

Production of Proline and Protease With Different Organic Wastes in Bacteria

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Abstract

Background: In this study, we investigated the proline and protease production of different bacteria in several organic waste materials. Our aim was to produce proline and protease economically in waste that is abundantly available while reducing its environmental impact.

Methods: 5 ml of different organic waste materials were placed in 10 ml grow tubes, inoculated and incubated for 24 h. Phosphate-buffered saline and 10% solutions of different organic wastes were added. These cultures were subsequently incubated at 37°C for 24 h. Cells were harvested at 24 h for L-proline assay. 1 ml of culture was transferred by pipette into an Eppendorf tube and centrifuged at room temperature. Cellular debris was removed by centrifuge and the supernatant was used for proline activity assays.

Results: Protease activity was determined using a modified method with casein as the substrate. We found that proline and protease can easily be produced economically using TCW, WCW and OWW organic waste.

Conclusion: We believe that this study will result in similar research leading to the economical use of these waste materials thus reducing their impact on the environment.

Background

Microbial degradation, or biodegradation, appears to be the most environmentally friendly method of removing hydrocarbons. Other methods can result in toxic compounds entering the environment.

P. aeruginosa floats with a single, polar, monotric flagellum rotating with proton motivating power. In the context of disease, this flagellar swimming motility is important in infection because the lack of swimming ability of the *P. aeruginosa* mutant causes a decrease in pathogenesis [1, 2].

Escherichia coli is a rod-shaped Gram (-) bacterium commonly found in the large intestines of warm-blooded animals [3] It is a model organism for the behavior of bacterial cell movement in mechanical and mass fluids. In particular, *E. coli* has been used as a prototypic micro-swimmer. The close motility of *E. coli* cells is important in the early stages of biofilm formation and pathogenic infection [4]. *E. coli* cells are important in the early stages of biofilm formation and pathogenic infection. *E. coli* cells have several extracellular, helical thread-like structures called flagella [4,5].

Bacillus cereus is a Gram (+), spore-forming, mobile, aerobic, rod-shaped and facultatively anaerobic bacterium. *Bacillus subtilis* is a soil bacterium that has a versatile metabolism and the ability to survive in various habitats. In nutritional research, it is known to enter the fusion motility as a cellular differentiation program when exposed to nutritional stress conditions [6,7].

Swarming migration has a bacterial action that can contribute significantly to the pathogenesis of *Bacillus* infection. Bacteria can be varied into prolonged, multi-core, hyper-flagellated swarmer cells that

can move away from the colony in a coordinated manner along a moist, solid surface or in a viscous environment [8].

Staphylococcus aureus is an anaerobic Gram (-) bacterium with a coccoid structure and causes widespread infections [9]. It has been shown that *S. aureus* colonies can passively propagate along the surface of soft agar plates using a surfactant in a process called propagation [10].

Proteases are a group of enzymes commonly found in plants, animals, and microorganisms. Commercially important, they are used in silver recovery, industrial peptide synthesis, the hydrolysis of proteins in the food industry and for various applications in the textile industry. Proteases are also involved in the degradation of dead plant and animal tissues in aquatic and terrestrial environments. Protease producing microorganisms can be extremely harmful to the environment. Bacterial proteases are more effective and practical than other cell proteases [11-16]. Proteases also play a role in numerous pathological processes. Microbial proteases act like virulence factors and are known to damage the defense proteins of the host organism. Commonly used microorganisms for protease production include *Streptococcus* sp., *Bacillus* sp., *Streptobacillus* sp., *P. aeruginosa*, and *E. coli* [17-20].

Salinity is an important abiotic stress mechanism affecting microorganism growth and productivity [21]. Under severe stress conditions, it can lead to growth inhibition or cell disruptions [22].

No microorganism can actively pump water in or out of a cell to compensate for water fluxes caused by changes in external osmotic conditions [23].

To survive these stresses most organisms have to stress-adaptation mechanisms. It's one of these things proline. To survive these stresses, most organisms have stress-adaptation mechanisms, one of which is proline [22, 24].

In microorganisms, proline is synthesized from L-glutamic acid via three enzymatic reactions [25]. The proline is not only involved in protecting the intercellular structure and regulating the cytosolic pH but also in promoting protein integrity and activating enzyme activities. Proline is particularly important in prokaryotic and eukaryotic cells and has been reported to act as an osmolyte [24,26]. Proline is an amino acid that increases in stress conditions, participates in the detoxification of free oxygen radicals and has protective properties that are important in resisting stress conditions [27].

In addition, *in vitro* studies have shown that proline has multiple functions in many organisms. It enhances the stability of proteins and membranes during freezing, dehydration, or high temperatures [2].

The aim of this study was to investigate the presence of *E. coli*, *B. cereus*, *S. aureus* and *P. aeruginosa* in various organic waste materials, to record survival levels and to measure the production of proline and protease.

Materials And Methods

Reagents

All chemicals were of the highest purity available commercially.

Microorganism

P. aeruginosa (ATCC 27853), *E. coli* (ATCC 25922), *S. aureus* (ATCC BAA 1026) and *B. cereus* (ATCC 10876) obtained from the ATCC and used this study.

Waste cheese whey

This waste was collected from commercial cheese factories in Malatya, Turkey.

Waste frying oil

Waste frying oil (WFO) was obtained and collected from the food Restaurant Malatya, Turkey.

Sugar beet molasses

This waste was collected from Malatya Sugar Factories in Malatya, Turkey. These wastes were filtered and then, they're autoclaved, and then used.

Other organic wastes

Others have supplied it ourselves because it is household waste.

Growth conditions

P. aeruginosa was cultivated in Luria- Bertani (LB) broth at 37 °C, static on incubator for overnight (O/N). 100µl of overnight cultures was setting OD₆₀₀ nm of 0,3 grown tube, and then filled with 5 ml in 10 ml tubes. Incubated was for 24 h of time on 37 °C. Bacterial growth was determined by measuring the absorbance at 600 nm (OD₆₀₀) by a visible spectrophotometer.

Proline assay

Bacteria were grown in a 10 ml flask containing 100 ml of LB medium. These cultures grew at 37 °C for 24 h. For L-proline assay, cells were harvested at 24 h growth phase. 1 ml culture was pipetted ependorf tube and centrifuged at 14.000 rpm for 20 min at room temperature, cellular debris was removed by centrifugation. Supernatant was then used for prolin activity assays [21].

Proline activity assay

After centrifugation supernatant was discarded and then on pellet 100 µl GTE buffer (glucose, Tris-HCl, EDTA) was added. Later room conditions were held for 1 min and then 200 µl lysis buffers was added and 5 min stay room temperature. Over 500 µl acidic ninhidrin was added. The tubes were kept in boiling

water for 30 minutes. After tubes were then cooled. The mixture was pipettes into new set tubes, and 2 ml benzene was added. The mixed in tubes gently vortexed and left at room temperature for 1 h until separation of the different two phases. The aqueous phase (i.e., the lower phase) was discarded by dipping a pipette through the organic phase add new tubes (i.e., the upper phase). Each sample reads at OD₅₂₀ nm against using benzene a blank [21, 28]. The proline concentration in samples was determined from a predetermined standard curve using proline.

Protease activity assay

Protease activity was determined using a modified method with casein as the substrate. The absorbance was read at OD₆₆₀ nm using Spectrophotometer [14, 16, 29, 30].

$\epsilon = (\Delta A^\circ / \text{min}) / 0,6896$ (This formula is used in calculation) [31].

Results

The main objective was to evaluate organic residues, examine their potential as media and to investigate the viability of certain parameters such as proline and protease production. NB was used for (+) control and PBS for (-) control. All study results were obtained after 24 hours.

Proline

We evaluated various organic waste materials found in the environment. The focus was on the production of proline and protease, which are commercially important and necessary to protect the environment.

Excluding the control media, Gram (+) bacteria showed higher proline production than Gram (-) bacteria. *B. cereus* produced 4.996 µg / ml proline in the presence of PLW (Fig. 1). The lowest production was seen in *P. aeruginosa* with 0.24 µg / ml in the presence of TWW (Fig. 7). However, on average Gram (-) bacteria in this study showed higher proline production. In our previous study in LB medium, we obtained 5.071 µg / ml in *E. coli* (Fig. 3) and 1.337 µg / ml in *P. aeruginosa* (Fig. 7). In the presence of 100 mM KCl in the same medium, we obtained 2.395 µg / ml in *E. coli* (Fig. 3) and 9.226 µg / ml in *P. aeruginosa* (Fig. 7).

In our study, the highest production was observed in *B. cereus* with molasses at 4.381 µg/ml and in the presence of WCW (Fig. 1) in *S. aureus* with 2.074 µg/ml (Fig. 5), excluding NB and PBS media used for control. The most efficient medium for *E. coli* was eggshell with 1.982 µg / ml (Fig. 3). For *P. aeruginosa*, it was TCW medium with 3.27 µg/ml (Fig. 7). Considering all environments, the most efficient in the study were eggshell and OWW. The most effective waste materials for proline production were TCW with 1.738 µg/ml average and molasses with 1.250 µg/ml average (Fig. 1). The most ineffective waste was TWW with an average of 0.859 µg / ml (Fig. 5). The most effective proline producing bacteria were *P. aeruginosa* with an average of 2.551 µg / ml (Fig. 7). All bacteria produced an average of 1.738 µg / ml proline in the presence of TCW ((Fig. 1 and 3). The lowest amount of organic waste was obtained in the

presence of TWW with an average of 0.859 $\mu\text{g} / \text{ml}$. While *P. aeruginosa* produced 2.551 $\mu\text{g} / \text{ml}$ proline on average in all environments (Fig. 7), *S. aureus* produced the lowest amount with 0.993 $\mu\text{g} / \text{ml}$ (Fig. 5). Since no research similar to our study was found in the literature, no comparison could be made with other studies. This study was conducted with different biological waste materials. We believe that microbiological proline production can be achieved practically and economically thus benefiting the environment.

As has been shown in previous tests (mostly on plants), proline is a response to stress in living organisms. For unknown reasons, negative changes were observed in *E. coli* and *B. cereus*. The biggest changes were seen in *S. aureus* (4.4-fold) (Fig. 5) followed by *P. aeruginosa* with 2.9-fold (Fig. 7). WCW did not materially increase stress response. This may result from the lack of adequate components for proline production in WCW and the weakness of the protein-produced metabolic pathways in bacteria.

When the control media are excluded, the lowest proline production was in TWW at 0.24 $\mu\text{g} / \text{ml}$ (Fig. 1) and the highest in TCW at 3.27 $\mu\text{g} / \text{ml}$ (Fig. 7). The difference was 13.6-fold.

When we look at proline production in the presence of organic waste, the highest value (+) were reached with 12.38 $\mu\text{g} / \text{ml}$ in NB medium (Fig. 5), which we used as control. 0.69 $\mu\text{g} / \text{ml}$ were obtained in (-) control PBS (Fig. 5). Inhibition was seen in TWW (0.24 $\mu\text{g} / \text{ml}$) (Fig. 1) and molasses (0.55 $\mu\text{g} / \text{ml}$) (Fig. 3). In the presence of the remaining organic waste materials (OWW, TCW, and WCW), values of 1.26, 3.27 and 2.02 $\mu\text{g}/\text{ml}$ was obtained, respectively (Fig. 5). However, no value could be obtained in the presence of WFO (not shown on the graph). This is interesting and may be due to the high carbohydrate load. These results show that the protein load in organic waste is important in proline production. TCW, WCW, and OWW are organic wastes that support proline production.

Protease

Protease is a virulence factor and a secondary metabolite. No protease production was detected in our study on EW and PLW so they are not shown on the graph. Unlike proline production, protease activity was higher in Gram (-) bacteria. However, the highest value in (-) control PBS was 0.104 U/ml while no results in (+) control NB were obtained in these experiments.

In our previous study with LB medium in *E. coli* and *Paeruginosa*, 0.401 U / ml and 0.2976 U / ml values were obtained, respectively. In the presence of 100 mM KCl, 0.9327 U / ml were obtained in *E. coli* and 0.295 U / ml in *P. aeruginosa*.

In our study, the highest protease production in WCW with high protein load was in *E. coli* (Fig.4). However, when we looked proportionally, it was clear that the highest difference was in *P. aeruginosa* at 67.5 times (Fig. 8). Why did *E. coli* produce the most despite this huge difference? This may have resulted from the metabolic load caused by the large genome of *P. aeruginosa* close to yeasts (Fig. 8). The lowest rate was seen in *S. aureus* (0.31) (Fig. 6) and the lowest difference in *B. cereus* (3.3-fold) (Fig. 2). We think this is because *B. cereus* is soil bacteria and protease activity may not be much needed in the soil

environment. However, other bacteria may have higher protease activity than *B. cereus* because of their ability to survive in living organisms.

The highest values were observed in *P. aeruginosa* at 51.347 U / ml (Fig. 8) and in *E. coli* in the presence of molasses at 47.182 U / ml (Fig. 4). The highest subsequent rate was 27.297 U / ml in the presence of OWW (Fig. 7). *B. cereus* reached the highest value in the presence of TCW with 8.394 U / ml (Fig. 2). *S. aureus* reached 3.717 U / ml (Fig. 6), again in the presence of molasses. The most efficient medium was molasses with a high carbohydrate load. The highest average for all four bacteria, 24.841 U / ml, was reached in the presence of molasses with a high hydrate load. Protease production in the last three environments was the lowest: WFO 0.261 with *B. cereus* (Fig. 2), 0.170 U/ml *P. aeruginosa* (Fig. 8) in the same organic waste and 0.159 U / ml *S. aureus* (Fig. 6). The lowest average was in the presence of WFO with 0.215 U / ml, excluding controls (Fig. 6). *E. coli* yielded the highest mean value of 13.326 U / ml in all media (Fig. 4). The lowest average production was in *S. aureus* at 1.9 U / ml (Fig. 6). The three most successful bacteria were *P. aeruginosa* with 51.35 U / ml (Fig. 8) and *E. coli* with 41.182 U / ml in the presence of molasses and *E. coli* with 27.297 U / ml in the presence of OWW (Fig. 4). The most efficient production waste environment was molasses with an average of 24.81 U / ml (Fig. 6) and OWW with an average of 8.288 U / ml (Fig. 8). When all waste materials were evaluated separately, the most effective production among bacteria was *E. coli* with an average of 13.326 U / ml (Fig. 4).

Protease activity was approximately 23-fold higher than proline activity. The OD₆₀₀ value (data not shown) increased by 314% and the increase in the number of living cells was reached 1,816%. According to these results, cells may be more viable and transparent.

We believe that protease production from organic waste is also important for environmental protection so we focused on the organic wastes mentioned above. Unlike proline production, the highest production was in the presence of molasses (51.35 U / ml) (Fig. 6). The OD₆₀₀ (1.36) value of molasses was found to be the highest in this study. The lowest value was (-) in control PBS (0.04 U / ml). The most significant values were then observed in TCW (3.84 U / ml) (Fig. 2) and WCW (2.93 U / ml) (Fig.4). Except for the control, the lowest value was 0.17 U / ml in WFO with a high oil load (Fig. 6).

Discussion

This study showed that proline and protease can both be produced economically and practically from TCW, WCW and OWW organic waste. We believe that this study will result in similar new research leading to the practical and economic use of waste material thus benefiting the environment. We think that this study will lead to similar new studies. At the same time, we think that the way to bring these wastes into the economy will be opened and the environment will be protected.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

HK and CCK designed this study. HK and CCK performed most experiments. Authors analyzed the data, wrote the manuscript, read and approved the final manuscript.

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Figures

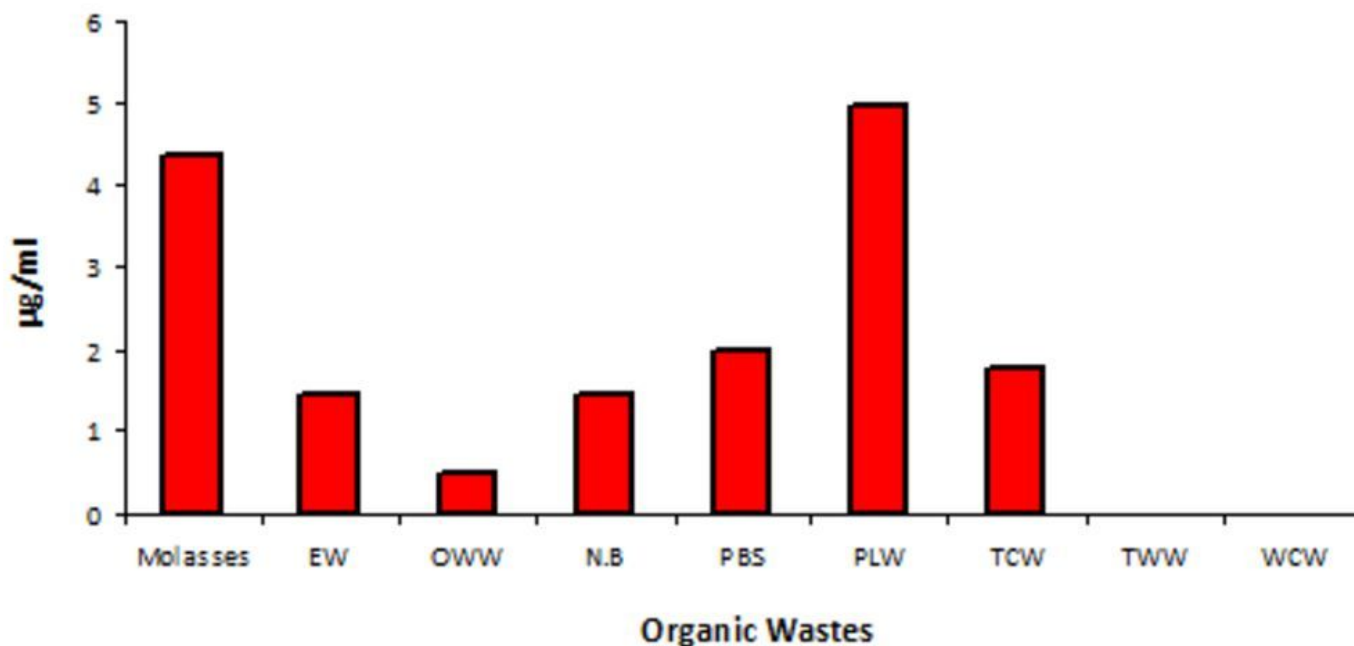


Figure 1

Proline levels of *B. cereus* in different organic wastes.

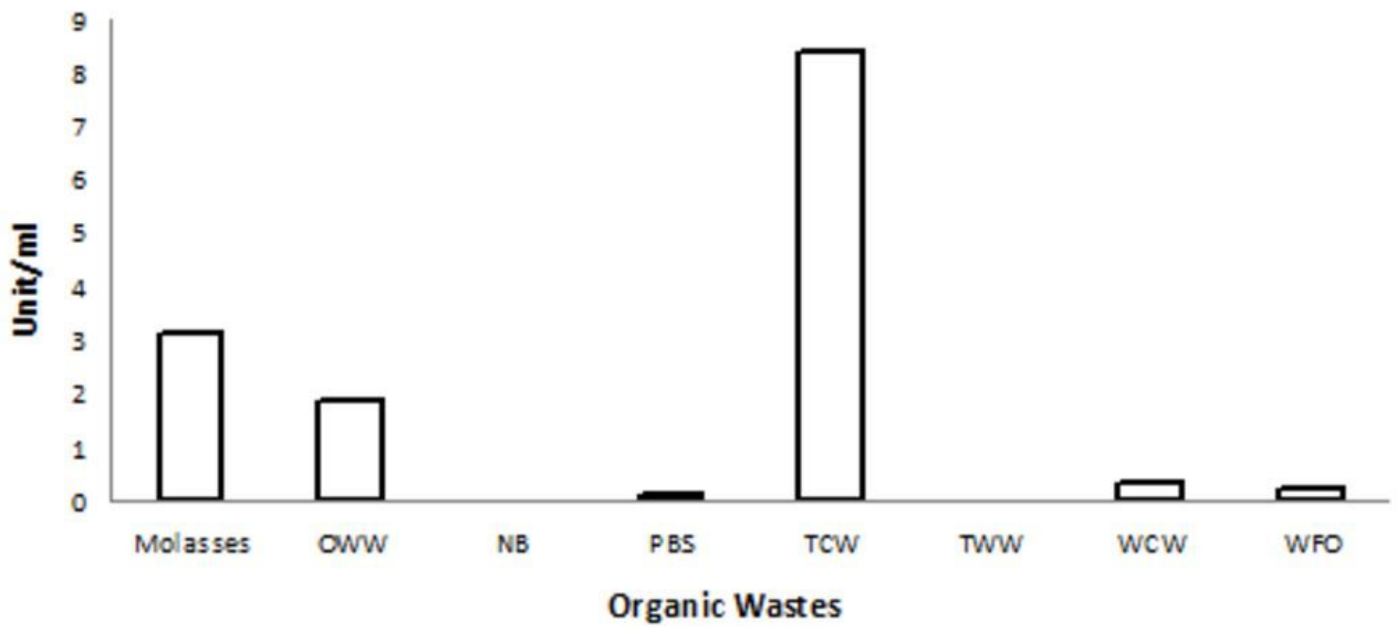


Figure 2

Protease levels of *B. cereus* in different organic wastes.

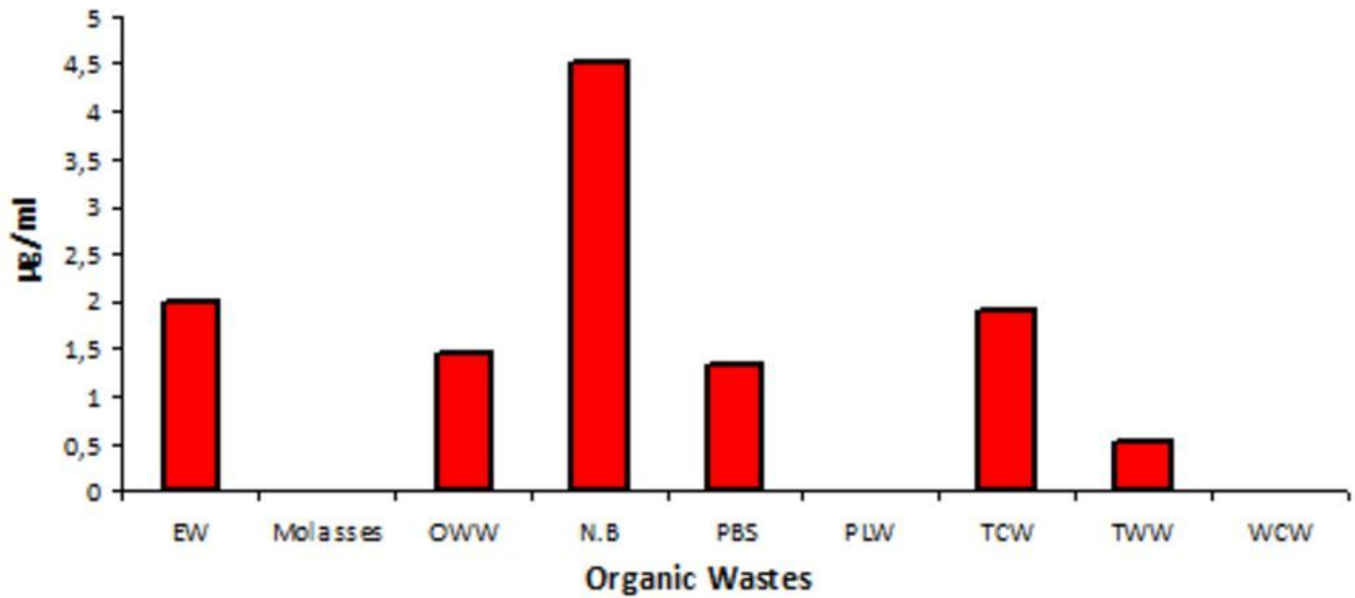


Figure 3

Proline levels of *E. coli* in different organic wastes.

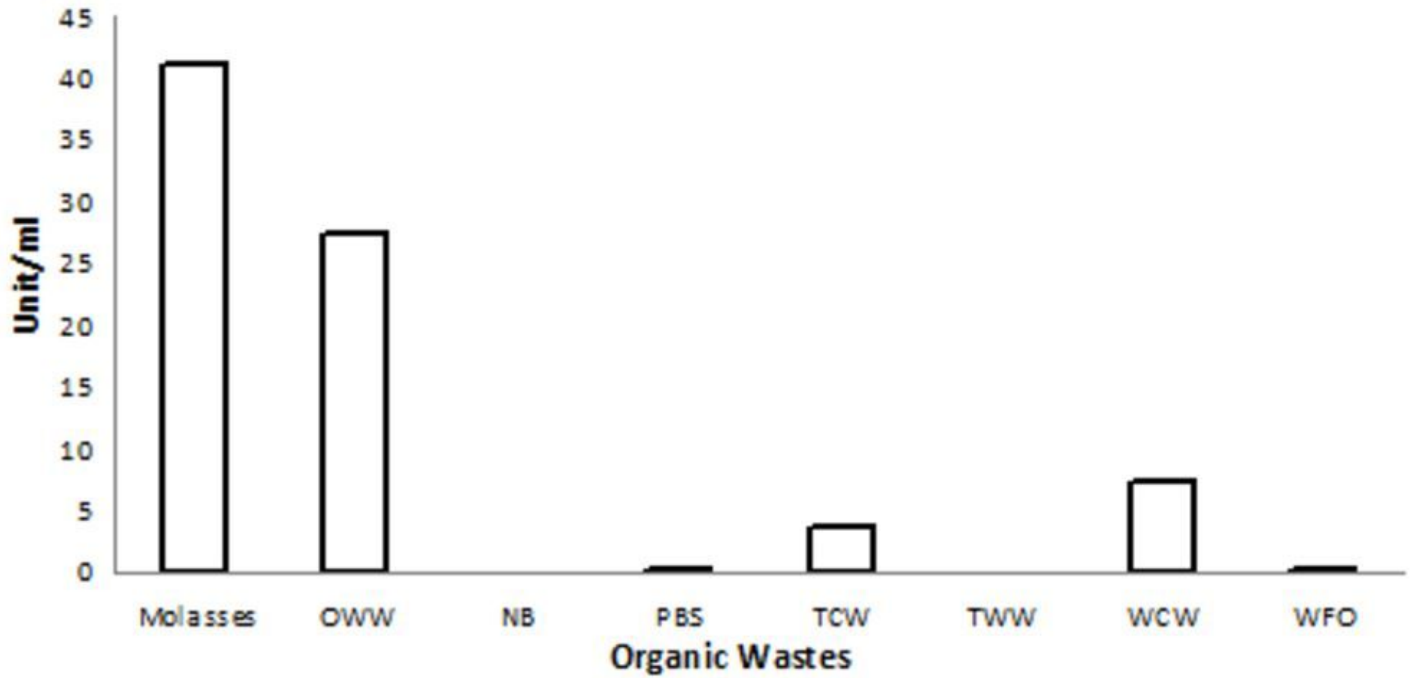


Figure 4

Protease levels of *E. coli* in different organic wastes.

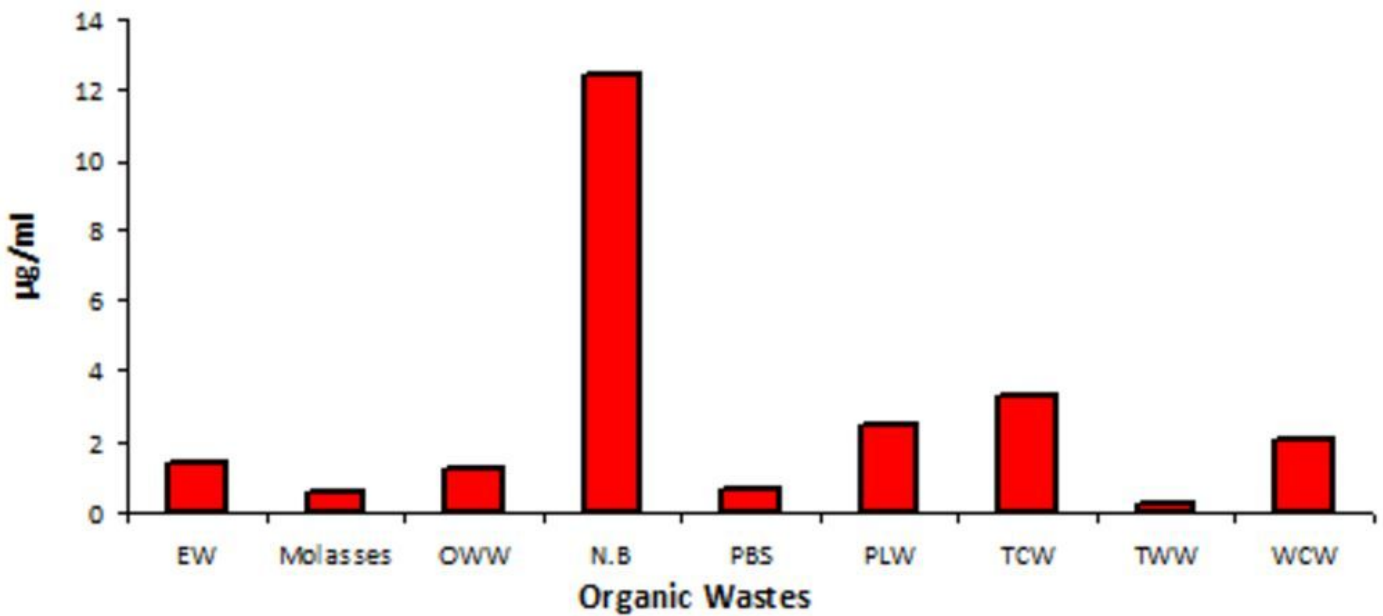


Figure 5

Proline levels of *S. aureus* in different organic wastes.

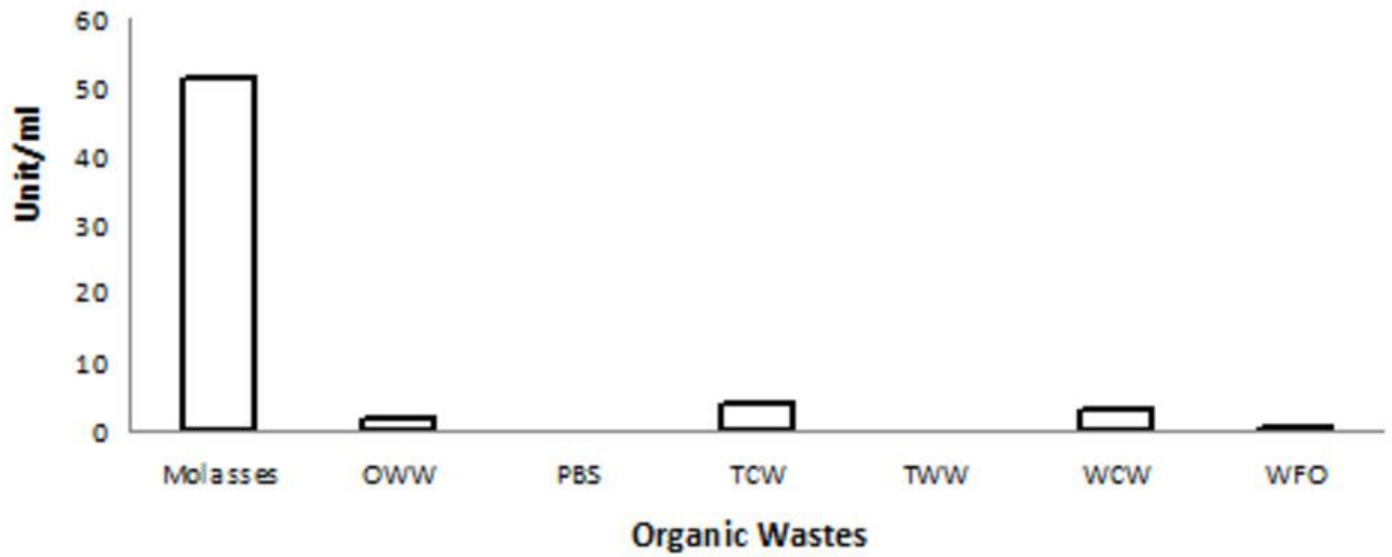


Figure 6

Protease levels of *S. aureus* in different organic wastes.

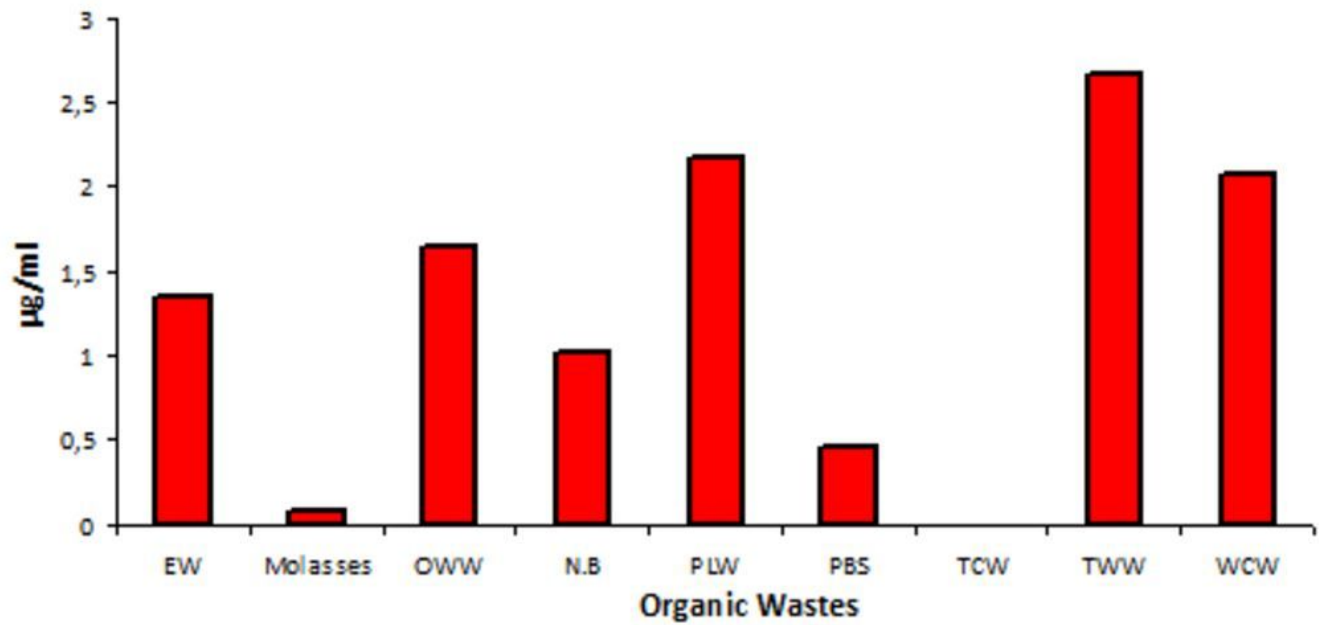


Figure 7

Proline levels of *P. aeruginosa* in different organic wastes.

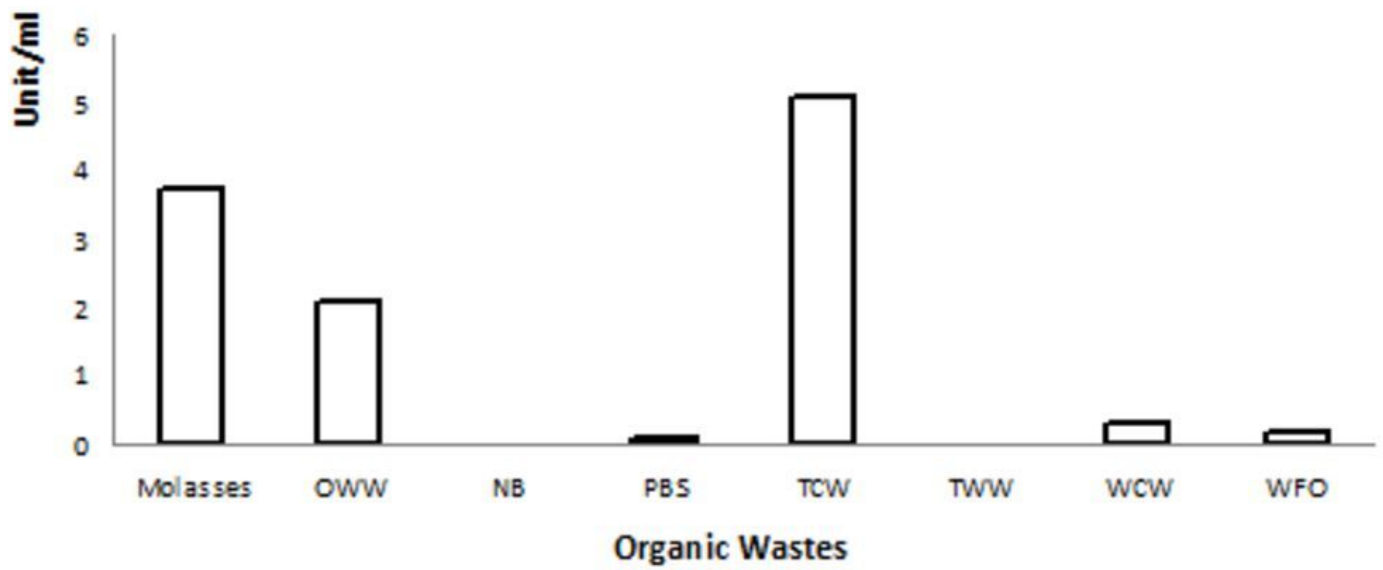


Figure 8

Protease levels of *P. aeruginosa* in different organic wastes.