"Isolation and Biochemical Characterization of Bacterial Autolysins as Enzyme-Based Antimicrobial Agents"

List of Authors:

1. Dr. Raja Kumar Parabathina., Orcid Id: 0000-0001-8157-4302
   Professor in Biochemistry,
   Institute of Biosciences and Technology,
   MGM University, Chh. Sambhajinagar-431003.
   Maharashtra, India.

2. Prathamesh Anil Dangre, Orcid Id: 0009-0007-6482-0955
   Institute of Biosciences and Technology,
   MGM University, Chh. Sambhajinagar-431003.
   Maharashtra, India.

3. Omkar Tukaram Chaudhari, Orcid Id: 0009-0007-9369-6607
   Institute of Biosciences and Technology,
   MGM University, Chh. Sambhajinagar-431003.
   Maharashtra, India.

4. Vishal Nandakumar Lolge., Orcid Id: 0009-0000-2609-8534
   DNA Labs CRIS,
   Dehradun, Uttarakhand, India.

*Corresponding author:
Dr. Rajakumar Parabathina,
Professor in Biochemistry,
IBT, MGM University, Chh. Sambhajinagar, Maharashtra, India

Abstract

Rising antibiotic resistance has renewed interest in enzyme-based antibacterial that target structures bacteria cannot easily redesign. Autolysins, the peptidoglycan-hydrolysing enzymes that bacteria normally use to remodel their own cell walls during growth and division, are one such candidate: applied externally, they can lyse susceptible cells rather than protect them. In this study, an autolysin was extracted from a locally isolated strain of Bacillus subtilis, confirmed biochemically through Gram staining, endospore staining, catalase, starch hydrolysis, indole, citrate, and motility tests. Two extraction routes were compared side by side: a 5 M lithium chloride treatment and a Tris-HCl buffer-induced autolysis followed by ammonium sulfate precipitation as a partial purification step. Protein content, measured by the Biuret method, was consistently higher in the Tris-HCl extract (OD540 = 0.365) than in the LiCl extract (OD540 = 0.213). A turbidity-reduction assay against a standardized cell suspension showed both extracts were catalytically active, with the Tris-HCl extract causing slightly greater lysis (~57%) than the LiCl extract (~51%). The picture reversed, however, in the agar well diffusion assay: the LiCl extract produced the larger zone of inhibition (~9 mm net), outperforming the Tris-HCl extract (~7 mm net) despite its lower protein yield. Optimal activity for both extracts occurred near pH 8.0 and 37°C. Taken together, the results suggest that protein yield and antibacterial potency are not the same thing for this enzyme. Tris-HCl extraction is preferable when the goal is maximum protein and lytic activity, whereas LiCl extraction appears to enrich for the antimicrobial active fraction. These findings support autolysin as a viable candidate for further development as an enzyme-based antimicrobial agent.

Keywords: *Autolysin; Bacillus subtilis; Peptidoglycan Hydrolase; Extraction; Precipitation; Antimicrobial*

1. Introduction

Antibiotic resistance has moved from a clinical inconvenience to one of the more pressing problems in global health. Bacteria continually find new ways around the drugs used against them, degrading the drug itself, altering the target it binds to, or simply pumping it back out of the cell, and each new workaround narrows the options available to clinicians (Davies & Davies, 2010; Ventola, 2015). The World Health Organization has flagged the shrinking antibiotic pipeline as a priority concern, which has pushed researchers to look beyond conventional small-molecule antibiotics altogether (WHO, 2014).

One alternative that has drawn steady attention is the use of enzymes that degrade the bacterial cell wall directly. Autolysins fall into this category. Bacteria produce them naturally, using them in carefully regulated amounts to loosen and remodel their peptidoglycan during growth, division, and daughter-cell separation (Shockman & Höltje, 1994; Vollmer et al., 2008). Under normal physiological conditions, this activity is tightly controlled an unregulated autolysin would simply destroy the cell that made it (Navarre & Schneewind, 1999). That same destructive capacity, though, becomes useful once the enzyme is extracted and applied externally: isolated autolysins can lyse susceptible bacterial cells on contact, particularly Gram-positive species whose peptidoglycan layer is thick and directly exposed rather than shielded by an outer membrane (Vollmer & Bertsche, 2008).

Peptidoglycan is also an unusually stable target from a resistance standpoint. It is essential and structurally conserved across Gram-positive bacteria, so there is comparatively little room for a cell to mutate its way out of being recognised (Fischetti, 2018). Because enzymatic lysis does not depend on active bacterial metabolism, autolysins and related hydrolases can act even on dormant cells, slow-growing persisters, and biofilm-embedded populations that are notoriously difficult to clear with conventional antibiotics (Schuch et al., 2014).

Much of the existing literature on peptidoglycan-degrading enzymes, however, centres on phage-derived endolysins and hen-egg lysozyme rather than bacterial autolysins specifically (Ibrahim et al., 2001; Schmelcher & Loessner, 2016; Schmelcher et al., 2012). Vollmer et al. (2008) and López and García (2004) laid out the structural role autolysins play in peptidoglycan turnover, and Shockman and Höltje (1994) demonstrated early on that purified autolysins can lyse susceptible cells when applied externally, but few studies combine isolation, biochemical characterization, and antimicrobial testing of a bacterial autolysin within a single, self-contained experimental design.

Kashyap et al. (2017) further showed that the activity of these enzymes is quite sensitive to environmental conditions such as pH and temperature, which makes basic biochemical characterization a necessary step before any autolysin can be seriously considered for downstream application. This study aims to help close this gap.

With this in mind, the present work set out to (i) isolate an autolysin-producing bacterial strain and confirm its identity as Bacillus subtilis through standard biochemical tests, (ii) extract and partially purify the autolysin using two contrasting methods and compare their biochemical properties, including pH and temperature optima, (iii) assess enzyme stability and substrate specificity in broad terms, and (iv) evaluate the resulting antimicrobial activity against a test bacterial strain.

2. Materials and Methods

2.1 Materials

Nutrient broth and nutrient agar, Luria broth, phosphate-buffered saline, Tris-HCl buffer (50 mM, pH 7.0), lithium chloride (5 M), ammonium sulfate, and the reagents required for Gram, endospore, catalase, starch hydrolysis, indole, and citrate testing were used throughout the study, alongside standard glassware, a shaking incubator, a refrigerated centrifuge, a UV-Vis spectrophotometer, a pH meter, and an autoclave for sterilization. A locally isolated bacterial culture served as the source organism and was maintained under aseptic conditions in a laminar airflow hood.

2.1 Biochemical identification of the isolate

Before any extraction work began, the isolate was screened using a standard biochemical battery to confirm its identity. Gram staining (crystal violet, iodine, alcohol decolourisation, safranin counterstain) and endospore staining (malachite green with heat, safranin counterstain) were both read under the microscope; catalase activity was checked by observing effervescence on addition of hydrogen peroxide; starch hydrolysis was assessed on starch agar flooded with iodine after incubation; indole production was tested in tryptone broth using Kovac's reagent; citrate utilisation was read on Simmons' citrate agar slants; and motility was assessed by stab-inoculation into semi-solid medium.

2.2 Culturing and cell harvesting

The confirmed Bacillus subtilis culture was grown in sterile nutrient broth at 37°C in a shaking incubator (120–150 rpm) until it reached mid-exponential phase, monitored by OD600 (target range 0.4–0.6), typically after 4–5 hours. Cells were then pelleted by centrifugation (~5000 rpm,

10 min), and the pellet was washed once in Tris-HCl buffer to remove residual medium before being split for the two extraction procedures described below.

2.3 Autolysin extraction

The washed pellet was divided into two equal portions. One portion was resuspended in 5 M lithium chloride and incubated at 4°C for 30–60 minutes with gentle mixing, after which the suspension was centrifuged (10,000 rpm, 10–15 min, 4°C) and the clear supernatant retained as the LiCl extract. The second portion was resuspended in Tris-HCl buffer (pH ~7.0) and incubated at 37°C for 30–60 minutes with shaking to induce autolysis, then centrifuged under the same conditions to yield the Tris-HCl extract.

2.4 Partial purification

Both supernatants were brought to roughly 60–80% ammonium sulfate saturation (0.4 - 0.5 g/mL) at 4°C with gentle stirring, then left overnight (~12 - 15 h) at 4°C to allow protein precipitation. The precipitate was recovered by centrifugation (10,000 rpm, 15 min, 4°C), and the resulting pellet was redissolved in a small volume (1 - 4 mL) of Tris-HCl buffer for downstream assays.

2.5 Protein estimation

Protein concentration in each extract was estimated by the Biuret method: 0.3–0.5 mL of sample was mixed with 1 mL of Biuret reagent, incubated at room temperature for 5–10 minutes, and read at 540 nm against a distilled-water blank.

2.6 Enzyme activity assay (turbidity reduction)

A fresh bacterial cell suspension was standardised to OD600 ≈ 0.8–1.0 in Tris-HCl buffer. Three tubes were compared: an untreated control, cells plus LiCl extract, and cells plus Tris-HCl extract (0.2–0.5 mL enzyme added to each). After 30–60 minutes at 37°C, OD600 was read again, and percentage lysis was calculated as (initial OD − final OD) / initial OD × 100.

2.7 Antimicrobial activity (agar well diffusion)

A lawn of the test organism was spread on nutrient agar plates, and 6 mm wells were cut with a sterile borer. Each well received 50–100 µL of enzyme extract; plates were incubated at 37°C for 24 hours and the resulting zones of inhibition measured in millimetres.

2.8 Effect of pH and temperature

To locate the pH optimum, enzyme extract was combined with substrate suspension prepared in citrate buffer (pH 4–6), phosphate buffer (pH 6–8), or Tris-HCl buffer (pH 8–10) and incubated at 37°C for 30 minutes before reading OD600. Temperature optimum was determined similarly,

incubating the same reaction mixture at 20, 30, 37, 45, 50, and 60°C for 30 minutes and comparing the resulting drop in turbidity.

3. Results

This section reports the outcome of each stage described above: isolate confirmation, extraction and partial purification, protein yield, and the two complementary activity assays with the LiCl and Tris-HCl extracts compared throughout.

3.1 Confirmation of the bacterial isolate

A battery of standard biochemical tests confirmed the isolate as Bacillus subtilis: purple, rod-shaped cells under Gram stain; visible endospores; immediate effervescence in the catalase test; a clear hydrolysis zone on starch agar; a negative indole result; a positive citrate response; and outward spreading growth in the motility test (Table 1, Fig. 1-7).

3.2 Culturing, harvesting, and extraction

The isolate was grown to mid-exponential phase and harvested by centrifugation for downstream extraction (Fig. 8). The pellet was then split for LiCl and Tris-HCl extraction and partially purified by ammonium sulfate precipitation (Fig. 9).

3.3 Protein estimation

Biuret assay readings were consistently higher for the Tris-HCl extract than for the LiCl extract, indicating greater total protein recovery by the buffer-induced method (Table 2).

The temperature-response values for both extracts, corresponding to the data presented later in Table 8, are summarized in Table 3.

3.4 Enzyme activity (turbidity reduction)

Both extracts produced a clear drop in OD600 relative to the untreated control, confirming that each contained catalytically active enzyme. The Tris-HCl extract achieved the greater reduction in turbidity overall (Table 4).

3.5 Antimicrobial activity (agar well diffusion)

The control well showed no measurable zone, while both enzyme extracts produced clear zones of inhibition, confirming antimicrobial activity in each. Here the ranking reversed relative to the protein and turbidity data: The LiCl extract produced the larger net zone of inhibition (Table 5, Fig. 11).

The turbidity-reduction enzyme activity results, corresponding to the data presented earlier in Table 4, are summarized again in Table 6.

3.6 Effect of pH and temperature on activity

Both extracts showed a similar bell-shaped response to pH, with the lowest OD600 readings — and therefore the highest apparent activity recorded at pH 8.0, and reduced activity at more acidic or alkaline pH values (Table 7).

Temperature followed the same pattern, with activity peaking at 37°C for both extracts and declining at higher or lower temperatures, dropping off sharply by 60°C (Table 8).

4. Discussion

Taken as a whole, the results confirm that a viable autolysin can be recovered from Bacillus subtilis by either extraction route, but that the two methods are not simply interchangeable versions of the same procedure each favours a different downstream property. The biochemical profile obtained for the isolate (Gram-positive rods, endospore formation, catalase positivity, starch hydrolysis, motility) is consistent with established descriptions of Bacillus species (Smith et al., 2000), which gives confidence that the subsequent extraction work was performed on the intended organism.

The higher Biuret absorbance seen for the Tris-HCl extract suggests that buffer-induced autolysis released more total protein than the LiCl treatment, possibly because the milder, near-physiological conditions preserved protein solubility and stability better than the high-ionic-strength LiCl environment (Scopes, 1994) the Biuret assay itself is a standard, well-validated method for this kind of comparison (Layne, 1957). This higher protein content tracked with slightly greater lytic activity in the turbidity assay, which is unsurprising if more autolysin protein was simply present in that fraction.

What is more interesting is that this advantage did not carry over into the agar well diffusion assay, where the LiCl extract produced the larger zone of inhibition despite containing less total protein. A plausible explanation is that the two extraction conditions do not release identical protein populations: LiCl treatment, working through ionic disruption of cell-wall associated proteins, may selectively enrich for an autolysin fraction (or a subset of isoforms) that diffuses more readily through agar or retains higher specific activity, even though the overall protein yield is lower. Turbidity reduction and zone-of-inhibition assays are, after all, measuring related but not identical things bulk lytic capacity against a suspended cell population versus diffusible antibacterial potency against a lawn culture and enzymes capable of degrading peptidoglycan have repeatedly

been shown to behave differently across these two readouts depending on their diffusion characteristics and stability (Schmelcher et al., 2012).

The pH and temperature profiles obtained here, with activity peaking near pH 8.0 and 37°C for both extracts, sit comfortably within the range typically reported for bacterial cell wall hydrolases (Shockman & Höltje, 1994) and align with the general observation that autolysin activity is strongly shaped by its environment (Kashyap et al., 2017). Practically, this means the enzyme's useful operating window overlaps well with physiological conditions, which is encouraging from an application standpoint. This study extracted and partially purified autolysin from Bacillus subtilis by two contrasting routes LiCl treatment and Tris-HCl buffer-induced autolysis and compared them directly on protein yield, lytic activity, and antimicrobial potency. Tris-HCl extraction gave the higher protein yield and the greater turbidity-reduction activity, while LiCl extraction, despite yielding less protein overall, produced the larger zone of inhibition in the agar well diffusion assay. Both extracts were active near pH 8.0 and 37°C. Read together, the two methods appear to serve different purposes: Tris-HCl extraction is the better choice when the priority is maximising protein recovery and general lytic activity, whereas LiCl extraction seems to enrich for the fraction most relevant to antibacterial performance. Either way, the results support autolysin as a workable candidate for further development as an enzyme-based antimicrobial.

The present dataset is a first pass rather than a finished characterization, and several extensions would strengthen it considerably. Full purification by dialysis, ion-exchange chromatography, and gel filtration would allow the active protein to be isolated cleanly from the mixture of species currently present in each crude extract. Molecular weight determination by SDS-PAGE, together with partial sequencing, would help clarify whether the LiCl and Tris-HCl extracts really do differ in composition, as the diffusion-assay results here suggest. A more finely resolved pH and temperature titration, combined with formal kinetic analysis, would sharpen the operating parameters reported above, and testing against a wider panel of Gram-positive and Gram-negative organisms would clarify how broadly this activity extends. Given the pressure that antibiotic resistance continues to place on treatment options, autolysins of this kind remain a reasonable avenue to pursue toward eventual industrial or pharmaceutical application.

Tables

Table 1. Biochemical characterization of the bacterial isolate.

Test	Observation	Result	Interpretation
Gram staining	Purple-coloured, rod-shaped cells	Positive	Gram-positive bacterium
Endospore staining	Green spores within red-stained cells	Positive	Endospore-forming organism
Catalase test	Immediate bubble formation	Positive	Catalase enzyme present
Starch hydrolysis	Clear zone around colony after iodine	Positive	Starch hydrolysed
Indole test	No red ring formed	Negative	Indole not produced
Citrate test	No colour change (medium remained green)	Positive	Citrate utilisation as noted
Motility test	Growth spreading away from stab line	Positive	Motile organism

Table 2. Protein content of the two extracts (Biuret method, OD540).

Sample	OD_{540}	Interpretation
LiCl extract	0.213	Moderate protein
Tris-HCl extract	0.365	Higher protein
Blank	0.000	Control

Table 3. Effect of temperature on turbidity reduction and enzymatic activity of LiCl and Tris-HCl extracts.

Temperature (°C)	LiCl OD_{600}	Tris OD_{600}	Turbidity Reduction (%)	Activity Level
20	0.52	0.50	~15	Low
30	0.42	0.38	~35	Moderate
37	0.25	0.20	~65	Optimal
45	0.35	0.30	~50	Decreasing
50	0.48	0.45	~25	Reduced
60	0.56	0.55	~8	Very Low

Table 4. Turbidity-reduction assay results.

Sample	Initial OD_{600}	Final OD_{600}	% Lysis	Interpretation
Control	0.052	0.048	~7.7%	Negligible activity
LiCl extract	0.140	0.068	~51.4%	Significant lytic activity

Tris-HCl extract	0.142	0.061	~57.0%	Highest lytic activity

Table 5. Zones of inhibition from the agar well diffusion assay.

Sample	Total zone (mm)	Net zone (mm)	Interpretation
Control	6 (well only)	0	No activity
LiCl extract	15–16	8.8–9	Highest activity
Tris-HCl extract	12–13	7	Moderate activity

Table 6. Enzyme Activity Assay (Turbidity Reduction Method).

Sample	Initial OD_{600}	Final OD_{600}	% Lysis	Activity Level
Control	0.052	0.048	7.7	Negligible
LiCl Extract	0.140	0.068	51.4	Significant
Tris-HCl Extract	0.142	0.061	57.0	Highest

Table 7. Effect of pH on enzyme activity (final OD_{600}).

pH	LiCl extract	Tris-HCl extract	Trend
4.0	0.54	0.52	Low
6.0	0.42	0.38	Moderate
7.0	0.28	0.22	High
8.0	0.20	0.15	Optimal
10.0	0.48	0.45	Low

Table 8. Effect of temperature on enzyme activity.

Temp (°C)	LiCl OD_{600}	Tris OD_{600}	Turbidity reduction (%)	Activity level
20	0.52	0.50	~15%	Low
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37	0.25	0.20	~65%	Optimal
45	0.35	0.30	~50%	Decreasing
50	0.48	0.45	~25%	Reduced
60	0.56	0.55	~8%	Very low

Figures:

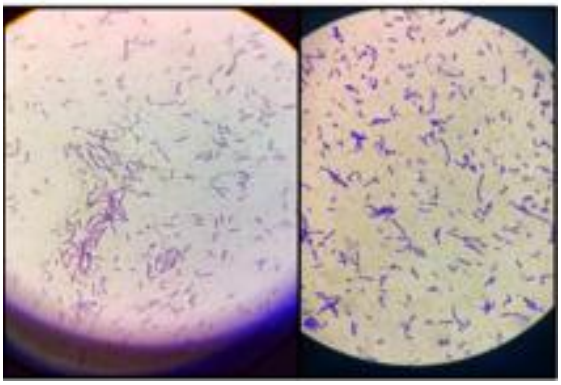

Fig.1. Gram Staining.

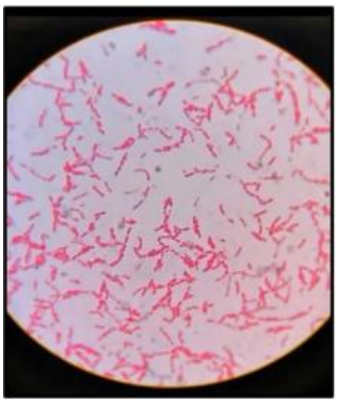

Fig.2. Endospore staining.

Fig.3. Catalase Test.

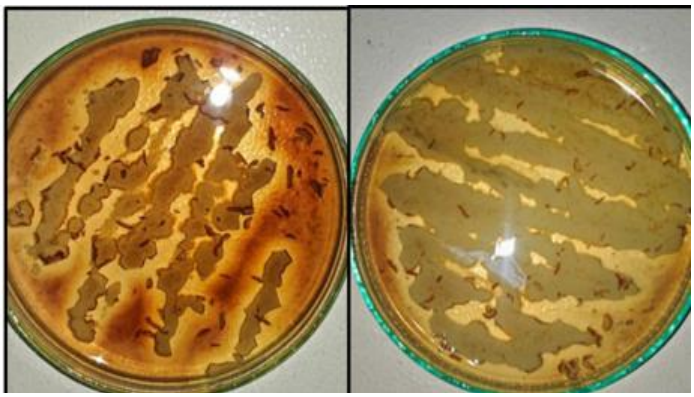

Fig.4. Starch Hydrolysis Test.

Fig.5. Motility Test.

Fig.6. Indole Test.

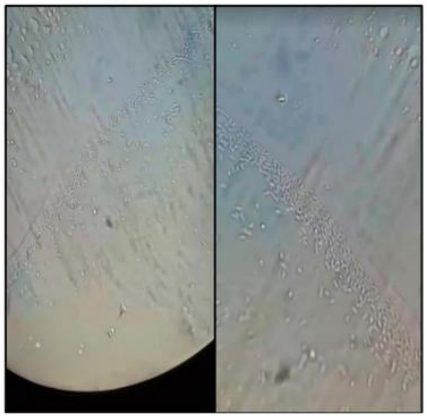

Fig. 7. Citrate Utilisation Test

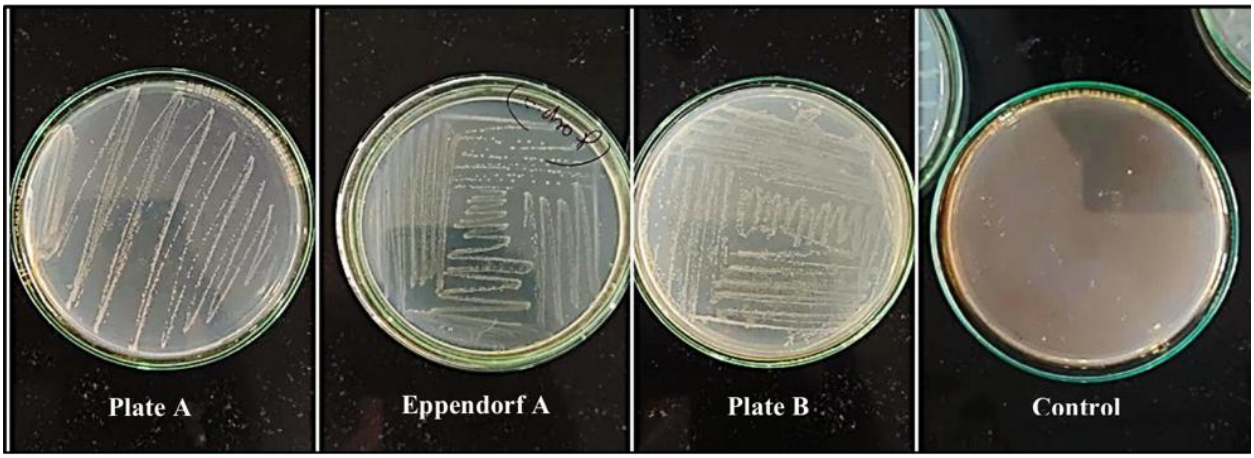

Fig.8. Streak Plate Method Culture.

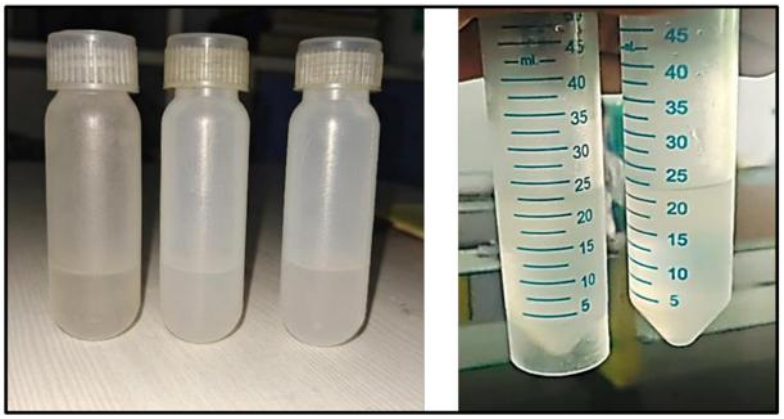

Fig. 9. Cell harvesting by centrifugation and partial purification of the autolysin fractions.

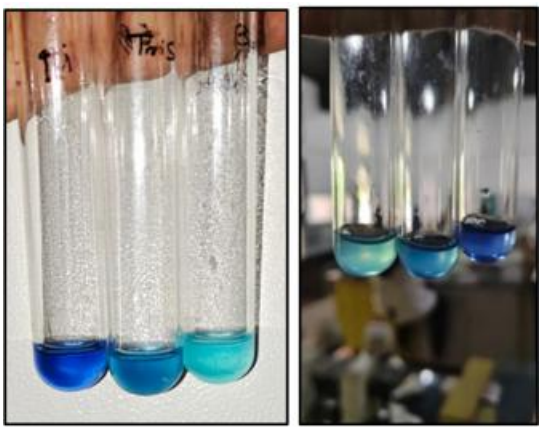

Fig. 10. Biuret protein assay for the LiCl and Tris-HCl extracts.

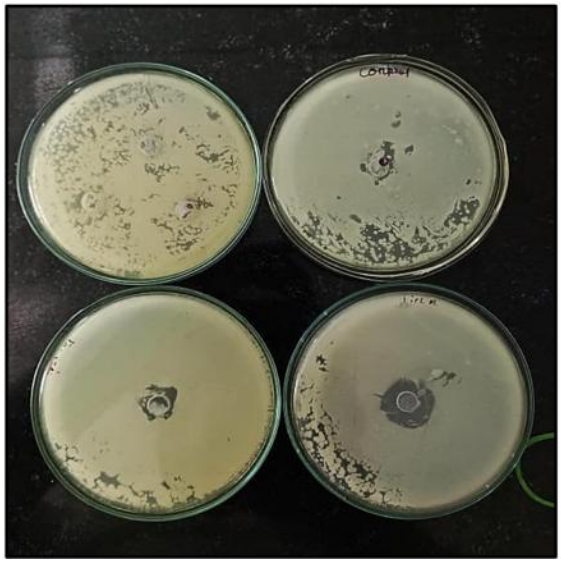

Fig. 11. Agar well diffusion assay showing zones of inhibition for control, LiCl, and Tris-HCl extracts.

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Conflict of Interest: all author has no conflict of interest about this research work.

Authors' Contribution:

Dr. Raja Kumar Parabathina: Designing the experiment and executing the protocols for the preparation of manuscript.

1. Prathamesh Anil Dangre: Worked on laboratory and executed the protocols.
2. Omkar Tukaram Chaudhari: Contributed in the laboratory and assisted for the preparation of manuscript.
3. Vishal Lolge: Contributed in the preparation of manuscript as well as procured the scientific information.

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