SCREENING AND PRODUCTION OF PROBIOTIC ENZYMES AND BIOMOLECULES FROM GUT MICROFLORA OF SCYLLA SERRATA (FORSKAL, 1775)

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ABSTRACT:

The gut micro flora of crustaceans found to have synergistic effects by inducing immune response and animal survival. Recently, it's been revealed that the micro biota in the stomach can supply us with essential nutrients like vitamins. Many strains of Bifidobacteria, Lactobacilli, Enterococci, Streptococci, Propionibacteria, *Bacillus* sp., *Escherichia coli*, and yeasts have been recognized for their effects on human health. However, Lactobacillus and Bifidobacterium at commercial level. Probiotic bacteria generate antimicrobial activity against pathogenic strain and neutralize toxic gut metabolites, there by restoring immunological function. The bacterial translocation and its metabolites induce intestinal permeability of nutrition. Thus contributes the healthy survival of the crustaceans in adverse marine environment, auto immune diseases among Human. The study aims to isolating probiotic bacteria and to quantify the beneficial biomolecules.

KEYWORDS: Probiotics, Gut micro flora, Crustaceans.

1. INTRODUCTION

In a nation like India, child malnutrition is a huge problem in terms of both public health and the nation's overall development as a whole. According to the most recent statistics, India is home to more than 40% of the world's malnourished children. The nutritional status in India is marked by malnourishment and morbidity, both of which exacerbate the situation that Indian children find them in as a result of their inadequate and adequate diet. According to estimates provided by National Family Health Survey (2005- 2006) more than half of all Indian children suffer from some form of malnutrition. (Jose, 2017)

Probiotics are defined by FAO/WHO (2002) as "living bacteria that, when given in the proper proportions, can have a positive effect on the host organism." Recently, probiotics have been developed that can assist a person who suffers from dysbiosis in re-establishing a healthy micro biome in their stomach (Kumar *et al.,* 2020). Lactic acid bacteria, also known as LAB, are gram-positive and catalase-negative, making them one of the most common types of probiotics. The proliferation of pathogenic strains in the host can be inhibited by the secretion of a variety of substances that are produced by probiotics. These compounds include hydrogen peroxide, bacteriocins, and organic acids. (Garcia-Gutierrez *et al.,* 2019). Probiotics can be made from a variety of different microorganisms (Grochowska *et al.,* 2019).

Lactobacillus and Bifidobacterium are the most common types of probiotic bacteria, and both have been shown to be good for human health. Science and medicine are currently focusing on how more and more people are using probiotics not just as supplements but also as real treatments for a variety of illnesses. The many effects of probiotics are being looked at and studied right now in a wide range of medical fields (Khalil, *et al.,* 2022). Antibiotic resistance has made it more important to look into probiotics and their products as possible antibiotic replacements. Pathogens are harmed by probiotics in a number of ways, including competitive exclusion, improved integrity of the intestinal barrier, and the production of strong antimicrobial substances like peptides (Besser *et al*., 2019, Fijan, 2016). So, many Lactobacillus strains have been found. Multi-drug resistant bacterial pathogens like MRSA (methicillin resistant *Staphylococcus aureus*) , *Streptococcus mutans*, *Escherichia coli, Pseudomonas aeroginosa, Klebsiella pneumonia, Shigella* spp and *Clostridium difficile.* (Kumar *et al*., 2016, Mannan *et al.,* 2017).

The goal of this research is to find Probiotic bacteria from mud crab *Scylla serrata* gut micro biota. After isolating the bacteria, standard tests are used to find and describe potential probiotic strains. These tests include evaluating the production of proteases, Xylanase, lactic acid, and folic acid.

2. MATERIALS AND METHODS

2.1 Isolation of crab gut

The crab species, *Scylla serrata*, was collected from Pazhaverkadu , Chennai. The digestive system was dissected out the gut in aseptic condition.

2.2 Preparation of samples

The nutrient broth was autoclaved at 150 lbs for 15 minutes. The gut was minced and inoculated into the nutrient broth. The sample was introduced to agar plates. Four colonies were morphologically identified as P1, P2, P3, and P4. The identified bacterial species were inoculated into Luria Bertani (LB) broth for 24 hours (Mannan *et al.,* 2017).

2.3 Characterization of isolated bacteria:

Biochemical tests like the Indole test, Voges-Proskauer test, Triple sugar iron test, Nitrate reduction test, Methyl red test, Motility test, Starch Casein test, Oxidase test, Catalase test, A carbohydrate test, and an amino acid decarboxylation test were used to figure out what kinds of bacteria were found (Mannan *et al.,* 2017).

2.4 Bacillus spp. Culturing:

Bacillus spp. was cultured in MRS agar, which contains (g/l)

5g of yeast extract, 10g of beef extract, 20g of dextrose, 1g of Tween 80 (polysorbate 80), 2g of ammonium citrate, 0.1g of magnesium sulfate, 5g of sodium acetate, 0.05g of 12.5g of magnesium sulfate, 2g of dipotassium hydrogen phosphate, and 12g of agar. *Bacillus* samples were inoculated into the MRS medium and incubated for 24 hours (Mannan *et al.,* 2017).

2.5 Extraction of xylan from oat husk:

10g of oat husk and 1g of NaOH were heated in 20ml of water for 90 minutes at 90°C and then cooled. The solution was given 2 ml of 60% methanol and spun at 2000 rpm for 10 minutes. The supernatant was thrown away, and then 3 ml of concentrated sulphuric acid and 0.05 g of NaOH were added. The mixture was then heated at 90 °C for 90 minutes. The pellet is given 5 ml of 60% methanol and centrifuged at 2000 rpm for 10 minutes. 10ml of 60% methanol was applied to the pellet and centrifuged for 10 minutes at 2000rpm. The pellet is treated with 10ml of pure methanol. The pellet was dried and kept in a safe place for future use (Adhyaru *et al.,* 2015).

2.5.1Screening for xylanase

The xylan was transferred to a xylan agar which consisted of (grams/litre) oat spelt xylan-5%, $MgSO_4.7H_2O$–50mg, NaCl-50mg, $Cacl_2$-10mg, Yeast extract-20mg, Peptone-50mg, and agar-15g. Then the medium was adjusted to pH 7.0. After 24 hours of incubation at 30°C, 0.2% Congo red was added to the agar, and it was then washed with 1 M NaCl. If there is a clear area around a colony, it is likely that xylanase is at work. Under a microscope, degradation of xylan fiber from an agar plate was observed. The development of xylanase degraded the fibers (Adesina and Onilude 2013).

2.6 Protease production:

The medium consisted of (grams/liter) K_2HPO_4-2g, Glucose-1g, Peptone-5g, Gelatin-15g, and Agar-15g. The medium was prepared based on the composition of the ingredients. The sample of *bacillus* was introduced and incubated for 24 hours (Sevinc and Demirkan, 2011).

2.7 Screening and production of proteases:

The plate was flooded with Coomassie brilliant blue, incubated for 5 minutes, and decanted. The zone of clearance was observed. The medium contains K_2HPO_4-2g, glucose-1g, gelatin (15 g), and peptone (5 g). The medium was prepared. The loop of *Bacillus* samples was put in the shaking incubator for 48 hours to grow (Sevinc and Demirkan, 2011).

2.8 Estimation of protease production:

Standard preparation

Standard samples were prepared with 10mg of casein dissolved in 10ml of distilled water. 200μl of sample is taken in S1, 400μl in S2, 600 μl in S3, 800 μl in S4, 1ml in S5. All the samples were made up to 2 ml by adding distilled water.

ii. Test sample preparation

Test sample T1 was made up of 1 ml of casein, 1 ml of water, and 500μl of the supernatant of the protease production medium was added. T2 was made up of 1 ml of casein, 1 ml of water, and 1 ml of the supernatant of the protease production medium. The samples were incubated for 30 minutes at room temperature.

5ml of alkaline copper was added to the samples above, and they were kept in the dark for 20 minutes. 10μl of Folin's reagent was added and incubated for 10 minutes. The samples were estimated by the spectrophotometer at 640 nm and recorded graphically.

Folic acid production

The medium has (in grams per liter) 2.5 grams of peptic digest of animal tissue, 5.5 grams of papaic digest of soybean meal, 5 grams of beef extract, 2.5 grams of casein enzymic hydrolysate, 5 grams of lactose, 2.5 grams of yeast extract, 0.5 grams of ascorbic acid, 0.225 grams of magnesium sulfate, and 19 grams of disodium-glycerophosphate. The medium was prepared. The *Lactococcus lactis* sample was inoculated and incubated for 72 hours (Laino *et al*., 2019).

2.9 Folic acid estimation

(i) Standard preparation:

100mg of folic acid is dissolved in 100ml of water. 200μl of sample is taken in S1, 400μl in S2, 600 μl in S3, 800 μl in S4, and 1ml in S5. All the samples were made up of 2 ml by adding distilled water.

ii) Test sample preparation:

Test sample T1 was made up of 500 µl of the folic acid sample. Test sample T2 was made up of 1ml of the folic acid sample, and both the samples were made to 2 ml by adding distilled water. Two millilitres of sodium nitroprusside solution and half a millilitre of concentrated ammonia were added to the above samples, and the dark yellow chromogen was taken up. The samples were estimated by the spectrophotometer at 390 nm and recorded graphically.

Lactic acid production

To 50ml of milk, the curd was added and incubated for hours. From the culture, 2 ml of sample is added to 2 ml of water. This sample was centrifuged at 2000 rpm for 15 minutes. 50 ml of milk was pasteurized by a double autoclaving process at 15 lbs for 15 minutes. Now, the 0.685 g pellet from the centrifuge was added to the milk sample, and it was put in a shaking incubator for 24 hours (Rupali, 2015).

ii) Test sample preparation:

Test sample T1 was made up of 500 µl of lactic acid. Test sample T2 was prepared with 1ml of the lactic acid sample, and both samples were made to 2ml by adding distilled water and 1ml of ferric chloride. The samples were estimated by the spectrophotometer at 390 nm and recorded graphically.

Lactic acid recovery

The broth was heated to 80 degrees Celsius before adding $Ca(OH)^2$ saturated solution drop by drop until the pH of the medium reached 10-11. Stir until the precipitates are formed. The extract was filtered with activated carbon and separated.

2.10 Estimation of standard lactic acid:

(i) Standard preparation:

Different concentrations of lactic acid (2ml, 2.2ml, 2.4ml, 2.6ml, 2.8ml) were taken and made up to 3ml by adding the distilled water. To all the samples, 1 mL of ferric chloride was added.

ii) Test sample preparation:

500 µl of pure lactic acid, 1.5 ml of distilled water, and 1 ml of ferric chloride were added. The samples were estimated by the spectrophotometer at 390 nm and recorded graphically.

3. RESULTS AND DISCUSSION:

Probiotics play a vital role in human and other farm animal health status. Probiotics greatly contribute to the improvisation of the immune response. In this study, enzymes from bacteria that are known to have probiotic effects are found and their enzyme activity is confirmed. The bacteria were isolated from the gut of *Scylla serrata*, a well-known mangrove animal. Figure 1 showed dorsal view of *Scylla serrata* and figure 2 showed the gut of the crab. *Bacillus subtilis* and *Lactococcus lactis*, which are the most common bacteria, are found (Figure 3).The biochemical characterization, carbohydrate utilization and amino acid utilization test confirms the *Bacillus subtilis* and *Lactococcus lactis* (Figure4,5 and 6, Table 1A, 1B,1C). *Bacillus subtilis* starch casein agar medium were used to confirm the *Bacillus subtilis* and MRS medium confirms the *Lactococcus lactis.* (Figure 7 and 8)

Fig 1 : ***Scylla serrata***

Fig 2: Gut sample of ***Scylla serrata***

Fig 3: Growth of bacteria from ***Syclla serrata*** **gut sample**

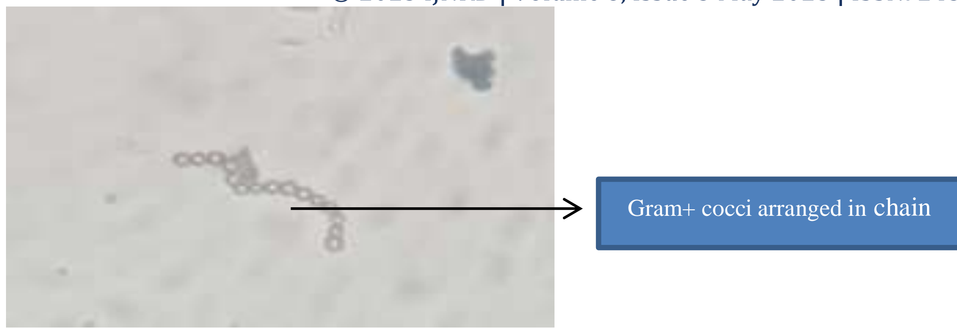


Fig 4: Gram's staining

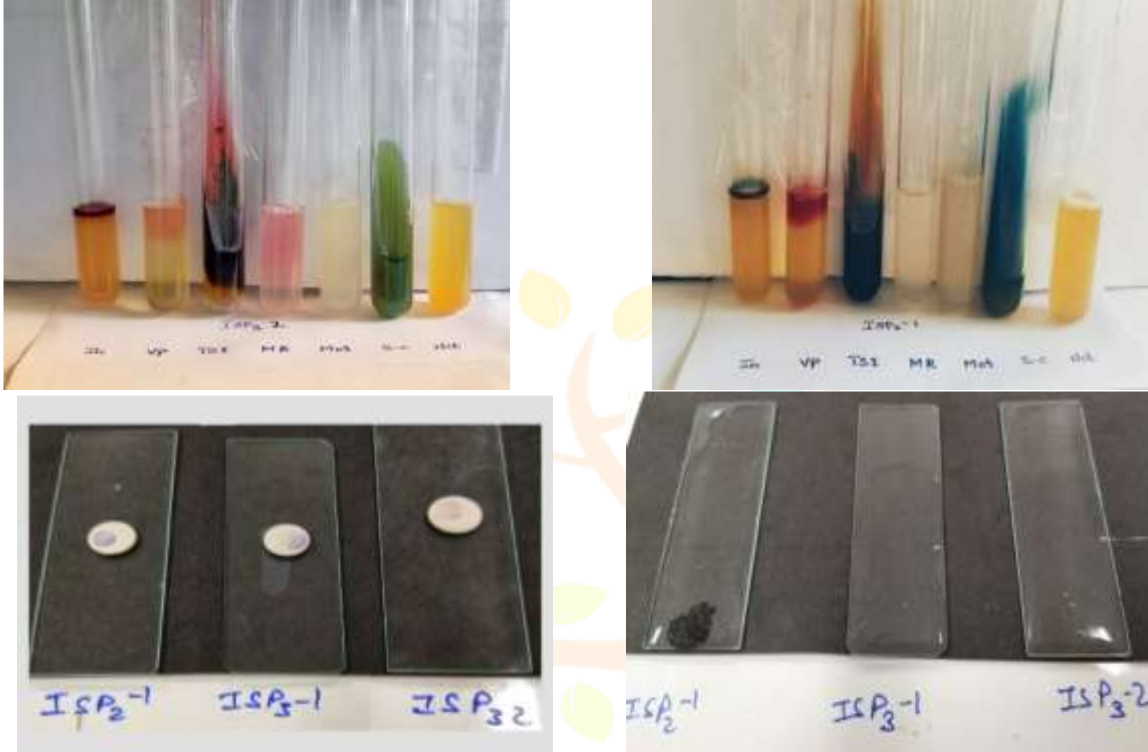


Fig 5: Biochemical Characterization

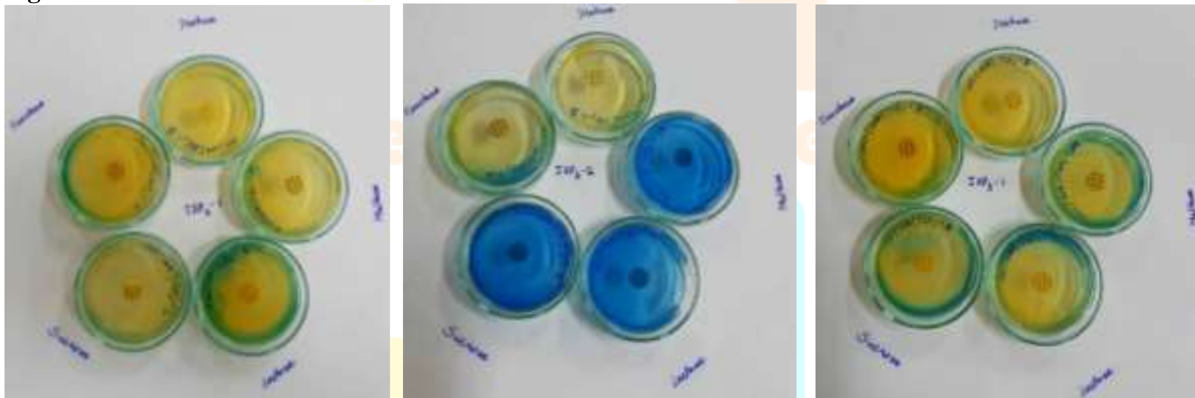


Fig 6: Carbohydrate Utilization

Fig 7: Growth on Starch Caesin Agar

Fig 8: Growth on MRS Agar

Fig 7 shows the zone of discolouration around the colonies confirms the starch degradation is positive
Fig 8 shows the medium sized, golden coloured colonies on MRS agar medium was confirmed as Lactic acid producing bacteria

Table 1A: Biochemical Characterization

S.NO	TEST	ISP_2-1	ISP_3-1	ISP_3-2
01	Gram's staining	+ rods	+ cocci in chain	+ cocci in chain
02	Motility test	Non - motile	Non - motile	Non - motile
03	Indole		+	+
04	Methyl red	++	++ (aerobic)	++ (aerobic)
05	Voges - Proskauer	++	++	+
06	Triple sugar iron	Acid slant	-ve slant (with H_2S production)	-ve slant
		Acid butt (H_2S production)	+ve butt (weak gas production)	Acid butt (full H_2S production)
07	Citrate	Slant –ve	+	+
		Butt +ve		
08	Starch casein	+	+	+
09	Oxidase	+	Weakly +ve	-
10	Catalase	Weakly +ve	+	+
11	Nitrate	-	-	-

Table 1B: Carbohydrate Utilization test

S.NO	TEST	ISP_2-1	ISP_3-1	ISP_3-2
01	Sucrose	+	Weakly + ve	Weakly + ve
02	Dextrose	+	Weakly + ve	Weakly + ve
03	Maltose	+	Weakly + ve	Weakly + ve
04	Lactose	Weakly + ve	Weakly + ve	Weakly + ve
05	Fructose	Weakly + ve	Weakly + ve	Weakly + ve

Table 1C: Amino acid Utilization test

S.NO	TEST	ISP_2-1	ISP_3-1	ISP_3-2
01	Proline	+	+	+
02	Lysine hydrochloride	Aerobic +ve	-	+
03	Arginine hydrochloride	+	+	+
04	Ornithine hydrochloride	Weakly +ve	Weakly +ve (aeobic)	Weakly +ve

Screening of Xylanase:

Xylan is a hemicellulose and heteropolysaccharide made up of monosaccharides like D-galactose, D-mannose, and L-arabinose, as well as organic acids like ferulic acid, acetic acid, and glucuronic acid, which are linked together by ester bonds and glycosidic bonds (Arendt *et al*., 2017). *Bacillus subtilis* was grown on xylan medium. The bacteria produces xylanase enzyme and degrades the xylan. The results were observed after the addition of Congo dye and the zone of discoloration was recorded as the positive result (Figure.8). For xylanase and cellulase activities, beech substrate and carboxyl-cellulase (C) were used as substrate and extracellular debranching support. Fatma and Giraffa, (2011), Said that Congo red dye can be used to test for cellulase activity and that it does not give any false negative results. In this study, the xylanase activity of the bacterium *Bacillus subtilis* was proven by treating it with Congo red and looking at the xylan strands under a microscope. (Figure.9) Around the xylan the zone of clearance showed after staining with Congo red, this confirms the degradation of xylan.

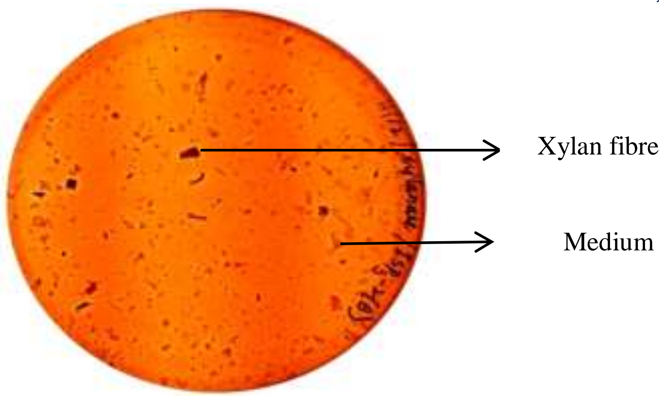


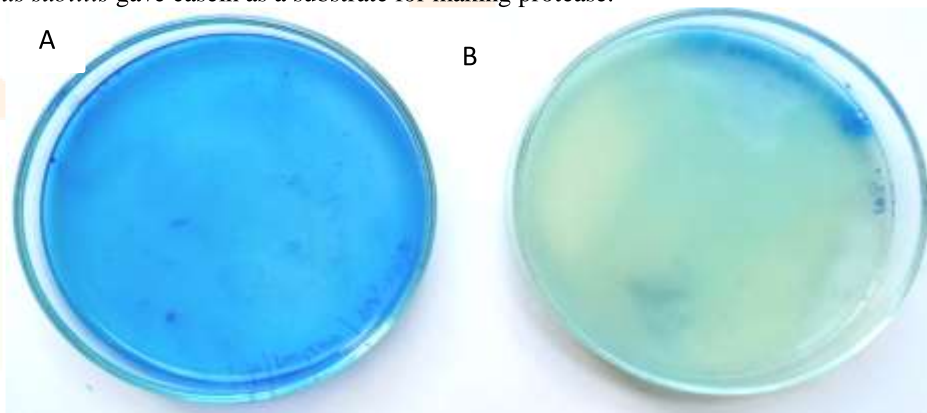
Fig 8: Drgradation of Xylan.

(A) Control of Xylan **(B) Bacillus subtilis treated Xylan**

Fig 9: Microscopic observation of Xylan.

3.1 Production of Protease:

Bacillus subtilis was also screened for the production of protease enzymes. The medium containing casein was used for the confirmation of protease production by the bacterium. After 24 hrs, the plates were flooded with coomassie brilliant blue dye. Various casein agar plates were used to test for protease activity. Even though hydrolysis led to the discovery of proteolysis in the original hydrolysis zone, we did not see protease reported for the detection of microbial protease production ((Adesina and Onilude 2013, Rupali, 2015). *Bacillus subtilis* needs only a small amount of nutrients and its physical parameters can be changed to make enzymes that are useful in industry (Lee *et al*., 2015). In this study, *Bacillus subtilis* was grown in a shake flask so that protease and xylanase enzymes could be made. The main substrate was xylan, and *Bacillus subtilis* gave casein as a substrate for making protease.



A. Control plate with no bacterium, discolouration. **B. Degradation of the protein, confirmed appears as blue in colour**

Fig 10: Screening of Protease

Estimation of the Protease:

The concentration of the casein protein degradation was colorimetrically recorded and graphically estimated (Figure.10, Table.2). The concentration of protease produced by *Bacillus Subtilis* was estimated after 48 hours as 0.204mg/ml.

Table : 2 Estimation of Protease:

S.NO	Total estimation, mg/ml
1.	0.204

3.3 Estimation of Folic acid

Probiotic and prebiotic-fortified yogurts may have health benefits, but it's important to keep in mind that yogurt's primary function may originate in disease prevention (rather than cure), where modulation of the gut microbiota may play a larger role in maintaining health than curing established diseases (Arendt *et al*., 2017). Since the bacteria responsible for making lactic acid also have the potential to make folate, that aspect of their metabolism is also being studied. *Lactococcus lactis* was taken from the gut of a *Scylla serrata* and grew in an

enriched medium for making folic acid and an enriched medium for making lactic acid. The concentration of folic acid produced was calorimetrically estimated [figure.11, Table 3]. After 72 hours, it was thought that the amount of folic acid made by *Lactococcus lactis* was found to be 19.23 mg/ml.

A: Initial growth on day – 1 **B: Growth after 72 hours.**

Figure.11: Production of folic acid

Table 3: Estimation of Folic acid:

S.NO	Total estimation, mg/ml
1.	19.23

3.4 Production of Lactic acid:

Form the enriched medium after 15 days of culture lactic acid was extracted by the downstream process. The concentration of lactic acid produced by *Lactococcus lactis* was estimated after 72 hours as 134mg/ml (Figure.12and 13, Table 4)

Figure. 12: Lactic acid production

A.Filteration **B. Filterate**

Figure.13: Downstream process of Lactic acid recovery

Table 4: Estimation of Lactic acid:

S.NO	Total estimation, mg/ml
1.	134

4.CONCLUSION:

Based on the present study *Bacillus subtilis* and *Lactococcus lactis* were isolated from the gut tissue of *Scylla serrata*, suggesting that these bacteria are part of the natural gut microbiota of this species. *Bacillus subtilis* and *Lactococcus* lactis exhibited the ability to utilize

simple nutrients, indicating their metabolic versatility and adaptation to the gut environment. The isolated strains of *Bacillus subtilis* and *Lactococcus lactis* were found to produce beneficial probiotic compounds such as xylanase, protease, folic acid, and lactic acid. Xylanase is an enzyme that breaks down xylan, a complex sugar found in plant cell walls. Its production by *Bacillus subtilis* and *Lactococcus lactis* suggests their potential role in aiding the digestion of plant material in the gut of *Scylla serrata.* Protease is an enzyme that helps in the breakdown of proteins. The production of protease by the isolated strains suggests their involvement in protein digestion and nutrient acquisition in the gut environment. Folic acid is a vital nutrient involved in various biological processes. The production of folic acid by the isolated strains highlights their potential contribution to the nutritional requirements of *Scylla serrata.* Lactic acid is a byproduct of carbohydrate fermentation and is known for its antimicrobial properties. The production of lactic acid by *Bacillus subtilis* and *Lactococcus lactis* suggests their ability to create an acidic environment in the gut, which can inhibit the growth of pathogenic bacteria.

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