	3.24
OmpA	-3.72	1.88	GLY75	2.245	-3.01	6.22	ASN145	2.045
2GE4			ASP56	2.043			THR114	2.178
							PHE143	1.176
Pal	-2.84	8.29	GLY142	1.649	-2.74	9.88	GLU90	1.649
1OAP			ALA135	1.662			ASN145	1.856
			LEU136	1.861				
OmpX	-2.47	15.42	ARG 133	2.239	-1.86	43.11	ARG133	2.143
1QJ8								

BE – Binding Energy, IC – Inhibition Constant, AAR – Amino Acid Residue, BL – Bond Length.

Conclusions

Studies on the NSNM and MIC proteins concluded that the outer membrane protein showed high binding affinities with NSNM and MIC. Additionally, researchers found that outer membrane protein (OmpF) is beneficial in survival mode under stress.

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Conflict of interest

The authors declare that they have no conflict of interest.

CRediT author statement

PS: Conceptualization, Methodology, Writing- Original draft preparation; MKS: Investigation, Data curation, Writing- Original draft preparation; AR and MY: Visualization, Investigation; RS: Supervision, Writing- Reviewing and Editing.

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