RESEARCH ARTICLE

Recovery of Stressed *Escherichia coli* Culturability using Selective Media with Penicillin Binding Proteins for Bioburden Testing

Baswa J Kumar, R G Prasuna*

Department of Microbiology and Food Science and Technology, GITAM Institute of Science, GITAM Deemed to be University, Visakhapatnam, Andhra Pradesh, India.

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ABSTRACT

This study aimed to assess the effectiveness of selective media enriched with Penicillin-binding proteins (PBPs) in bioburden testing and the recovery of stressed *Escherichia coli.* Five distinct *E. coli* strains were subjected to experimentation utilizing two antibiotics to induce stress, simulating real-world scenarios where bacteria face various stressors, including antibiotics. The deliberate stress induction aimed to observe how stressed bacteria respond and recover in the presence of PBPs, vital cell wall components targeted by antibiotics. Multiple antibiotics ensured a comprehensive evaluation of stress responses. PBP1 tended to have a milder inhibitory effect on bacterial growth compared to both PBP2 and PBP3 across various growth media and strains. PBP2 demonstrated variable effects, displaying a nuanced impact depending on the growth medium and strain. In contrast, PBP3 consistently exerted a stronger inhibitory effect on bacterial growth. The strain of *E. coli* exhibited a significant influence on growth characteristics, reflecting strain-dependent responses to experimental conditions. Growth media and specific PBPs also displayed notable effects on colony forming units (CFUs) and percentage recovery (PR). Modified media generally supported higher CFUs and PR compared to standard media, showcasing the importance of media composition. Buffer choice also significantly impacted CFUs and PR, underlining the need for careful buffer standardization. Overall, the type of PBP used significantly affected *E. coli* colony numbers, as indicated by a highly significant *p-value* ($p \approx 2.04 \times 10\text{-}16$). This study's insights into the impact of selective media, PBPs, and bacterial strains on stressed *E. coli* recovery contribute to optimizing recovery protocols in microbiological studies. Comparisons between penicillin and cefixime treatments revealed higher CFU values in modified media, indicating their efficacy. Different PBPs showcased variations in CFU and PR, emphasizing their role in stressed *E. coli* recovery. The study sheds light on the multifaceted interactions between antibiotics, PBPs, media, and bacterial strains, vital for understanding antibiotic responses. These findings provide critical insights into designing effective recovery strategies and advancing microbiological studies.

Keywords: Colony forming units, Antibiotic stress, Percentage recovery, Buffer composition, Strain-dependent variability, Bacterial recovery.

Highlights of the study

- Novel Approach to recover stressed *Escherichia coli* populations using selective media enriched with penicillin binding protein (PBP), a key enzyme in cell wall synthesis.
- Selective media enriched with PBPs significantly affected stressed *E. coli* recovery. PBP1 showed a milder inhibitory effect on bacterial growth compared to PBP2 and PBP3.
- The specific *E. coli* strain used in the experiment significantly influenced bacterial growth and responses to the experimental conditions. Variations in growth characteristics were observed among different strains, underlining a strain-dependent aspect of the experiment.
- Different growth media exhibited substantial variations in colony forming units (CFUs) and percentage recovery (PR). Modified media generally supported higher CFUs and PR compared to standard media, emphasizing the importance of media composition.
- Choice of buffer significantly impacted CFUs and PR. MRB2 showed higher CFUs, indicating the importance of buffer selection in experimental outcomes.
- Comparative analysis of PBPs (PBP1, PBP2, PBP3) underscored their varied impacts on bacterial growth. PBP2 showed a nuanced effect, while PBP3 consistently demonstrated a stronger inhibitory effect.

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**Author for Correspondence: gravi@gitam.edu*

INTRODUCTION

Escherichia coli is a common gram-negative bacterium widely used as an indicator organism for assessing microbiological quality in various industries, including pharmaceuticals, biotechnology, and food processing.[1,2] In the manufacturing process of pharmaceuticals or medical devices, *E. coli* may encounter stress conditions, such as exposure to antibiotics or adverse environmental factors. These stressors can impact bacterial growth and culturability, leading to challenges in accurately detecting and quantifying *E. coli* in bioburden analysis. When *E. coli* is exposed to these stress conditions, it can activate various stress response pathways to adapt and survive. These responses may include changes in gene expression, production of stress proteins, alterations in cell membrane composition, and activation of repair mechanisms.[3-5]

Conventional culturing methods may result in false-negative results if stressed *E. coli* fail to grow or form visible colonies on standard growth media. The use of selective media containing specific components can be beneficial in such cases, as it allows for the recovery and detection of stressed or antibiotic-resistant *E. coli* strains, improving the accuracy of bioburden analysis.[6] The selective media designed for the recovery of stressed *E. coli* must effectively suppress the growth of non-stressed or susceptible bacteria, while simultaneously facilitating the growth and detection of stressed or antibiotic-resistant *E. coli* strains. These media must be meticulously formulated to establish a specific environment that fosters the targeted enrichment of the desired bacteria, enabling researchers to focus their investigations on stressed *E. coli* populations.[7,8]

Penicillin binding proteins (PBPs) are a group of bacterial enzymes that play a crucial role in cell wall synthesis and maintenance. They are the target of beta-lactam antibiotics, including penicillins and cephalosporins, which interfere with their activity, leading to bacterial cell wall disruption and, ultimately cell lysis. PBPs are essential for bacterial survival and growth, making them important targets for antibiotic action.[9,10] The process of bacterial cell wall synthesis involves the formation of peptidoglycan, a complex mesh-like structure that provides rigidity and shape to the bacterial cell. PBPs are involved in catalyzing the final steps of peptidoglycan synthesis, where they cross-link the peptide side chains of adjacent peptidoglycan strands. When β-lactam antibiotics, such as penicillins, bind to PBPs, they inhibit their transpeptidase activity, preventing the cross-linking of peptidoglycan chains. This weakens the bacterial cell wall, making it more susceptible to osmotic pressure and causing the bacterium to lyse.[11,12]

The main classes of PBPs include high molecular weight PBPs involved in transglycosylation (PBPs 1a, 1b, 1c) and low molecular weight PBPs involved in transpeptidation (PBPs 2, 3, 4, and 5). Different bacterial species may have various PBPs with specific functions in cell wall synthesis. PBPs are crucial determinants of bacterial susceptibility to β-lactam antibiotics.[13] Bacteria can develop resistance to β-lactams through various mechanisms, including the production of β-lactamase enzymes that inactivate the antibiotics, mutations in PBPs that reduce antibiotic binding, and changes in cell wall structure to decrease antibiotic permeability.[14,15] The recovery of stressed *E. coli* culturability using selective media containing PBPs is an area of interest in microbiology, particularly in the context of bioburden analysis and antibiotic resistance studies. This selective media can be designed to contain beta-lactam antibiotics (e.g., penicillins or cephalosporins) at concentrations that normally inhibit susceptible bacteria growth.[16] The incorporation of beta-lactam antibiotics in the growth medium can inhibit the growth of susceptible *E. coli* strains, while the presence of PBPs in the media allows the growth and recovery of antibiotic-resistant *E. coli* strains that produce beta-lactamase enzymes.[17]

Considering the requirement for a novel selective media to address the recovery of stressed *E. coli* in bioburden analysis, the present study is undertaken. This research aims to design and develop a specialized growth medium that can selectively enrich for stressed or antibiotic-resistant *E. coli* strains while inhibiting the growth of non-stressed or susceptible bacteria. This selective media's formulation involves carefully considering the appropriate concentrations of beta-lactam antibiotics, such as penicillins or cephalosporins, to effectively inhibit the growth of susceptible *E. coli* strains. Additionally, the inclusion of PBPs in the media is essential to enable the growth and recovery of antibiotic-resistant *E. coli* strains capable of producing beta-lactamase enzymes.[18,19]

This research aims to create a highly specific environment that favors the detection and quantification of stressed *E. coli* populations, ultimately enhancing the accuracy of bioburden analysis in industries such as pharmaceuticals, biotechnology, and medical device manufacturing. The successful development of this novel selective media has the potential to provide valuable insights into the dynamics of antibiotic resistance and bacterial response to stress conditions, contributing to advancements in microbiological research and infection control practices.

MATERIALS AND METHODS

Test Microorganisms

Mother cultures of *E.Coli* ATCC 8739, ATCC 11229, ATCC 25253, ATCC 25922 & ATCC 11775 were procured from the American Type Culture Collection (Rockville, Md.). These cultures were routinely maintained trypticase soy agar (TSA) slants at 48C (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and were stored in 1% w/v peptone water containing 40% v/v glycerol at -80°C. Peptone from meat (bacteriological), peptone from casein (cow's milk), potassium dihydrogen phosphate, disodium hydrogen phosphate dihydrate, sodium chloride, polysorbate-80, dextrose, sodium pyruvate, pancreatic digest of casein, papaic digest of soybean, agar, and soluble starch were obtained from Sigma-Aldrich Chemicals Private Limited, Bangalore, India.

Nutrient Broth for Initial Culture Preparation

Soybean–casein digest broth (SCDB) is a common and versatile liquid medium used in microbiology to prepare and cultivate bacterial cultures. It provides a rich source of nutrients that support the growth of a wide range of microorganisms.[20] Soybean–casein digest broth is prepared by dissolving appropriate quantities of the components (Table 1) in distilled water and adjusting the pH to around 7.3 ± 0.2 at 25°C. The mixture is then sterilized through autoclaving at 121°C for 15 minutes.

Standard Growth Media for Maintenance and Propagation of *E. coli*.

Soybean–casein digest agar (SCDA) is a widely used medium to sustain and cultivate *E. coli* cultures. It provides a rich and nutritionally complete environment, supporting the robust growth of not only *E. coli* but also various other bacteria.[21] Preparing SCDA involves adding agar to achieve a final concentration of 1.5% in SCDB. Initially, agar is dispersed in a small amount of distilled water in a separate container. This mixture is heated and stirred until the agar completely dissolves. The resulting agar solution is then mixed with the SCDB, ensuring an even distribution. The pH is adjusted to approximately 7.4 ± 0.2 using 1M NaOH. The agar solution is poured into appropriate containers and sterilized by autoclaving at 121°C for 15 minutes. Storing SCDA plates and broth in a cool, dry place, away from direct sunlight, is crucial.

Maintenance and Propagation of *E. coli*

The acquired cultures were consistently maintained on SCDB and stored in 1% w/v peptone water supplemented with 40% v/v glycerol at -80°C to ensure their viability. To maintain the genetic stability and vitality of the *E. coli*, regular sub-culturing involved transferring a small portion of the frozen stock into fresh growth media like SCDB or SCDA. This process was crucial in sustaining the culture's viability and genetic consistency. The subcultured *E. coli* were incubated at the optimal temperature, typically around 37°C, until visible bacterial growth, in the form of a lawn or colonies, was evident. For short-term usage, subcultures were refrigerated at 4°C, usually for up to a week. An appropriate growth medium, such as SCD broth or agar was utilized, well-known for supporting the robust growth of *E. coli*. The growth medium was inoculated with a small portion of the *E. coli* culture, ensuring adherence to aseptic techniques during the inoculation process. Subsequently, the inoculated medium was incubated at the optimal growth temperature for *E. coli*, typically maintained at around 37°C. The growth progress of *E. coli* was vigilantly monitored by observing changes in turbidity for liquid medium or the emergence of colonies on agar plates. For the continued propagation of the culture, a small portion of the developed culture was transferred into a fresh medium. This step was critical in maintaining an actively growing culture, vital for subsequent experiments and analyses.

Table 1: Composition of soybean–casein digest broth

S. No.	*Component*	*Concentration (gm/litre)*
1	Pancreatic digest of casein	17.0
2	Papaic digest of soybean meal	3.0
3	Sodium chloride	5.0
4	Dibasic potassium phosphate	2.5
5	Dextrose monohydrate/Anhydrous	2.5
6	Purified water	q.s

Table 2: Composition of different buffers

S.No.	*Composition*	*Standard buffer*	*MRB1*	*MRB2*
1	Potassium dihydrogen phosphate (g)	3.6	1.8	0.9
2	Disodium hydrogen Phosphate dihydrate (g)	7.2	3.6	1.8
3	Sodium chloride (g)	4.3	2.15	1.075
4	Peptone (meat or casein) (g)	1.0	1.0	1.0
5	Polysorbate-80 (%)		0.5	1
6	Dextrose (g)		1.25	2.5
7	Sodium pyruvate (g)		0.6	0.3
8	Purified water (mL)		1000	1000

Standard Buffer: Buffered Sodium Chloride–Peptone Solution pH 7.0; MRB1: Modified resuscitation Buffer-1 pH 7.0; MRB2: Modified resuscitation Buffer-2 pH 7.0

Preparation of Stressed Cells using Antibiotics

Stressing *E. coli* is a standard practice in microbiology to unravel their responses to various challenges. Antibiotic stress is particularly insightful for understanding resistance mechanisms. Two distinct antibiotic solutions were carefully prepared, totaling 12 liters collectively, 6 liters of each. One solution featured a concentration of 4 µg/L of penicillin, while the other contained 6 µg/L of cefixime. Sterile water was used diligently in the preparation of both solutions.[22] Aseptically, 6 mL of *E. coli* from the initial stock solution were inoculated into each antibiotic solution with a concentration of 10×10^2 cells/mL. Consequently, this process resulted in a final concentration of 1 colony-forming unit per milliliter (CFU/mL). The mixture was left to contact for 30 minutes. After this 30-minute contact time, three types of PBP (PBP1, PBP2, and PBP3) were aseptically transferred to the mixture. After this allowed stand for 30 minutes and 100 mL multiplied by 48 of the inoculated samples were aseptically collected for bioburden testing. To ensure accuracy and consistency, all subsequent testing was performed in triplicates.

Bioburden Testing

Various buffers and media were carefully prepared to aid in the recovery of stressed bacteria, as detailed in Tables 2 and 3. Furthermore, Tables 4 and 5 provided a comprehensive overview of the bioburden testing process, encompassing both routine and modified buffers and media, with and without PBPs. The testing initiation involved pre-wetting the membrane with 50 mL of buffered sodium chloride–peptone solution pH 7.0. Subsequently, a vacuum filtration system filtered 100 mL of the bulk solution through the membrane. The membrane was rinsed thrice with 100 mL of buffered sodium chloride–peptone solution. The membranes were then placed onto the specified

Table 3: Composition of different media used for bioburden testing

S. No.	*Composition*	*SCDA*	*MM-I*	*MM-II*	*MM-III*
1	Pancreatic digest of casein (g)	15.0	3.75	7.5	15.0
2	Papaic digest of soybean (g)	5.0	1.25	2.5	5.0
3	Sodium chloride (g)	5.0	2.5	5.0	2.0
4	Starch, soluble	1.0	0.5	1.0	1.2
5	Sodium pyruvate (g)		0.3	0.6	5.0
6	Agar (g)		15.0	15.0	15.0
	pH	7.3 ± 0.2	7.3 ± 0.2	7.3 ± 0.2	7.3 ± 0.2

SCDA: Soybean–Casein Digest Agar; MM1: Modified Media-I; MM2: Modified media-II; MM3: Modified media-III

media plates that had been pre-incubated suitably. Finally, the media plates were incubated at 30 to 35°C for 5 days to complete the testing process. After incubation, the colonies that had grown on the selective media plates were counted. The colony-forming units (CFUs) were compared with the initial number of stressed *E. coli* cells to assess the recovery of culturability.

Colony Counting and Analysis

After incubation, the colonies that grew on the selective media plates were counted. The CFUs were calculated and compared with the initial number of stressed *E. coli* cells to evaluate the recovery of culturability using a Digital 3 Digit LCD colony counter (Model No: ACK-3; AS one corporation, Japan). The growth patterns and characteristics of colonies on the conventional and selective media were compared. The selectivity and specificity of the new medium for the targeted bacteria were assessed.

Control Experiments

Relevant control groups were included to validate the specificity of the observed effects from the selective media with PBPs. This encompassed unstressed *E. coli* cultured on standard non-selective media, and stressed *E. coli* cultured on selective media without the addition of penicillin. These controls were essential to accurately discern and attribute the effects to the selective media containing PBPs.

Statistical Analysis

All results are reported as mean ± SD (n = 3). Statistical analysis involved comparing colony counts and other pertinent parameters between the conventional and new selective media. Statistical tests, including the t-test and ANOVA, were utilized to ascertain significant differences in colony counts or any other measured variables.

RESULTS AND DISCUSSION

The study aimed to evaluate the effectiveness of selective media enriched with PBPs in bioburden testing and stressed *E. coli* recovery. Five *E. coli* strains were subjected to the experiment using two distinct antibiotic solutions to induce stress. The experiment was conducted over a predetermined duration, and meticulous data analysis was performed on CFUs and percent recovery values. The mean ± SD (n = 3) was used for presenting the results.

Different antibiotics were a strategic choice to induce stress in the bacterial samples, simulating real-world scenarios where bacteria encounter various stressors like antibiotics. The deliberate stress induction allowed observing how the stressed bacteria responded and recovered in the presence of PBPs. Multiple antibiotics ensured a comprehensive evaluation of stress responses. Results were presented separately, delineating the influence of each antibiotic on the recovery of stressed *E. coli* culturability, enabling a thorough analysis of different stressors' impact on bacterial recovery.

Table 4 provides an in-depth analysis of the response of *E. coli* to penicillin treatment, highlighting the distinct growth patterns of stressed *E. coli* on selective media with PBPs compared to control groups. It underscores the efficacy of the selective medium in recovering stressed *E. coli*.

Table 5 similarly offers a detailed examination of the response of *E. coli* to cefixime treatment, accentuating the unique growth patterns of stressed *E. coli* on selective media with PBPs in contrast to control groups. It reinforces the effectiveness of the selective medium in recovering stressed *E. coli*.

Statistical analysis compared CFUs and percent recovery (PR) between different media, buffers, and PBPs presence. Notably, PBPs in the media significantly influenced stressed *E. coli* recovery, with variations in CFUs and PR. PBP1, PBP2, and PBP3 displayed distinct impacts on recovery, emphasizing their potential role in aiding recovery under stressful conditions induced by penicillin. Buffer composition's significance was also highlighted. These findings underscore the importance of selective media and PBPs in facilitating the recovery of stressed *E. coli*, offering insights into optimizing recovery protocols in microbiological studies. Further statistical analyses substantiated these observations, enhancing the understanding of selective media impact on stress-induced bacteria.

Effect of Different Media on CFUs and PR

To understand the effect of different media on CFUs and PR, data was analyzed. Generally, MM1, MM2, and MM3 tend to have higher CFUs compared to SDCA across various PBPs for most bacterial strains. MM1 often shows the highest CFUs, indicating better bacterial growth compared to SDCA. SDCA tends to have lower CFUs compared to MM1, MM2, and MM3. The choice of PBP variant seems to influence CFUs to some extent, but the variation isn't as prominent as observed with the choice of media. The differences in CFUs between

Table 4: The results of *E. coli* treated with penicillin

S.No.	Media	Buffer	PBP	*ATCC 8739*		*ATCC 11229*		*ATCC 25253*		*ATCC 25922*		*ATCC 11775*	
				CFU	*PR*	*CFU*	*PR*	*CFU*	*PR*	*CFU*	*PR*	*CFU*	*PR*
1	SDCA	Standard	-	2.33 ± 1.15	2	2.67 ± 0.58	2	1.67 ± 1.53	1	0.67 ± 1.15	1	0.33 ± 0.58	0
2	SDCA	Standard	PBP1	42.33 ± 0.58	46	39.67 ± 2.08	46	30.33 ± 2.08	34	23.33 ± 2.08	26	30.67 ± 2.08	35
3	SDCA	Standard	PBP2	37.33 ± 0.58	41	36.33 ± 1.15	43	26.33 ± 1.15	30	21.67 ± 2.31	23	30.33 ± 2.52	35
4	SDCA	Standard	PBP3	31.33 ± 2.08	34	34.33 ± 1.53	40	26.33 ± 0.58	30	19.67 ± 1.53	21	27.33 ± 0.58	31
5	SDCA	MRB1	-	1.33 ± 1.53	1	2.33 ± 0.58	2	1.33 ± 1.15	1	1.33 ± 0.58	1	0.33 ± 0.58	0
6	SDCA	MRB1	PBP1	30.33 ± 2.08	33	36.67 ± 1.53	43	28.33 ± 1.53	32	34.33 ± 2.08	38	38.33 ± 3.06	44
7	SDCA	MRB1	PBP2	22.33 ± 2.08	24	36.33 ± 2.08	43	26.33 ± 2.08	30	32.33 ± 3.79	36	37.67 ± 2.31	43
8	SDCA	MRB1	PBP3	23.33 ± 2.08	25	38.67 ± 1.53	45	23.67 ± 1.53	26	29.33 ± 2.08	32	36.67 ± 1.53	42
9	SDCA	MRB2	-	1.67 ± 2.08	2	1.33 ± 0.58	1	1.33 ± 0.58	1	0.33 ± 0.58	0	1.67 ± 2.08	1
10	SDCA	MRB2	PBP1	25.33 ± 2.08	27	31.67 ± 2.52	37	27.67 ± 1.53	31	30.67 ± 1.53	34	32.67 ± 2.08	37
11	SDCA	MRB2	PBP2	25.67 ± 1.53	27	31.33 ± 1.53	37	26.67 ± 1.15	30	33.33 ± 1.53	37	30.67 ± 2.08	35
12	SDCA	MRB2	PBP3	20.67 ± 2.52	22	28.33 ± 2.52	33	21.33 ± 1.53	24	31.67 ± 2.08	35	29.67 ± 1.53	34
13	MM1	Standard	-	0.67 ± 0.58	0	2.33 ± 1.15	2	1.33 ± 1.53	1	2.33 ± 0.58	2	1.33 ± 1.15	1
14	MM1	Standard	PBP1	32.33 ± 1.53	35	37.67 ± 1.15	44	35.33 ± 1.53	42	39.33 ± 2.08	44	37.67 ± 0.58	43
15	MM1	Standard	PBP2	33.67 ± 1.53	36	35.67 ± 1.53	42	39.33 ± 1.53	46	38.33 ± 0.58	43	33.33 ± 0.58	38
16	MM1	Standard	PBP3	37.67 ± 1.53	41	36.33 ± 1.53	43	40.33 ± 2.08	48	37.67 ± 0.58	42	38.67 ± 1.15	44
17	MM1	MRB1	-	1.33 ± 1.15	1	1.33 ± 0.58	1	1.33 ± 1.53	1	1.33 ± 0.58	1	1.67 ± 0.58	1
18	MM1	MRB1	PBP1	56.33 ± 1.53	62	60.33 ± 2.52	72	56.67 ± 1.53	65	57.33 ± 2.08	64	58.33 ± 1.53	68
19	MM1	MRB1	PBP2	52.33 ± 2.08	57	49.33 ± 0.58	59	51.67 ± 1.53	59	53.33 ± 2.08	60	54.33 ± 0.58	63
20	MM1	MRB1	PBP3	44.33 ± 2.08	48	41.67 ± 1.53	49	49.33 ± 1.15	56	53.33 ± 2.89	60	50.33 ± 2.52	58
21	MM1	MRB2	-	2.33 ± 2.08	2	1.67 ± 1.53	2	1.33 ± 1.15	1	1.33 ± 0.58	1	1.67 ± 1.53	1
22	MM1	MRB2	PBP1	49.67 ± 1.53	54	50.67 ± 0.58	60	48.33 ± 2.31	55	50.33 ± 3.06	56	48.67 ± 2.08	56
23	MM1	MRB2	PBP2	41.33 ± 1.53	45	44.00 ± 2.65	53	55.67 ± 1.53	63	49.33 ± 1.15	55	42.67 ± 1.15	49
24	MM1	MRB2	PBP3	33.67 ± 1.53	36	39.67 ± 1.53	46	41.67 ± 2.08	47	48.33 ± 1.53	54	44.67 ± 2.52	51
25	MM2	Standard	-	1.33 ± 1.53	1	2.33 ± 1.15	2	0.67 ± 0.58	0	1.33 ± 1.15	1	2.67 ± 1.15	2
26	MM2	Standard	PBP1	27.67 ± 1.53	30	30.33 ± 1.53	36	29.33 ± 0.58	34	30.33 ± 1.53	34	29.33 ± 1.53	34
27	MM2	Standard	PBP2	29.33 ± 2.08	32	33.33 ± 2.08	39	33.33 ± 2.08	39	29.33 ± 0.58	32	33.67 ± 2.52	38
28	MM2	Standard	PBP3	29.67 ± 2.08	32	31.33 ± 1.15	37	28.33 ± 1.53	33	33.67 ± 3.21	37	37.67 ± 1.53	43
29	MM2	MRB1	-	1.33 ± 1.53	1	2.33 ± 1.15	2	2.00 ± 1.73	2	1.33 ± 0.58	1	1.33 ± 1.53	1
30	MM2	MRB1	PBP1	44.33 ± 2.08	48	40.67 ± 1.15	48	45.33 ± 2.08	52	49.67 ± 2.31	55	50.33 ± 3.06	58
31	MM2	MRB1	PBP2	38.33 ± 3.06	42	38.67 ± 3.51	45	39.67 ± 2.08	45	45.33 ± 2.89	51	41.67 ± 1.53	48
32	MM2	MRB1	PBP3	32.33 ± 1.53	35	38.33 ± 2.08	45	39.33 ± 0.58	45	45.33 ± 2.52	51	39.67 ± 2.08	45
33	MM2	MRB2	-	1.67 ± 2.08	1	1.33 ± 1.53	1	1.33 ± 1.53	1	1.67 ± 1.15	1	1.67 ± 1.53	1
34	MM2	MRB2	PBP1	41.33 ± 3.06	45	40.67 ± 2.31	48	43.33 ± 2.31	50	42.33 ± 2.52	47	39.33 ± 2.08	45
35	MM2	MRB2	PBP2	34.33 ± 2.52	37	33.67 ± 1.53	39	38.67 ± 1.53	44	38.33 ± 2.08	43	39.33 ± 0.58	45
36	MM2	MRB2	PBP3	32.33 ± 2.52	35	31.67 ± 1.53	37	36.33 ± 2.08	41	34.67 ± 1.15	38	38.67 ± 2.08	44
37	MM3	Standard	-	0.67 ± 1.15	0	1.67 ± 1.53	1	1.67 ± 1.53	1	0.67 ± 1.15	0	2.33 ± 1.53	2
38	MM3	Standard	PBP1	29.33 ± 3.06	32	27.33 ± 1.53	32	27.33 ± 1.53	32	33.67 ± 4.16	37	29.67 ± 2.52	34
39	MM3	Standard	PBP2	27.67 ± 1.53	30	30.33 ± 1.15	36	22.67 ± 1.53	26	30.33 ± 0.58	34	33.33 ± 2.08	38
40	MM3	Standard	PBP3	31.33 ± 1.53	34	29.67 ± 1.53	34	31.00 ± 1.00	36	31.67 ± 1.53	35	32.67 ± 3.06	37
41	MM3	MRB1	-	1.67 ± 1.53	1	2.33 ± 0.58	2	1.33 ± 1.53	1	1.33 ± 0.58	1	1.67 ± 1.53	1
42	MM3	MRB1	PBP1	35.33 ± 1.53	38	42.33 ± 3.06	50	49.33 ± 1.53	56	47.33 ± 1.53	53	42.67 ± 2.08	49
43	MM3	MRB1	PBP2	33.33 ± 1.53	36	37.33 ± 2.08	44	43.67 ± 3.21	50	46.33 ± 1.53	52	37.33 ± 1.53	43
44	MM3	MRB1	PBP3	30.33 ± 3.21	33	34.67 ± 1.15	40	40.67 ± 2.08	46	40.33 ± 1.53	45	27.67 ± 2.52	31
45	MM3	MRB2	-	2.33 ± 2.08	2	1.67 ± 1.53	1	1.33 ± 1.15	1	1.33 ± 1.53	1	1.67 ± 0.58	1
46	MM3	MRB2	PBP1	37.33 ± 2.52	41	43.67 ± 3.21	51	34.67 ± 1.53	39	41.67 ± 2.08	46	39.33 ± 2.08	45
47	MM3	MRB2	PBP2	33.67 ± 1.53	36	36.33 ± 1.53	43	33.33 ± 1.53	38	40.67 ± 3.21	45	37.33 ± 0.58	43
48	MM3	MRB2	PBP3	32.67 ± 3.21	35	32.67 ± 3.21	38	31.67 ± 2.52	36	36.33 ± 1.15	40	35.33 ± 1.53	41

CFU: Colony Forming Units; PR: Percent recovery

Table 5: The results of *E. coli* treated with cefixime

S.No.	Media	Buffer	PBP	*ATCC 8739*		*ATCC 11229*		*ATCC 25253*		*ATCC 25922*		*ATCC 11775*	
				CFU	*PR*	*CFU*	*PR*	*CFU*	*PR*	*CFU*	*PR*	*CFU*	*PR*
1	SDCA	Standard	-	2.33 ± 1.15	2	0.67 ± 0.58	0	2.33 ± 1.53	2	1.67 ± 0.58	1	1.67 ± 0.58	1
2	SDCA	Standard	PBP1	37.33 ± 2.89	41	30.33 ± 3.51	36	35.67 ± 1.3	40	26.33 ± 1.15	29	28.33 ± 2.31	32
3	SDCA	Standard	PBP2	33.33 ± 1.53	36	27.33 ± 2.52	32	33.67 ± 1.53	38	31.67 ± 1.53	35	27.33 ± 2.52	31
4	SDCA	Standard	PBP3	31.00 ± 2.65	33	26.67 ± 2.08	31	31.33 ± 2.08	36	28.33 ± 2.08	31	25.67 ± 1.53	29
5	SDCA	MRB1	-	4.67 ± 2.52	4	1.33 ± 0.58	1	3.33 ± 1.15	3	1.67 ± 0.58	1	0.33 ± 0.58	0
6	SDCA	MRB1	PBP1	29.67 ± 2.08	32	31.33 ± 2.52	37	30.33 ± 1.15	34	31.33 ±2.52	35	29.33 ± 1.53	34
7	SDCA	MRB1	PBP2	25.33 ± 1.53	27	29.33 ± 2.52	34	24.67 ± 2.08	27	22.33 ± 2.08	25	22.33 ± 2.08	25
8	SDCA	MRB1	PBP3	23.67 ± 2.52	25	21.33 ± 1.53	25	24.33 ± 1.53	27	23.67 ± 0.58	26	23.67 ± 0.58	27
9	SDCA	MRB2	-	3.67 ± 1.53	3	1.33 ± 1.15	1	1.67 ± 2.08	1	1.67 ± 2.08	1	1.67 ± 2.08	1
10	SDCA	MRB2	PBP1	27.33 ±	30	32.33 ± 3.06	38	28.67 ± 1.53	32	29.67 ± 0.58	32	29.67 ± 0.58	34
11	SDCA	MRB2	PBP2	21.33 ± 2.08	23	22.67 ± 1.53	26	28.33 ± 2.08	32	23.67 ± 1.53	26	26.67 ± 1.53	30
12	SDCA	MRB2	PBP3	21.67 ± 1.53	23	23.33 ± 1.53	27	20.33 ± 1.53	23	21.33 ± 3.21	23	21.33 ± 3.21	24
13	MM1	Standard	-	4.33 ± 2.08	4	1.33 ± 0.58	1	2.67 ± 2.08	2	1.33 ± 0.58	1	1.33 ± 0.58	1
14	MM1	Standard	PBP1	37.67 ± 0.58	41	31.67 ± 1.53	37	35.33 ± 0.58	44	38.33 ± 1.53	43	29.67 ± 2.52	34
15	MM1	Standard	PBP2	35.33 ± 1.53	38	29.33 ± 2.08	34	38.33 ± 2.31	44	37.67 ± 1.15	42	33.67 ± 2.89	38
16	MM1	Standard	PBP3	35.33 ± 2.52	38	27.33 ± 2.08	32	36.33 ± 3.79	41	39.33 ± 1.53	44	37.67 ± 1.53	43
17	MM1	MRB1	-	4.33 ± 2.08	4	1.33 ± 0.58	1	3.33 ± 2.08	3	1.33 ± 0.58	1	1.33 ± 0.58	1
18	MM1	MRB1	PBP1	49.33 ± 1.15	54	44.33 ± 2.08	53	48.33 ± 1.15	55	46.33 ± 2.08	52	54.33 ± 4.73	63
19	MM1	MRB1	PBP2	43.33 ± 1.53	47	42.67 ± 1.53	50	38.67 ± 2.08	44	37.33 ± 2.08	42	48.67 ± 1.15	56
20	MM1	MRB1	PBP3	40.33 ± 2.52	44	39.33 ± 3.21	46	42.33 ± 2.08	48	33.67 ± 2.08	37	37.33 ± 1.53	43
21	MM1	MRB2	-	4.33 ± 1.53	4	1.33 ± 1.53	1	3.33 ± 1.53	3	1.33 ± 1.53	1	1.33 ± 1.53	1
22	MM1	MRB2	PBP1	42.67 ± 1.53	46	39.33 ± 4.04	46	39.67 ± 2.52	45	38.33 ± 1.53	43	46.67 ± 4.16	54
23	MM1	MRB2	PBP2	35.33 ± 2.52	38	38.67 ± 2.31	45	42.67 ± 2.31	48	40.33 ± 0.58	45	45.67 ± 2.31	52
24	MM1	MRB2	PBP3	30.33 ± 2.08	33	33.33 ± 2.31	39	30.67 ± 2.31	34	35.33 ± 1.53	39	38.33 ± 1.15	44
25	MM2	Standard	-	3.33 ± 2.31	3	2.67 ± 1.15	2	1.33 ± 1.15	1	2.67 ± 1.15	2	0.67 ± 1.15	0
26	MM2	Standard	PBP1	38.33 ± 2.08	42	33.67 ± 2.08	39	30.67 ± 2.31	34	36.33 ± 1.53	40	33.67 ± 1.15	38
27	MM2	Standard	PBP2	39.33 ± 4.16	43	35.67 ± 2.08	42	29.33 ± 1.53	33	37.33 ± 1.53	42	37.33 ± 1.53	43
28	MM2	Standard	PBP3	35.67 ± 1.53	38	31.67 ± 1.53	37	31.67 ± 1.53	36	36.33 ± 2.08	40	38.67 ± 0.58	44
29	MM2	MRB1	-	2.67 ± 1.53	2	1.33 ± 1.53	1	4.67 ± 1.53	4	2.33 ± 1.15	2	1.67 ± 1.15	1
30	MM2	MRB1	PBP1	39.33 ± 3.21	43	38.33 ± 1.53	45	42.67 ± 2.52	48	40.33 ± 2.52	45	36.67 ± 1.15	42
31	MM2	MRB1	PBP2	39.33 ± 1.15	43	37.67 ± 2.08	44	32.33 ± 2.08	37	33.67 ± 0.58	37	36.67 ± 2.31	42
32	MM2	MRB1	PBP3	32.67 ± 1.53	35	30.33 ± 0.58	36	29.33 ± 1.15	33	31.33 ± 2.31	35	35.33 ± 1.15	41
33	MM2	MRB2	-	2.67 ± 1.53	2	1.67 ± 1.15	1	2.67 ± 1.53	2	1.67 ± 1.15	1	1.67 ± 1.15	1
34	MM2	MRB2	PBP1	40.33 ± 3.06	44	39.33 ± 2.08	46	37.67 ± 2.08	43	36.33 ± 0.58	40	37.33 ± 1.53	43
35	MM2	MRB2	PBP2	36.67 ± 2.52	40	30.67 ± 1.53	36	34.33 ± 2.08	39	33.33 ± 1.53	37	31.67 ± 1.53	36
36	MM2	MRB2	PBP3	31.67 ± 1.53	34	33.33 ± 1.53	39	30.33 ± 1.53	34	32.33 ± 2.52	36	25.67 ± 1.15	29
37	MM3	Standard	-	0.67 ± 0.58	0	0.67 ± 0.58	0	0.67 ± 0.58	1	1.33 ± 0.58	1	2.33 ± 1.53	2
38	MM3	Standard	PBP1	28.67 ± 1.53	31	26.33 ± 1.15	31	19.33 ± 2.08	22	27.33 ± 1.15	30	31.33 ± 1.15	36
39	MM3	Standard	PBP2	25.33 ± 1.53	27	28.33 ± 1.53	33	23.67 ± 1.15	26	26.67 ± 2.08	29	26.67 ± 2.08	30
40	MM3	Standard	PBP3	26.67 ± 2.52	28	25.67 ± 1.53	30	25.67 ± 1.53	29	25.67 ± 1.53	28	25.67 ± 1.53	29
41	MM3	MRB1	-	2.33 ± 0.58	2	0.67 ± 1.15	1	1.67 ± 1.53	1	1.67 ± 1.53	1	1.67 ± 1.53	1
42	MM3	MRB1	PBP1	37.33 ± 2.08	41	38.33 ± 1.53	45	36.67 ± 1.53	41	35.33 ± 1.53	39	38.33 ± 1.15	44
43	MM3	MRB1	PBP2	32.67 ± 3.06	35	36.33 ± 2.08	43	33.33 ± 4.04	38	33.33 ± 4.04	37	33.33 ± 4.04	38
44	MM3	MRB1	PBP3	33.33 ± 2.89	36	36.67 ± 1.53	43	30.33 ± 2.89	34	25.67 ± 2.08	28	25.67 ± 2.08	29
45	MM3	MRB2	-	3.33 ± 2.08	3	1.67 ± 2.08	1	1.67 ± 2.08	1	1.67 ± 2.08	1	1.67 ± 2.08	1
46	MM3	MRB2	PBP1	35.67 ± 1.53	38	38.33 ± 1.53	45	33.67 ± 2.89	38	34.33 ± 2.52	38	37.67 ± 0.58	43
47	MM3	MRB2	PBP2	33.67 ± 2.08	36	36.33 ± 1.53	43	33.67 ± 1.53	38	32.33 ± 2.52	36	34.67 ± 2.52	40
48	MM3	MRB2	PBP3	30.67 ± 1.53	33	33.33 ± 3.21	39	32.33 ± 1.53	37	31.33 ± 1.53	35	27.33 ± 2.08	31

CFU: Colony Forming Units; PR: Percent recovery

PBP variants are relatively minor compared to the differences observed between different media types. There are variations in CFUs among different bacterial strains within each media type and PBP variant. ATCC 25253 and ATCC 25922 often show higher CFUs compared to ATCC 8739 and ATCC 11229 across various media types and PBPs.

PR is a measure of how many bacteria were able to grow and form colonies on a specific medium compared to the total number of bacteria. PR tends to follow a similar trend to CFUs, where MM1, MM2, and MM3 generally yield higher PR compared to SDCA. The choice of PBP variant and bacterial strain also influences PR within each media type. In summary, MM1 yields higher CFUs and PR, indicating better bacterial growth than SDCA and other modified media (MM2 and MM3). The choice of PBP variant and bacterial strain also influences CFUs and PR, but the effect of media is more prominent. Additionally, different bacterial strains exhibit variations in CFUs and PR within each media and PBP variant.

The *p-value* for the ANOVA test regarding the different media is very small ($p \approx 0.00063$), indicating a statistically significant effect of the growth media on the mean CFU. In practical terms, this suggests that the choice of growth media significantly influences the number of *E. coli* colonies formed.

Effect of Different Buffers on CFUs and PR

The effect of different buffers on CFUs and PR can be observed from the provided data. Generally, MRB1 and MRB2 tend to result in higher CFUs compared to the standard buffer across various PBPs for most bacterial strains. MRB2 often shows the highest CFUs, indicating better bacterial growth compared to the standard buffer and MRB1. The standard buffer tends to have lower CFUs compared to MRB1 and MRB2. The choice of PBP variant also seems to influence CFUs, with different PBP variants resulting in varying CFUs within each buffer. The differences in CFUs between PBP variants are relatively minor compared to the differences observed between different buffers. There are variations in CFUs among different bacterial strains within each buffer type and PBP variant. ATCC 25253 and ATCC 25922 often show higher CFUs compared to ATCC 8739 and ATCC 11229 across various buffers and PBPs. PR tends to follow a similar trend to CFUs, where MRB1 and MRB2 generally yield higher PR compared to the standard buffer. The choice of PBP variant and bacterial strain also influences PR within each buffer type.

The *p-value* for the ANOVA test concerning the buffer condition is ($p \approx 0.002$), which is less than the typical significance level of 0.05. This indicates a statistically significant effect of the buffer on the mean CFU of *E. coli*. The buffer used in the experiment plays a significant role in influencing the growth of *E. coli*. Different buffer conditions can affect the CFU, possibly due to variations in pH, ion concentrations, or other factors that can influence bacterial growth. Given the significant effect of the buffer, it's crucial to carefully consider and standardize the buffer conditions when conducting experiments related to *E. coli* growth. Consistent and appropriate buffer choices can help ensure accurate and reliable results in microbiological studies.

Comparison between PBP1, PBP2 and PBP3

Comparing the influence of PBPs 1, 2, and 3 on mean CFU of *E. coli* across diverse growth media provides valuable insights into their impact on bacterial growth and susceptibility to penicillin.

For PBP1, a consistent trend of increased mean CFU is observed compared to the standard condition across various growth media and strains. This suggests that PBP1 tends to exert a milder inhibitory effect on bacterial growth compared to the standard condition without a specific PBP.

On the other hand, PBP2 exhibits varying effects across different growth media and strains. It appears that PBP2 can either enhance or inhibit bacterial growth depending on the growth medium and strain, illustrating a nuanced impact.

In contrast, the utilization of PBP3 shows a consistent trend of reducing mean CFU compared to the standard condition across most growth media and strains. This suggests that PBP3 typically exerts a more pronounced inhibitory effect on bacterial growth compared to the standard condition without a specific PBP.

In summary, PBP1 generally demonstrates a lesser inhibitory effect on bacterial growth compared to both PBP2 and PBP3, a pattern consistent across most growth media and strains. PBP2 demonstrates variable effects, indicating a nuanced impact on bacterial growth based on the specific growth medium and strain. Conversely, PBP3 tends to exhibit a stronger inhibitory effect, resulting in reduced mean CFU in most cases.

The *p-value* obtained from the ANOVA test comparing the different PBPs is exceedingly small ($p \approx 2.04 \times 10^{-16}$), signifying a highly significant influence of the PBP on mean CFU. In simpler terms, the type of PBP used significantly affects the number of *E. coli* colonies.

Effect of Strain

The *E. coli* strain plays a crucial role in determining the mean CFU across various growth media and in the presence of different PBPs, constituting a fundamental aspect of this experiment. The *p-value* from the ANOVA test for the *E. coli* strain is remarkably low ($p \approx 0.000$), underscoring an extremely significant effect of the strain on *E. coli*'s mean CFU.

This experiment's specific *E. coli* strain significantly influences bacterial growth, as evidenced from the CFU counts. Variations in growth characteristics and responses to the experimental conditions are observed among different strains, illustrating the strain-dependent nature of the experiment's outcomes.

Across diverse media types, CFUs exhibit substantial variations. Notably, in media MM1, PBP1 tends to yield higher CFUs compared to other PBPs within the same media. Furthermore, the PR demonstrates variations based on the media type and the specific PBP applied. Different PBPs manifest diverse effects on CFUs and PR within identical media. For instance, PBP1 consistently leads to higher CFUs and PR in most cases. Interestingly, media with standard

buffers occasionally display higher CFUs and PR in contrast to those with specific PBPs. Different *E. coli* strains (ATCC 8739, ATCC 11229, ATCC 25253, ATCC 25922, ATCC 11775) showcase distinct responses concerning CFUs and PR across varied media, PBPs, and buffers. The standard deviations indicate noticeable variability in CFUs and PR, emphasizing the experiment's inherent diversity and complexity.

Comparison of Results

In both penicillin and cefixime treatments, higher CFU values are generally observed in modified media compared to standard media. Modified media seem to support a higher recovery rate (PR) compared to standard media in both treatments. Different PBPs show variations in CFU and PR across different media. Generally, higher CFU and PR values are observed in the presence of specific PBPs compared to the absence of PBPs. Different strains of *E. coli* (ATCC 8739, ATCC 11229, ATCC 25253, ATCC 25922, ATCC 11775) exhibit variations in CFU and PR in response to penicillin and cefixime across various media and PBP conditions. Standard media generally have lower CFU and PR than modified media for penicillin and cefixime treatments. Buffers seem to influence CFU and PR, with variations observed across different buffers within the same media. In the cefixime treatment, lower CFU values and higher PR values are observed compared to the penicillin treatment. These comparisons provide insights into the efficacy of penicillin and cefixime on *E. coli* under various conditions, highlighting the importance of media, buffers, PBPs, and bacterial strains in determining antibiotic responses.

CONCLUSION

This study reveals significant insights into the influence of selective media enriched with PBPs on stressed *E. coli* recovery and the differential responses among various strains. Key conclusions include: The study underscores the efficacy of selective media enriched with PBPs in recovering stressed *E. coli*. PBP1 exhibited a milder inhibitory effect on bacterial growth compared to PBP2 and PBP3, providing a potential avenue for targeted stress induction without severe growth suppression. The strain of *E. coli* used significantly influenced bacterial growth and responses. Different strains showcased variations in growth characteristics, highlighting the importance of considering strain-specific responses in similar experiments. Diverse growth media yielded varying CFUs and PR. Modified media consistently supported higher CFUs and PR compared to standard media, emphasizing the pivotal role of media composition in bacterial recovery. Choice of buffer had a notable impact on CFUs and PR, with MRB2 proving more conducive for bacterial growth. The study underscores the need for careful buffer selection to optimize recovery outcomes. Comparative analysis of PBPs revealed distinct effects on *E. coli* growth. PBP2 exhibited a nuanced impact, showcasing its potential for fine-tuning stress induction strategies, while PBP3 consistently exerted a stronger inhibitory effect. The ANOVA tests demonstrated highly significant effects of PBPs and growth media on mean CFU. This statistical significance reaffirms the critical role of these factors in bacterial recovery. The findings have broad implications for microbiological studies, guiding the optimization of stress-induced bacterial recovery protocols.

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