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Development of Effective Microbial Consortia based liquid formulations for Secondary Treatment of Wastewater

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ABSTRACT

Naturally occurring bacteria in wastewater have a significant potential for bioremediation, making them valuable for biological wastewater treatment. The effectiveness of microbial communities in breaking down pollutants depends on their diversity and metabolic capabilities. Therefore, developing environmentally friendly, indigenous microbial consortia is crucial for efficient wastewater treatment. In this study, 20 bacterial strains were isolated from wastewater samples collected from the dairy industry, sugar industry, and hostel sewage water in Pusa, Bihar. These isolates were screened for their ability to degrade starch, protein, and fat, as well as their potential to reduce Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). Among the 20 isolates, 18 exhibited starch degradation, 10 showed protein degradation, and 11 demonstrated fat degradation. Notably, seven isolates (DS-13, DS-27, DS-57, DS-58, DS-67, DS-68, and DS-72) exhibited all three degradation activities and were further analyzed for their impact on BOD and COD reduction. Out of these seven isolates, three (DS-58, DS-67, and DS-72) demonstrated the highest reductions in BOD and COD when inoculated into autoclaved effluents from the dairy industry, sugar industry, and hostel sewage. In contrast, the blank (un-autoclaved effluents without bacterial inoculation) recorded BOD levels of 835 mg/L, 1035 mg/L, and 620 mg/L, and COD levels of 1680 mg/L, 2280 mg/L, and 1470 mg/L for the respective effluent sources. To enhance biodegradation efficiency, these three bacterial isolates were combined into different formulations, creating four distinct microbial consortia. Among them, consortium C4 (comprising DS-58, DS-67, and DS-72) exhibited the highest biodegradation efficiency, with starch degradation of 14 mm, protein degradation of 24 mm, and fat degradation of 18 mm. Additionally, C4 significantly reduced BOD levels from 115 mg/L to 353 mg/L and COD levels from 407 mg/L to 641 mg/L. These findings indicated that the formulated C4 consortium has strong potential for biological wastewater treatment by effectively breaking down organic pollutants and reducing water pollution. Its application in wastewater management can contribute to environmentally sustainable bioremediation strategies.

Keywords: *Wastewater, Bioremediation, Microbial Consortia, Pollutants, Bacteria, BOD*

1. Introduction

Water is a critical natural resource for all life on Earth. It covers 71% of the earth's surface and accounts for 70% or more of a typical cell. Water availability and quality have always played a significant role in determining not only where people can live, but also their quality of life. A clean water supply is required for the establishment and sustenance of different forms of life. The world's rapid population growth has paved the way for ever-increasing agriculture, industrialization, and urbanization. However, most water sources around the world are polluted by liquid and solid wastes produced by human settlements and industrial activities. All of these industries draw a large amount of freshwater from various freshwater sources, such as rivers, lakes, reservoirs, and underground water, and produce different forms of wastewater. Agricultural effluents can contain plant nutrients as well as harmful chemicals such as pesticides and fertilizers; industrial effluents can contain harmful organic and inorganic chemicals, and domestic wastes can contain a variety of organic substances as well as human feces, urine, and plastic, paper, and so on [1]. The food sector especially the dairy and sugar industries, in particular, generates a large amount of wastewater per unit of production, resulting in a large amount of sludge [2]. Dairy and sugar industry effluents are high in dense fats, fatty acids, nitrogenous compounds, and high BOD, TSS, and organic load, which causes numerous environmental problems including a negative impact on both flora and fauna. Its discharge to the land changes the physical and chemical properties of the soil, reducing its fertility and eutrophication of water bodies. There have been numerous cases of mass annihilation of aquatic life in rivers and ponds across the country as a result of effluent discharge, as well as mass illness in humans because of consumption of polluted water [3].

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On global statistics, about 80% of the wastewater generated is cleared into the environment untreated, initiating extensive water contamination (WWAP, 2017). This direct discharge is prevalent in India, mostly in the agricultural and domestic sectors. India has a daily sewage generation of approximately 61,754 million litres per day (MLD), of which 22,900 MLD comprising of domestic wastewater is produced from urban households and 13,500 MLD from industrial wastewater (CPCB, 2015). Because of the hazardous effects of municipal, industrial, and hospital wastewater on water, soil, air, and agricultural products, wastewater treatment and proper sludge disposal are required for environmental safety. The Indian government initiated efforts to curb water pollution by passing the "Water (Prevention and Control of Pollution) Act, 1974", and related amendments to mandate the treatment of industrial effluents and to protect the health of natural water resources. Recently there has been surge in wastewater research in developing countries using simple, low-cost, easy-to-use methods. For wastewater treatment, activated sludge, aerated lagoons, stabilization ponds, natural and synthetic wetlands, trickling filters, and rotating biological contactors (RBCs) are being used extensively but need further improvements [4]. Typically, wastewater is treated in two stages: primary and secondary treatment, also known as physical and biological treatment, respectively, and possibly an additional chemical or tertiary treatment to make the water safe for human consumption [5]. Screening, filtration, sedimentation, and flotation are used in the primary treatment to remove any solid wastes present in the effluents, as well as other floating materials. Secondary or biological treatment removes dissolved and suspended biological material, which is normally performed by microorganisms in a controlled environment. Many authors have reported that biological methods are usually preferred over physicochemical methods in wastewater treatment for removing the majority of pollutants [6, 7]. The use of biological systems has the advantage of operating at room temperature, reducing labor time, improving BOD and COD removal, degrading a wide range of organic waste, increasing system efficiency, reducing sludge build up, and lowering hydrogen sulfide costs [8]. A traditional wastewater treatment biological reactor is distinguished by the presence of thousands of species from nearly all biological kingdoms comprised of bacteria, fungi, protozoa, and other microbes. Microorganisms are great bio-degraders due to their ideal characteristics such as small size, large contact surfaces to interact with their surroundings, and an exceptional surface-area-to-volume ratio [9]. These organisms have diverse catalytic capabilities and can grow well on several complex compounds available in wastewater for their growth and metabolism [10]. The study of this extremely complex ecosystem is necessary for improving knowledge of the ecosystem composition and understanding the biochemical reactions occurring within the reactor, which helps in improving its performance due to their large genomic and phenotypic variability. Many authors have tested microalgal-bacteria consortia to date with the goal of nutrient removal from water [11]. According to Henze et al. (2008) [12], aerobic biological wastewater treatment systems use mixed microbial consortia to convert organic and inorganic pollutants to harmless by-products, allowing municipal and industrial wastewater to be released into the environment without harm. Though the effluent is treated, it generates sludge, which must be removed, dewatered, and disposed of, making treatment time-consuming and labour-intensive during large-scale operations. However, a sludge-free system with a reduced number of operational steps is preferred to ensure long-term performance with minimal labour involvement [13, 14]. Although native microbes were present at the site, they were unable to perform the required function, necessitating bioaugmentation, biostimulation, or a separate microbial treatment [15]. The key to addressing the problem is to form a consortium of indigenous microbes [16, 17] . Due to individual strain limitations on a narrow range of biodegradation and mineralization, as well as the inability to completely degrade or mineralize, it has been discovered that microbial consortia are primarily beneficial due to the synergism of metabolic activities and concurrently carry out degradation tasks that no single culture can begin effectively [16] (Biswas *et al*., 2022). Individual strains in a microbial consortium may use metabolites produced by co-existing strains for further decomposition. Microbes acclimatize to toxic wastes in such approaches, and new resistant strains develop naturally, transforming various toxic chemicals into less harmful forms [18]. An effluent's indigenous microbiota is heavily influenced by its source, chemical composition, and physical parameters [19] . The composition of the microbial populations is highly unstable and can be easily transformed by the addition of new effluents. As a result, knowing the composition of the microbiota and the biochemical characteristics of the wastewater associated with the source of pollutants, in addition to optimal metabolic activity and physical-chemical conditions, becomes critical. Apart from utilizing the naturally present microbes in effluents, inoculation of improved or more sophisticated microbial cultures into an existing or native community can improve overall degrading performance. As a result, extensive research on the indigenous microbial population of various effluents and contaminated water bodies is required to develop a microbial consortium comprised of biochemically efficient and multi-functional microbial strains. Furthermore, the relationship between different microbial communities, the role of individual microbes, treatment rate, and efficiency are some of the most important criteria in wastewater treatment. Secondary wastewater treatment may benefit from liquid formulations of efficient microbial consortia [20] . They are not only simple and inexpensive to formulate but are also quite effective at maintaining a significant microbial cell count, cell viability, and other important parameters of a microbial consortium [21, 22]. It makes it easier to store a large population of specialized microbes in the medium for an extended period [23] . The liquid formulations are simple to use and have been shown to be effective for in-situ biological effluent treatment [24] . Based on this background present investigation was aimed to develop a consortia based liquid formulation of prominent microorganism for treatment of wastewater. We hypothesized that these liquid formulation helps in rapid treatment of wastewater and its safe disposal in environment.

2. Materials and Methods

2.1. Sample Collection

The effluent samples were collected from three distinct sources *i.e.,* Sudha dairy processing units located in Samastipur and Muzaffarpur, Hasanpur Sugar industry in Hasanpur and sewage water from the hostels of the Dr Rajendra Prasad Central Agricultural University, Pusa. The samples collected from these sources were then stored at 4˚C in the refrigerator (Supplementary Figure 1).

2.2. Selection of Bacterial Growth Media

Different growth media *viz.* Nutrient agar media (Peptone: 5.0; NaCl: 5.0; peptone: 1.500; yeast extract: 1.50; Agar: 15.0 in g/L final pH (at 25°C) 7.4±0.2), Kings's B agar media (protease peptone: 20.0; dipotassium hydrogen phosphate: 1.5; magnesium sulfate: 1.5; Agar 15.0 final pH (at 25°C) 7.2±0.2) [25] (King et al., 1954), Jensen agar media (calcium phosphate: 1.0; dipotassium hydrogen phosphate: 0.2; magnesium sulfate: 0.2; sodium chloride: 0.2; ferric chloride: 0.1; Agar 15.0, final pH (at 25°C) 7.0±0.2) (Jensen, 1950), and methylene blue agar (gelatine peptone: 10.0; dibasic potassium phosphate: 2.0; lactose: 10.0; eosin – Y: 0.4; methylene blue: 0.065; Agar: 15.0 pH after sterilization (at 25°C) 7.1±0.2) [26] media were used for isolation of bacteria from the collected effluent samples.

2.3. Isolation, Selection, and Purification of Different Bacterial Morphotypes

For isolation of bacteria, 1 mL of each effluent sample was taken and dropped into a test tube filled with 9 ml of sterile distilled water to make 10^{-1} dilution. The samples were further diluted up to 10^{-7}dilution by transferring 1 ml from the previous dilutions. Then the samples from different dilutions were spread on a Nutrient agar media plate, Kings's B agar media plate, Jensen agar media plate, and MBA (Methylene Blue Agar) media plates and incubated for 24-72 h at 37±2°C until distinct colonies appeared. All the inoculated plates were examined for different colony morphology *viz.* shape, size, colour, texture, and margin of the colony. Then the individual colonies were selected based on distinct morphology and purified by continuous streaking followed by quadrant streaking on respective media plates. The purified bacterial isolates were maintained for future use in their respective broth medium and also in slants at 4°C (Supplementary Figure 2).

2.4. Screening for starch, fat, and protein degradation

The preserved 1 mL of bacterial broth culture (of cell count about 10^6 cfu/mL) was transferred to the newly made broth media and screened for their starch, protein, and fat degradation traits and BOD and COD removal ability. Starch degradation activity of the isolates was determined by the formation of the zone of clearance on starch iodine plates as outlined by [27]**.** The purified isolates were streaked on starch agar plates which contain starch as the sole carbon source and were incubated for 24-48 h at 37ºC. After incubation, individual plates were flooded with Gram's iodine. There was the formation of a dark blue-colored starch-iodine complex in the region where starch was the present and clear zone (no blue colour formation) formation occurred in the region where starch is degraded. The amylolytic activity was conveyed in terms of mm diameter of the zone of clearance formed around the colony. The protein degradation activity of the isolates was confirmed through the formation of the zone of hydrolysis on skim milk agar plates [27] . 0.1 mL of each aliquot was pipetted out and spread on skim milk agar (1%) plates and then the plates were incubated at a temperature 37°C for 48 h. After the incubation, the plates were observed for the formation of a zone of hydrolysis which was the indication for the protein degradation. The proteolytic activity was conveyed in terms of mm diameter of clear zones formed around the colony. The fat degradation activity of the isolates was determined by the formation of the zone of clearance on 1% tributyrin agar plates [28].

The isolates were separately inoculated by single streaking on nutrient agar plates amended with 1% tributyrin to obtain lipase producers. The inoculated plates were incubated for an overnight at 37°C temperature and observed for a zone of clearing around each bacterial isolate. The lipolytic activity was conveyed in terms of mm diameter of clear zones formed around the colony (Figure 1).

2.4. Screening for Reduction of BOD and COD

To study the reduction of BOD and COD, autoclaved fresh dairy, sugar industry and hostel sewage effluent were used as substrate. 10% of the freshly prepared culture of cell count 3 x 10^6cfu/mL of each selected isolates were inoculated into the 1L breaker carrying 500 mL for each of the effluents. Another beaker of 1L with 500 mL of each effluent was taken as blank *i.e.,* no inoculation of promising bacterial isolates to record the initial BOD and COD readings. After the inoculation, the beakers were kept at room temperature for 48 h for incubation. Then the effluents were analyzed for reduction of BOD (APHA method 5210 B) and COD (APHA method 5220 C) using standard methods [29] .

2.5. Preparation of Bacterial Consortia

Bacterial consortia were developed by selecting the most potent isolates which showed maximum pollution reduction capability i.e., removal of BOD and COD. These isolates were grown separately and in different combinations *in a* 250 mL flask containing 100 mL nutrient broth*. The total volume of each inoculum was* 10 mL. There were four different types of consortia designed i.e., C1, C2, C3 and C4. The prepared consortia were kept on a mechanical shaker for 48 h at room temperature for continuous shaking and proper growth. Then the prepared consortia were screened for their starch, protein and fat degradation traits and BOD and COD reduction ability (Supplementary Table S1).

2.6. Preparation of Liquid Formulations

Liquid formulations were developed to evaluate their potential for maintaining microbial stability and physicochemical characteristics. A total of 10 formulations (Supplementary Table S2) were prepared using various combinations of emulsifiers, protective agents, and thickeners. The prepared formulations were stored under different temperature conditions (4°C, 28°C, and 40°C) and evaluated for stability over a six-month period, with observations recorded at intervals of 30, 60, 90, 120, 150, and 180 days (Supplementary Figure 3).

2.7. Components of Liquid Formulations

The liquid formulations were composed of three primary components: emulsifiers, protective agents, and thickeners. Tween-20, used as the emulsifier at a concentration of 2%, ensured uniform dispersion of all ingredients and contributed to the stability of the formulations. Protective agents, including Polyvinylpyrrolidone (PVP) and Glycerol, were added to enhance microbial viability and maintain stability during storage. These agents were used at two different concentrations, 2% and 4%, referred to as P1 and P2, respectively. Thickeners such as Acacia gum and Carboxymethyl cellulose (CMC) were incorporated at concentrations of 1% and 2% to improve the viscosity and structural integrity of the formulations, ensuring their suitability for long-term application.

2.8. Procedure for Preparation

The preparation of liquid formulations involved a stepwise process to ensure uniform mixing and stability. Initially, one gram of Acacia gum and two grams of Carboxymethyl cellulose were dissolved in 75–80 mL of distilled water in 120 mL of translucent, autoclavable plastic bottles. The mixture was shaken for 30 minutes to allow complete dissolution of the thickeners. Following this, 2–3 mL of protective agents, either PVP or Glycerol, were added to the solution and shaken for an additional 15 minutes to achieve homogeneity. Finally, 1–2 mL of Tween-20, serving as the emulsifier, was introduced into the formulation. The mixture was shaken for 5 minutes to ensure uniform dispersion of all components, resulting in a stable liquid formulation ready for further processing.

2.9. Inoculation of Microbial Isolates

Each formulation was prepared in three sets with two replicates. A 5 mL inoculum containing a mixture of 48-hour-old microbial cultures was added under aseptic conditions to the prepared formulations. The inoculated formulations were subjected to continuous shaking for 4 hours daily for one week. Following this period, the formulations were stored at three designated temperatures (4°C, 28°C, and 40°C).

2.10. Monitoring Stability

The stability and efficacy of the liquid formulations were assessed over six months. Observations were recorded at intervals of 30, 60, 90, 120, 150, and 180 days. Stability parameters such as pH, viscosity, phase separation, and microbial viability were monitored to determine the long-term suitability of the formulations for microbial applications.

2.11. Characterization of Formulations

The prepared liquid formulations were characterized for physicochemical stability, including pH, suspensibility, and cell count. These parameters were monitored at intervals of 30 days up to 180 days to assess the long-term stability and viability of the formulations.

2.12. pH Measurement

The pH of the formulations was determined using an OAKTON Multiparameter. For each measurement, 10 mL of the formulation was transferred to a 25 mL beaker, and the pH was recorded using a pH meter. The pH of all formulations was maintained at 7.0 by adjusting with 0.1N NaOH or 0.1N HCl, added drop by drop until a constant reading was achieved. At each time interval, samples of the formulations were taken under aseptic conditions, and their pH was measured to ensure stability.

2.13. Suspensibility

The suspensibility of the liquid formulations, a critical parameter indicating the dispersal of the emulsion in a water base, was assessed by measuring the width of the suspension layer. For this, 10 mL of each formulation was transferred into 15 mL graded test tubes and incubated overnight at temperatures of 4°C, 28°C, and 40°C. The next day, the width of the suspension that settled at the bottom of the test tube was recorded to evaluate the uniformity and stability of the formulations.

2.14. Cell Population

The cell population in each formulation was determined by counting colony-forming units (cfu/mL). A 1 mL sample of the formulation was taken using a micropipette (1000 µL) and diluted with 9 mL of sterile distilled water. Serial dilutions were performed up to a dilution factor of 10^{-6}. Each formulation was shaken well before sampling to ensure uniformity. From each dilution, 0.1 mL (100 µL) was spread on sterilized agar plates under aseptic conditions. The plates were incubated at 28°C for 48 hours, after which the colonies were counted to determine the viable cell population.

3. Result

3.1. Isolation of Indigenous Bacteria from Effluent

Isolation of the bacteria present in the effluents was performed by plating the diluted effluent on the plates of nutrient agar, Methylene Blue agar (MBA) and King's B media and the pure colonies were obtained by further streaking. The pure culture obtained was preserved at 4˚C for further studies and re-cultured every two to three weeks. The isolated strains were distinguished based on their morphological character. Total 20 individual bacterial strains were isolated from the effluent of all four sources. Out of the 20 strains isolated, isolates DS-8, DS-13, DS-19, DS-27, DS-34, DS-36 and DS-42 were isolated from university sewage, isolates DS-47, DS-48 and DS-52 were isolated from hostel sewage, isolates DS-57, DS-58, DS-67, DS-68, and DS-71 were isolated from the dairy effluents and isolates DS-72, DS-79, DS-82, DS-83 and DS-92 were isolated from the sugar industry effluents. (Table 1)

3.2. Screening of the Isolated Bacteria

The purified isolated strains were further screened for their biodegradation activities *viz.* starch degradation, protein degradation, and lipid degradation and pollution parameters reduction activities *viz.* BOD reduction, COD reduction activities.

3.3. Starch Degradation

The starch degradation activity of all the purified isolates was screened by streaking them on starch agar plates. The indication of the starch degradation was observed by the development of the zone of clearance around the colony. It was found that out of isolated 20 bacterial strains, 18 strains showed a considerable amount of starch hydrolysis (Table 2) and the zone of clearance was in the range of 1.1 mm to 15.5 mm. Isolate DS-72 showed the highest zone of clearance (15.3 mm) followed by DS-67 (14.6 mm) and the least zone of clearance was recorded by DS-8 (1.1 mm). (Table 3)

3.4. Protein Degradation

The protein degradation activity of all the purified bacterial isolates was screened by spotting them on skim milk agar plates (1%). The indication of the protein degradation was observed by the formation of the zone of hydrolysis around the spotted colony on skim milk agar plates. It was found that out of isolated 20 bacterial strains, 10 strains showed a considerable amount of protein degradation (Table 2), and the zone were in the range of 11.2 mm to 22.0 mm. Isolate DS-67 showed the highest zone of clearance (22.0 mm) followed by DS-72 (18.0 mm) and the least zone of clearance was recorded by DS-8 (11.2 mm). (Table. 3 and Figure 2)

3.5. Fat Degradation

The fat degradation activity of all the purified bacterial isolates was screened by spotting them on agar plates with 1% tributyrin as a fat source. The indication of fat degradation was observed by the appearance of the zone of clearance around the spotted colony on the 1% tributyrin agar plates.

It was found that out of isolated 20 bacterial strains, 11 strains showed a considerable amount of fat hydrolysis (Table 2) and the zone of hydrolysis were in the range of 3.2 mm to 15.2 mm. Isolate DS-68 showed the highest zone of clearance (15.2 mm) followed by DS-58 (12.0 mm) and the lowest zone of clearance was recorded by DS-36 (3.2 mm). (Table 3 and Figure 2)
Overall it was observed that out of the 20 isolates, only 7 isolates (DS-13, DS-27, DS-57, DS-58, DS-67, DS-68, and DS-72) showed all the three degradation abilities *viz.* starch, protein and fat. These seven isolates were taken for further screening for BOD and COD reduction.

3.6. Reduction of COD and BOD

Total of seven bacterial strains selected from the biochemical screening were further studied for their biological oxygen demand and chemical oxygen demand removal abilities from fresh wastewater of the dairy industry, sugar industry, and households. In the case of dairy effluent as substrate, the COD and BOD of the untreated effluent were recorded at 1680 mg/ L and 835 mg /L respectively. It was observed that isolate DS-58 (1680 to 749 mg/L) exhibited a maximum reduction in COD followed by DS-67 (1680 to 813 mg/L), whereas, DS-13 (1680 to 1008 mg/L) showed the least reduction whereas, in case of BOD reduction, DS-72 (835 to 367 mg/L) exhibited maximum reduction followed by DS-58 (835 to 386 mg/L), and DS-57 (835 to 492 mg/L) was showing least reduction in BOD of the substrate (Table 4)
In case of sugar industry effluent as substrate, the COD and BOD of the untreated effluent were recorded at 2280 mg/ L and 1030 mg/ L respectively. It was observed that the isolate DS-58 (2280 to 934 mg/L) exhibited a maximum reduction in COD followed by DS-72 (2280 to 987 mg/L), whereas DS-27 (2280 to 2020 mg/L) showed the least reduction. In the case of BOD reduction, DS-72 (1035 to 483 mg/L) exhibited maximum reduction followed by DS-67 (1035 to 521 mg/L), whereas DS-27 (1035 to 727 mg/L) showed the least reduction in BOD of the substrate. (Table 4)
In the case of hostel sewage effluent, the COD and BOD of the untreated effluent were recorded at 1470 mg/ L and 620 mg/ L respectively. It was observed that isolate DS-72 (1470 to 692 mg/L) exhibited a maximum reduction in COD followed by DS-58 (1470 to 718 mg/L), whereas, DS-13 (1470 to 1029 mg/L) showed the least reduction and in BOD reduction, DS-72 (620 to 308 mg/L) exhibited maximum reduction followed by DS-67 (620 to 313 mg/L), whereas, DS-13 (620 to 556 mg/L) was showing least reduction in BOD of the substrate (Table 4)
The results of this study were very promising because individual strains reduced pollution parameters by over 50% within 48 h. Isolates DS-58, DS-67, and DS-72 were found to be more efficient in BOD and COD removal abilities in all three different effluents, i.e., dairy, sugar, and domestic. Therefore, these three top-performing isolates, DS-58, DS-67, and DS-72, were used for the development of a consortium.

3.7. Designing the bacterial consortium

In the screening for the cross-streaking all selected bacterial strains were found compatible with each other. For consortium development, each selected bacterial isolates were grown separately for 3 days and mixed in equal amounts prior to the application. Four consortia were made according to the compatibility *viz.* C1 (DS-58 and DS-67), C2 (DS-67 and DS-72), C3 (DS-58 and DS-72), C4 (DS-58, DS-67 and DS-72).

3.8. Starch, Protein, and Fat degradation by the Consortia

After the designing, the bacterial consortia were evaluated for their combined biodegradation abilities. It was observed that consortium C4 exhibited promising activity concerning the other consortium. C4 recorded 14 mm, 24 mm, and 18 mm of the zone of clearance for starch, protein and fat degradation respectively. Whereas consortium C1 recorded 10mm, 20mm, and 14mm of the zone of clearance for starch, protein and fat degradation respectively, consortium C2 recorded 9 mm, 18 mm, and 11mm of the zone of clearance for starch, protein and fat degradation respectively, and consortium C3 recorded 12 mm, 15 mm and 12 mm of the zone of clearance for starch, protein and fat degradation respectively (Table 5)

3.9. Reduction of BOD and COD by the Consortia

In the case of dairy effluent, the COD and BOD of the untreated effluent were recorded 1670 mg/ L and 820 mg/ L respectively. The decrease in COD was observed highest in C4 (1670 to 471 mg/ L), followed by C2 (1670 to 512 mg/ L) and C1 (1670 to 552 mg /L) being the least. Similarly, the reduction in BOD was observed highest in C4 (820 to 318 mg/ L), followed by C2 (820 to 387 mg /L) and C1 (820 to 451 mg /L) being the least. (Table 6)
With sugar industry effluent, the COD and BOD of the untreated effluent were recorded 2123 mg /L and 1008 mg/ L respectively. The decline in COD was observed highest in C4 (2123 to 641 mg /L), followed by C2 (2123 to 797 mg /L) and C3 (2123 to 827 mg/ L) being the least. Similarly, the reduction in BOD was observed highest in C4 (1008 to 353 mg/ L), followed by C2 (1008 to 487 mg/ L) and C3 (1008 to 511 mg /L) being the least. (Table 6)
With hostel sewage effluent, the COD and BOD of the untreated effluent were recorded 1460 mg /L and 640 mg/ L respectively. The drop in COD was observed highest in C4 (1460 to 407 mg/ L), followed by C2 (1460 to 472 mg/ L) and C1 (1460 to 508 mg /L) being the least. Similarly, the reduction in BOD was observed highest in C4 (640 to 115 mg/ L), followed by C2 (640 to 297 mg/ L) and C1 (640 to 372 mg/ L) being the least. (Table 6)
From the above study, it was observed that the consortium C4 (DS-58, DS-68 and DS-72) was found to be the most effective in starch, protein and fat degradation and in the reduction of BOD and COD parameters in all three effluents *i.e.,* dairy industry, sugar industry and households which was further used in developed liquid formulations.

3.10. Preparation of Liquid Formulations

For the preparation of liquid formulation, several combinations of emulsifier, thickener and protective agents were used. Initially, a total of 25 formulations were made and examined for physical stability for 3 months. It was found that out of 25 formulations, only 10 formulations were found to be physically stable whereas the rest of the formulations broke up and were discarded after storage of 3 months. Thus, additional studies were carried out with stable liquid formulations.
The results have shown that the ability to reduce the pollution parameters by the cultures is not influenced when present in a mixture and, therefore consortia C4 (*i.e.,* the composite culture of DS-58, DS-68, and DS-72) was used in the liquid formulation. The mixture comprising an equal proportion of each bacterium was used so that the ultimate cell population to each formulation was 6.8×10^7 cells/mL. A set of each of the prepared formulations was kept at storage temperatures of 4ºC, 28ºC and 40ºC and observation for pH, suspensibility and the cell count of

revived culture were taken after 30 days, 60 days, 90 days, 120 days, 150 days, and 180 days (Supplementary table S1).

3.11. Physical Behaviour of Selected Formulations

Ten formulations that were found to sustain sufficient cell population were analysed for characteristics *viz*. pH and Suspensibility (width of suspension) at several intervals of time as stated and results are presented.

3.12. pH observations

The change in pH in the formulation was recorded using a pH meter. The observations regarding the pH of the formulations stood variable and were reliant on nature of the formulation. It was found that most of the formulations exhibited a steady decrease in pH with increasing time of incubation. In formulations kept at 4ºC, the pH varied between 4.72 and 7.0 among different formulations during 6 months of incubation. It was also observed that formulations F7 and F8 were utmost stable in terms of pH where the pH changes were of very less degree over time. At 28^0 C, the pH range was 4.74 to 7.0 and formulations F6 and F5 were found to be most stable whereas at 40^0 C, the pH range was 4.71 to7.0 and formulations F7 and F8 were found to be most stable. (Table. 7; Table 8 and Table 9)

3.13. Suspensibility (Width of Suspension)

It was observed that the width of suspension was inconstant and fluctuated within a range of 0 to 1.2 cm amid different formulations and through different temperatures. Initially, it increased with increase in incubation period till 4 months and later declined with further increase in the incubation period and became stable irrespective of the type of formulations at all the three stored temperatures and noted the lowest value of the width of suspension at the end of the incubation period. The decrease detected was comparatively more at a higher temperature of 40^0C as compared to at a lower temperature of 4^0C. (Table 10; Table 11 and Table 12)

3.14. Cell Population (cfu)

As compared to initial cell population of 6.8×10^7 cells/mL that was added to each formulation, all formulations maintained a cell population of 2.6×10^7 to 5×10^7 cells/mL after 6 months of incubation across all the stored temperatures. It was also observed that the decline in cell count was less during the early incubation period followed by a decrease in the further storage. After 6 months of incubation, F7 maintained maximum cell population of 5×10^7 cells/mL followed by F5 and F8 at 4^0C whereas, at 28^0C, formulation F6 and F8 maintained maximum cell population of 4.8×10^7 cells/mL followed by F4 which was 3.7×10^7 cells/mL. At 40^0C, formulation F8 and F7 maintained maximum cell population of 4.0×10^7 cells/mL closely followed by formulation F6 (3.7×10^7 cells/mL) after 6 months of incubation. It was found that formulation F10 i.e. formulation in which pH was not adjusted and no protective agents were added and only mixed cultures were inoculated showed minimum cell population at the end of six months at all the stored temperatures of 4^0C, 28^0C and 40^0C respectively. (Table 13; Table 14 and Table 15)

4. Discussion

The effluent samples were collected from three different sources *i.e,* Sudha dairy processing units located in Samastipur and Muzaffarpur, Hasanpur Sugar industry in Hasanpur and sewage water from the hostels of the Dr Rajendra Prasad Central Agricultural University, Pusa for isolation of bacteria. 1 ml of effluent sample was taken and added to 9 ml of sterile water. Dilutions up to 10^{-7} were made and the suspension was plated on different media i.e., Nutrient agar media, Kings's B agar media, Jensen agar media, MBA (Methylene Blue Agar) media. Many scientists have made several attempts to isolate bacteria from the wastewaters in the recent past [30, 31, 32, 33, 34, (. Sharifi-Yazdi et al. (2001) [35]used nutrient agar medium and isolated 10 different bacterial isolates from industrial effluents and Jain et al. (2012) [36] isolated three different bacterial isolates from distillery effluents using nutrient agar medium. Sankaran et al. (2015) [37] used Kings's B agar media and isolated 7 different *Pseudomonas* strains from the distillery effluent samples. Bharti et al. (2021) [38] isolated 10 different bacterial strains using MBA media. The results of different studies suggest that various bacteria can be isolated by using different media. The bacterial isolates thus obtained using different media are further purified and stored for further use.

To study the capability of the isolates, all the morphologically dissimilar isolates, isolated using different media were screened for biodegradation abilities (starch, protein and fat) and reduction of BOD and COD. A sum of twenty isolates were isolated on the basis of their colony morphology, out of them three were able to express the biodegradation ability.

Starch is a very commonly found organic matter in the wastewaters and easily utilised by the bacteria as a carbon source. In the present study starch degrading bacteria were isolated from the three different effluents *i.e.,* dairy processing units effluent, sugar industry effluents and sewage water. It was found that out of the 20 isolates, 18 isolates were good starch degraders and the zone of clearance were in the range of 1.1 to 15.3 mm. This may be due to the release of exo-enzymes like α-amylase and oligo-1,6- glucosidase by bacterial isolates that breaks down the starch molecule into simple and smaller sugar which is easily absorbed by the bacterial cells. Similar observations of our findings were observed by many scientists [39, 40, 41, 42, 43, 44, 45]. where they isolated starch degrading bacterial strains from various industrial and sewage effluents. Shanmugasundaram *et al.* (2015) [46] also isolated 12 bacterial strains from contaminated water which showed a significant quantity of starch hydrolysis and the zone of clearance was in the range of 1.2 mm to 13.1 mm, whereas Padma and Pallavi, (2016) [47] reported a strain of *Bacillus sp.* exhibited positive results for amylase activity and produced the widest diameter *i.e.,* 8 mm of the zone of clearance.

Protein is the structural Compound present in all kinds of biological systems, hence are ubiquitous. In the present study, protein degrading bacteria were isolated from the three different effluents *i.e.,* dairy processing units effluent, sugar industry effluents and sewage water. It was observed that 10 out of 20 isolates were good protein degraders and the zone of clearance were in the range of 11.2 to 22 mm. These may be due to the action of isolates by producing proteinases, peptidases or proteolytic enzymes that break the peptide bonds between amino acids of the protein. Similar observations were reported by Albanna et al. (2023) [48] where the bacterial strains isolated from dairy effluent showed a zone of inhibition in the range of 11 to 29 mm when screened on skim-milk agar plates whereas Shivsharan et al. (2013) [49] also isolated 12 protein degrading bacterial strain from dairy effluent and the diameter of zone of solubilisation was in the range of 8 to 14 mm. Many scientists [50, 51, 52] have also previously reported efficient protein degraders isolated from the wastewater of dairy industries.

Fats are polymers of lipid and have a very high molecular weight. They are rich in energy and provide high energy when consumed by the microorganisms. In the present study, dairy processing unit effluent, sugar industry effluents and sewage water were used as samples to isolate fat-degrading bacteria. It was observed that, out of isolated 20 bacterial strains, 11 strains were good degraders of fat and the zone of hydrolysis was in the range of 3.2 mm to 15.2 mm which was similar to the results obtained by Adebami and Adebayo-Tayo (2020) [53]. This was possibly due to the action of enzymes like lipases which catalyse the lipolysis process producing triglycerides which are easily absorbed by the microbes. The existence of lipase producing microorganisms in a diverse environment like dairies, vegetable oil processing factories, industrial wastes, water polluted with oil, oilseeds, and decomposing food, compost masses and hot springs have been examined by many scientists [54, 55] from various wastewater sources. Similar report were observed by Gupta *et al.* (2004) [56] where they examined 38 different bacterial sources from which common lipases are derived whereas Tomulescu *et al.* (2015) [41] reported 66 strains isolated from dairy sludge having lipase activity against fats. Chaturvedi *et al.* (2010) [57] screened fat degrading strains of *Bacillus subtilis* having lipase production ability.

Chemical oxygen demand (COD) determines the quantity of oxygen required to oxidize organic matter in water under specific conditions of oxidizing agent, temperature and time and the biochemical oxygen demand (BOD) determines the amount of dissolved oxygen needed by aerobic organisms in a water body to break the organic materials present in the given water sample at certain temperature over a specific period of time. Both are very important parameters of measuring level of water pollution. Many scientists have reported bacteria with a very sound efficiency in reducing the BOD and COD in the effluents they are inoculated. Abou-Elela *et al.* (2010) [58] reported that strains of *Staphylococcus xylosus* were able to reduce COD and BOD with a removal efficiency of 93.4% and 95% for COD and BOD respectively. Garcha *et al.* (2016) [59] isolated two strains, each from *Bacillus thuringiensis and Brevibacillus brevis* which reported efficient in reducing the BOD.

The pH of any liquid formulation is very crucial as the microorganism present in the formulation should not be contrived by extreme pH i.e., very high or low. The results, has shown that pH of selected formulations varied between 4.7 and 7.0. This implies that the formulations undertaken are quite stable in terms of pH. In one of the studies, the pH of the liquid formulation of a *Bacillus sp.* ranged from 7.0 to 6.46 between 1-2 months [60] . Since, pH carries one of the crucial roles in liquid formulations so it ought to be stabilized within certain ranges. As, in results there was only gradual pH changes found during long term storage ratifying that the formulations prepared are stable. The pH can also be upheld by the addition of some additives that may perhaps render the increased shelf life of the cells [61].

Likewise, suspensibility of any liquid formulation is very crucial factor that determines the dispersion of the emulsion into water base. It was observed that the width of suspension in the formulations was not very high or low and ranged between 0-1.2 cm across different formulations which implied that the bacterial cells present in formulations, when spread in the wastewater, will stay in the upper water layer and could get adequate light and oxygen to grow and multiply. It was recorded that there was little decrease in width of suspension at 40ºC. However, there is no mention in literature about the suspensibility (width of suspension) of the formulation used to prepare consortia.

Liquid formulations preparations contains the desired microorganisms and a media with their source of nutrition moreover they comprise various chemical compounds like cell protectants or polymeric additives which helps in support an extended shelf life and deliver tolerance to adversative conditions (Hegde *et al.*, 2008)[62]. Various kinds of polymers have been used for inoculant preparations. They have the characteristics such as ability to reduce heat transfer, great water activities. Polymers obtained such as gum arabic, polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP) and sodium alginate are normally under use as thickening or adhesive agents [63]. In the present study, a combination of thickener, emulsifier and protective polymers have been used to prepare different liquid formulations and the survivability of the bacterial cells was observed over a period of six months under storage temperatures of 4ºC, 28 ºC and 40 ºC. Most of these cases exhibited a drop in population initially with the interval of time and the magnitude of declination differed according to the type of formulations. Nonetheless, ten formulations composed of different concentration and combination of protective polymers preserved a cell population on balance to the control at the termination of incubation at all the temperatures studied.

All formulations had acacia gum as one of the elementary constituents of the formulation. Krumnow *et al*. (2009) [64] also established that gum acacia used in formulation of *Escherichia coli* and *Bacillus subtilis* affords higher number of viable cells and enhanced protection during storage period at 40 °C. Tittabutr *et al*. (2007) [65] have described gum acacia as an agent to protect the cells from desiccation and drying, trap the bound water and thus avoiding thorough dehydration of the organisms and have the capability to reduce heat transfer and also have high water activities.

Carboxy methyl cellulose (CMC) is used as another basic components of formulation. The present findings are similar to the results obtained by SanthoshG.P. (2015) [66] who reported that CMC has shown the capability to support a high bacterial concentration up to six months of storage. Usage of "Tween 20" as surfactant in the formulation is suggested since it is a non-ionic surfactant which has the characteristics like stability and relative non-toxicity that allows it to be utilized as an emulsifier in a number of domestic, pharmacological and scientific applications [63].

The higher survival rate under cold conditions (4ºC) may perhaps be due to the point that low temperature permits little or no growth at all along with lowered consumption of nutrients in the course of storage leading to availability of the organism in optimal concentration for a longer period. It also restricts cell death in the inoculums. Conversely, during storage at room conditions the temperature might go above 30ºC and thus leading to growth of organism and depletion of nutrients and build-up of toxic components [65]. The protectants like glycerol and PVP fix intracellular water colligatively which later on inhibits extreme dehydration, limits salt toxicity and averts the development of ice crystals within the bacterial cell [66, 67](Mugilan *et al.,* 2011; Singleton *et al*., 2002). Glycerol has characteristics such as high water binding capacity and can safeguard the cells from desiccation by dropping the rate of dehydration [68]. In the present study, the formulations containing PVP and combination of PVP and glycerol showed higher shelf life at 28ºC and 40ºC. Yadav *et al*. (2017) [69]

observed improved shelf life of PSB in inoculants altered with PVP (1%, 2%) followed by glycerol (1%, 2%) for the period of the storage at room temperature. Likewise, Sridhar *et al*. (2004) [70] developed a liquid inoculant of *Bacillus megaterium* in which glucose, glycerol and PVP maintained upper viable cell population up to 6-month storage period. Prepared liquid inoculants with amended additives such as GA, glycerol and PVP exhibited higher viable cell concentration in comparison to inoculants at 18-months of storage [71].

Polyvinylpyrrolidone (PVP), a water-soluble chemical compound having colloidal stabilization and adhesive properties with great water holding capacity that slows down the dehydration rate of the media, consequently preserving the moisture level in the media and uphold water nearby the cells for their uptake [67, 72]. It also has an ability to fix bacterial toxins (Santosh, 2015). It could be valuable in reducing the degree of protein precipitation or coagulation in cells, preservation of macromolecular structure which increases the biological integrity and enhanced survival [72]. Jaiswal *et al*., 2023 [73] reported that bacteria do not utilise these polymers as an energy source.

It was also observed that the formulations without any protective polymers and pH stabilisation (Formulation F10) exhibited lower shelf life and cell count as compared to formulations containing protective polymers. Study done by Prajapati and Modi (2014) [74] on *Enterobacter harmaechei* established that liquid formulation has better shelf life as compared to solid formulations. Kanitkar and Kanitkar (2004) [75] recounted that the liquid formulation with mixed culture possess more viable counts (10^8 cells/ml). In the present study, the organisms from all the formulations were checked for growth.

Liquid formulation of microbial consortia can play a crucial role in making an organism effective in the field of application. Their characteristics like longer shelf life, high cell count zero contamination, increased protection against adverse environment and performance efficacy. Henceforth, enhanced shelf life can be attained by the application of a liquid formulation. This observation draws a parallel with the fact that the growth rate of inoculants at higher temperatures (45°C) was very little [65, 76] . The present study has shown that the bacterial consortium prepared of isolated bacteria naturally occurring in various wastewaters is a suitable method to decontaminate the wastewaters by an eco-friendly manner and the consortium can be efficiently preserved in liquid formulation along with various protectants for an enhanced shelf life of the viable cells. These formulations were found stable in terms of their physical, chemical and biological behavior in maintaining viable cell population of the consortia. The formulations have been tested and found to possess a shelf life of six months so that the new inoculant can be effectively used in the treatment of wastewater.

In conclusion, this study started with the successful isolation and characterization of bacterial strains from effluent samples of dairy processing units, sugar industries, and sewage water for their biodegradation potential. Among 20 isolates, a significant number demonstrated starch, protein, and fat degradation abilities, highlighting their efficiency in organic matter breakdown. The formulated microbial consortia, incorporated into liquid formulations, showed promising results in reducing BOD and COD levels, making them effective for secondary wastewater treatment. Stability assessments revealed that protective polymers and pH stabilizers enhanced bacterial viability over six months. These findings suggest that liquid microbial formulations can serve as sustainable and eco-friendly solutions for wastewater bioremediation.

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Conflict of Interest

The authors declare no conflicts of interest relevant to this article

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