Optimization of *Bacillus clausii* on Apple Waste and its Synergistic Effects Against Pathogenic Bacteria

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Abstract

The probiotic Bacillus clausii is good for human health. The ability to generate spores is one of its primary characteristics; the ensuing endurance to heat, acid, and salt ensures safe passage through the human gastrointestinal tract without cellular loss. The study is aimed at the optimization of Bacillus clausii on apple waste and its synergistic effects against pathogenic bacteria. Bacillus clausii has been taken from Abasyn University, Peshawar. Further, the subculturing, and biochemical characterization have been done using standard microbiological procedures. The optimization of Bacillus clausii is done using apple waste. Mueller Hinton Agar is used for the antimicrobial susceptibility assay as well as the synergistic effect between Salmonella and E. coli. Bacillus clausii is identified as Gram positive, showed a creamy white appearance, had rod shaped microorganism, and underwent various biochemical tests. It is found that the growth of Bacillus clausii is maximum at 37℃, at pH 9, and a glucose concentration of 3gram with shaking conditions at 150 rpm. Moreover, Bacillus clausii showed resistance to cefotaxime, and cefixime, while highest sensitivity against ciprofloxacin and meropenem. After 24 hours of incubation, no bacterial inhibition is observed against Salmonella. No synergistic effect between E. coli and Bacillus clausii is observed against Salmonella typhi clinical isolates. In conclusion, Bacillus clausii grows at a maximum at a temperature of 37℃, with a PH of 9, the glucose concentration is 3 grams. Moreover, no synergistic effect is observed against Salmonella typhi bacteria.

Keywords: Bacillus clausii; Apple Waste; Synergistic Effects; Pathogenic Bacteria.

Introduction

Bacillus clausii is a facultative alkaliphilic rod bacterium, Gram positive and capable of forming endospores, used as human probiotics (Abbrescia et al., 2014). The Bacillaceae family includes *Bacillus clausii*. It might be isolated from many sources, such as soil and marine habitat, as

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well as the alkaline environments. Bacillus species are involved in many different processes, ranging from their function as biological control agents to their probiotic properties in the case of *B. subtilis*, *B. clausii*, *B. coagulans*, and other strains (Sorokulova et al., 2008). For instance, *B. licheniformis* is one of several strains that have important medical and commercial implications (Senok et al., 2005). Certain Bacillus species are important for the economy because they generate proteins, including cellulases, xylanases, amylases, and antacid proteases (Muras et al., 2021).

Probiotics are live bacteria that, when taken in sufficient amounts, help maintain the initial microbiota in the stomach, which can be disturbed by loose stools or the ingestion of toxins (Rashmi, & Gayathri, 2017). Probiotics typically constrain in the gastrointestinal tract, where they impact the intestinal microflora. In the gastrointestinal tract, various types of microorganisms colonize in the human (Zeashan et al., 2020). From the human gut, probiotics are naturally isolated and have the capability to survive passage via gut (Hoyles et al., 2012).

Bacillus licheniformis and *Bacillus clausii* isolated from the feces of healthy adult human feces show their ability to survive in the GIT tract (Lopetuso et al., 2016). In combination with antibiotics, the strains of *B. clausii* bacteria used during antibiotic therapy to reduce the side effects on the gastrointestinal tract (GIT) (Plomer et al., 2020; Nista et al., 2004). Some of the *Bacillus clausii* probiotic strains *such as* T (CNCM I-273), O/C (CNCM I-276), SIN (CNCM I-275) and N/R (CNCM I-274) named as a market name with Enterogermina® by Sanofi are well endured, safe, and efficaciously used from the last few decades (Abbrescia et al., 2014; Lopetuso et al., 2016). Furthermore, the *Bacillus clausii* probiotics are consumed globally, and their wide availability makes it prominent. Other species that make probiotic are *Bifidobacterium* spp., and *Lactobacillus* spp. also have been reported (Elshaghabee et al., 2017).

Bacillus clausii also produces some antibacterial peptides that inhibit the growth of pathogenic bacteria involved in different infections, such as *Enterococcus faecalis, Salmonella typhimurium, Listeria monocytogenes, Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Shigella flexneri* (Rochín-Medina et al., 2018). Fruit peels make up the majority of the agricultural industrial waste that is released into the environment. Six bacterial isolates, such as, *B. megaterium, Bacillus amyloliquefaciens, B. licheniformis, B. nealsonii, B. safensis* and *B. horikoshii* (Batoolet al., 2015). A study from Pakistan reported that using phytobiotics with probiotics (*Bacillus clausii*) inhibits some selected strains of bacteria (Mushtaq et al., 2024). A number of microorganisms serve as probiotics, including *Bacillus clausii*. To overcome this problem, *Bacillus clausii* needs to be optimized by utilizing the available apple

waste. Furthermore, utilizing such waste material, decreases environmental pollution because such waste material can support the growth of many pathogenic bacteria that possess an environmental threat. Keeping in view the above problems, research has been conducted to optimize *Bacillus clausii* utilizing apple waste as a substrate. The hypothesis of the current study is the optimization of *Bacillus Clausii* using the apple waste and their antibacterial potential against pathogenic bacteria involved in various infections. The current study aimed to optimize *Bacillus Clausii* on apple waste and its synergistic effects against pathogenic Bacteria.

Material and Methods

This is basically a cross-sectional study. The experimental work is performed in Microbiology Laboratory in the Department of Allied Health Sciences, Iqra National University, Hayatabad, Peshawar, from March 2023 to August 2023. The study isolates are received from Abasyn University and the clinical isolates are obtained from Hayatabad Medical Complex, Peshawar. *Bacillus Clussii* is transferred to Microbiology Laboratory, Iqra National University Peshawar immediately after collection and then inoculated the isolate on Nutrient Agar media plates. After that, the plates are incubated at 37ºC for 24 hours for biochemical identification.

Optimization of Bacillus clausii

For the extract of apple waste, boiled the apple in water. After that the cheese cloth is used for extraction purpose. In a flask, 20 ml of apple waste and 80 ml of distilled water is added using a graduated cylinder, which made a 100 ml medium for culturing purposes. Then the sugar, NaOH and HCl are added as required per experiment as these are the variables for each experiment. As sample collecting tube is used, which would be dipped in the media in flask, in such a way that the other half portion is outside the flask and the other half is dipped in the flask. Then covered properly the flask opening by using the cotton, and the remaining half of the sample collecting tube is folded by paper clip. Placed the prepared flask in a shaking incubator by setting the temperature and revolution per minute (rpm) according to each experimental requirement, as these are variables.

Sugar Concentration for Maximal Bacillus clausii Growth

Sugar is added to 3 different flasks, i.e.1gram in flask 1st, 2grams in second flask 2nd and 3grams in 3rd flask 3, respectively. Then poured the

preserved *Bacillus clausii* samples into the flask and a control sample are taken from the flask. Flasks are further kept at 37^0C for 72 hours.

Temperature Optimization

All the apple waste containing flasks is kept at different temperatures, e.g. flask 1 is kept at 25℃, flask 2 is kept at 37℃ and flask 3 is kept at 8℃. Flasks are kept at 37℃ after 72 hours.

Ph Optimization

The pH is maintained by adding NaOH and HCl drop by drop. Flask 1 is pH 5, flask 2 is pH 7 and flask 3 had pH 9. *Bacillus clausii* is introduced into the flasks and then the flasks are kept at 37^0C in the incubator for 72 hours.

Spectrophotometry

The optical density is measured at a wavelength of 600nm. For this purpose, the Eppendorf tubes are used to collect the sample from the flask up to 72 hours after each 12 hours. A sample is taken and then each Eppendorf tube is labeled by writing time and experiment number.

Antimicrobial Activity

Muller-Hinton agar (MHA) media used for the evaluation of antibacterial activity of *Bacillus claussi*. Previously published criteria for antimicrobial activity assays have been used (Bauer et al., 1966). Bacterial lawn is made, and then the plates are streaked using sterile cotton swab. The plates are then incubated for 24 hours at 37℃. The results are analyzed, and zones of inhibition are measured in mm. A panel of five antibiotic discs are used i.e. cefotaxime (CIX, 30 μg), cefixime (CFM, 5 μg), ciprofloxacin (CIP, 5 μg), amoxicillin (AML 10 μg), and meropenem (MEM, 10 μg).

Criteria for Multi-drug Resistance (MDR) Organisms

Bacteria showed resistance to three or more antimicrobial classes are termed MDR (Falagas et al., 2006; Paterson & Doi, 2007).

Synergistic Effect

A lawn of *Salmonella typhi* is made on the plate of Mueller Hinton agar. The wells are made in the center of plates for *E. coli* and *Bacillus clausii* then the cultures of *E. coli* and *Bacillus clausii* are inoculated into the wells and then incubated at 37℃ for 24 hours to observe their synergistic effects (Valgas et al., 2007).

Results

Isolates identification and Characterization

The study isolates of *Bacillus Clausii* received from Abasyn University is already identified through standard microbiological procedures. Initially confirmed through Gram staining and further confirmation is done through various biochemical tests. The isolates for synergistic activity are collected from the Hayatabad Medical Complex (HMC), Peshawar. The isolates are obtained from the pathology lab, as *E. coli* from Urinary tract infection (UTIs) from female patients of 48 years and *Salmonella typhi* from a male patient of 28 years old obtained from blood culture analysis. The isolates are identified using standard microbiological procedures for Gram positive and Gram negative bacteria. characterized as multi-drug resistant (MDR) as per previously published criteria in the material and methods portion.

Three experiments are run for the purpose of optimization of *Bacillus clausii* on Apple Waste.

Experiment No.1 (Effect of Glucose Concentration on Bacillus clausii)

For experiment No.1, 20 ml of apple juice and 80 ml of water is added to a flask and 1, 2, and 3 gram of glucose are also added, and it is placed in incubator. This is run at 150 revolutions per minute (rmp) at 37℃. Results showed that after 36 hours of incubation at 37℃ the maximum number of cells is $114x10^5$ per ml in the flask of 3 grams of glucose. Maximum growth is observed at 3 grams of glucose concentration. The study results showed that increasing the glucose concentration can enhance the growth of *Bacillus clausii* as shown in Table 1.

Table 1: Number of cells using different glucose concentration.

Time duration of sample taking	3Gram	2 Gram	1 Gram
0	1780000	1730000	1650000
6	2950000	6210000	2410000
12	8150000	3950000	2290000
18	9170000	4120000	2310000
24	10700000	5140000	2430000
30	11100000	5800000	2410000
36	11400000	6000000	2380000
42	10100000	6200000	2250000
48	9110000	5200000	2010000

Experiment No.2 (Bacillus clausii Growth on Different Temperatures)

For experiment No. 2, 20 ml of apple juice and 80ml is added to a flask, and they are kept at temperatures of 8^0C, 25^0C and 37℃ and are

placed in an incubator that is run over at 150 rmp at 37℃. Table 2 shows that after 24 hours of incubation at 37℃, the number of cells is $108x10^5$ per ml in the flask at 37ºC. Temperature is the main factor for the growth of any microorganisms. The study revealed that 37^0C is the optimum temperature for the growth of *Bacillus clausii*. At this temperature their metabolic activities are maximum.

Table 2: Growth of Bacillus clausii using different temperature.

Time duration of sample taking	37ºC	25ºC	8ºC
0	1780000	1730000	1650000
6	2410000	2950000	6210000
12	2290000	3450000	8150000
18	2310000	4120000	9170000
24	2430000	5140000	10700000
30	2410000	5800000	10800000
36	2380000	6000000	10400000
42	2250000	6200000	10100000
48	2010000	5800000	9110000

Experiment No.3 (Growth of Bacillus clausii Using Different pH)

For experiment No. 3, 20ml of apple extract and 80 ml of water are added to a flask and pH is maintained at 5, 7, and 9 by adding and is placed in an incubator which is run at 150rmp at 37℃. Table 3 shows that after 30 hours of incubation at 37℃ the number of cells is $154x10^5$ per ml in the flask at pH 9. The results revealed that these organisms are alkaliphiles and best grow at pH 9.

Table 3: Growth of Bacillus clausii using different pH.

Time duration of sample taking	pH9	pH7	pH5
0	1780000	1730000	1650000
6	2410000	2950000	6210000
12	2550000	3850000	8150000
18	2670000	4120000	9170000
24	3200000	5740000	11500000
30	2810000	5800000	15400000
36	2750000	7000000	16000000
42	2250000	6200000	15100000
48	2010000	5800000	14210000

Growth of Bacillus clausii Under Optimized Conditions

Bacillus clausii is incubated under optimized conditions, i.e., sugar concentration 3grams, temperature 37℃ and pH 9. Maximum growth is obtained after 30 hours under optimized conditions. In this experiment all the variables are investigated for the growth of *Bacillus clausii*. It is observed that after 30 hours *Bacillus clausii* is cultured at

glucose concentration 3grams, temperature 37℃ and pH 9 and maximum growth is observed as shown in Table 4.

Table 4: Growth of Bacillus clausii under optimized conditions.

Time duration of sample taking	Growth
0	1780000
6	2410000
12	2550000
18	2970000
24	3600000
30	3910000
36	3650000
42	3250000
48	3010000

Antibiotic Susceptibility Assay Results

In the Muller-Hinton agar medium, five antibiotics are applied against the *Bacillus clausii* lawn over the petri dish to observe the susceptibility pattern. After 24 hours, it shows resistance to cefotaxime (CIX, 30 µg), and cefixime (CFM, 5 µg), where *Bacillus clausii* shows sensitivity against ciprofloxacin (CIP, 5 µg) of about 28 mm, amoxicillin (AML 10 µg) of about 15 mm and meropenem (MEM, 10 µg) of about 26 mm using CLSI guidelines 2023.

Antibiotic Susceptibility Profile of E. coli and Salmonella typhi Isolates

The highest sensitivity of *E. coli* isolates is observed against fosfomycin, followed by amikacin, piperacillin/tazobactum and meropenem, while resistance to cefotaxime, ceftriaxone, cefepime, ceftizidime, ampicillin, Doxycycline, gentamicin, and nitrofurantoin. In the case of *Salmonella typhi* the isolates showed resistance to ciprofloxacin, followed by sulphamethxozole trimethoprim, levofloxacin ampicillin and ceftriaxome, while sensitive towards piperacillin/tazobactum, meropenem, imipenam, Doxycycline and azithromycin. Both the isolates are MDR.

Synergistic Effect

A lawn of *Salmonella typhi* is prepared on the plate of Mueller Hinton Agar (MHA). The wells are made in the center of plates for *E. coli* and *Bacillus clausii* then the cultures of *E. coli* and *Bacillus clausii* are inoculated into the wells and then incubated at 37^0C for 24 hours. After 24 hours of incubation, no bacterial inhibition is observed against *Salmonella*. There is no observed synergistic effect between *E. coli* and *Bacillus clausii* against *Salmonella*.

Discussion

Bacillus clausii is now widely used as a probiotic and also in the treatment of intestinal health to relieve gastrointestinal discomfort and its immunomodulatory outcomes. In the current study, *Bacillus clausii* optimization is done using apple iste using various conditions such as temperature, pH, and glucose concentrations, as well as their synergistic effect of pathogenic bacteria causing gastrointestinal distress. In the current investigation the clinical isolates are confirmed as multi-drug resistant pathogens, showed resistance to cefotaxime, ceftriaxone, cefepime, ceftizidime, ampicillin, Doxycycline, gentamicin, and nitrofurantoin in case of *E. coli* and sulphamethxozole trimethoprim, levofloxacin ampicillin and ceftriaxome to *Salmonella typhi,* same findings are also reported previously from Pakistan (Haq et al., 2022), demonstrated almost similar patterns.

Another report (Batool et al., 2021) from Pakistan has demonstrated the same resistance pattern in the case of *Salmonella typhi* isolates, supporting the current study findings. *Bacillus clausii* growth is observed at maximum pH 9 in the currents study. Similar findings are also reported (Rani et al., 2018) that these bacteria grow at various pH condition from 6-9, which supports the current study findings. It is known that *Bacillus clausii* as a spore can resist the stomach pH and germinate in the intestine in vegetative form (Urdaci et al., 2004). Another study also reported the optimum growth of *Bacillus clausii* at pH 9 (Kageyama et al., 2007).

In the current study, the active temperature and pH for the growth of *Bacillus clausii* are 9 and 37℃. Similar findings are observed previously (Thakur et al., 2021), supporting the current study. These findings correlate with a study by (Fanimo et al., 2003), that optimized *Saccharomyces Cerevisiae* on apple waste. They utilized apple waste, and the yeast is optimized using glucose concentration. Another report by (Ahire et al., 2020) suggested that optimized levels are achieved by adding the glucose at a final concentration of 2g/L at the initial fed-batch culture, while in the current study maximum colony is observed at 600nm on the final of 3 grams glucose concentration, which is slightly different from the previous findings. The optimization of various glucose concentrations depends upon the strain type and various other environmental factors etc.

Another similar study by Tabandeh et al. (2011) has reported that *Bacillus clausii* growth is observed at 37℃ and pH 8 at 160 rpm for 30 hours. Another investigation reported the maximum growth at PH 9 by (Oskouie et al., 2008). Another study reported by (Kabeer, & Mushtaq, 2023), observed that the bioactive compounds produced by *Bacillus clausii* against *E. coli* are very good at a maximum of 37mm in diameter,

which is not similar to the current study. Another investigation demonstrated that *Bacillus clausii* is effective in the treatment of children having persistent diarrhea (Dang et al., 2024). A study from Pakistan reported that using photobiotic with probiotics (*Bacillus clausii*) inhibits some selected strains of bacteria (Mushtaq et al., 2024). In this study, no synergistic effect is observed by *Bacillus clausii* against *E. coli* and *Salmonella typhi*, which depends upon the in vivo and in vitro efficacy of the *Bacillus clausii* strains. This helps us in treating the complicated infection caused by these pathogenic bacteria as a probiotics nature of this bacteria.

Conclusion

Bacillus clausii has shown a maximum growth at temperature at 37°C, pH 9, and the glucose concentration is 3 grams. Moreover, *Bacillus clausii* showed resistance against cefotaxime, and cefixime, while showed sensitivity against ciprofloxacin of about 28 mm zone of inhibition, amoxicillin of about 15 mm and meropenem of about 26 mm. Moreover, there is no synergistic effect shown between *Bacillus clausii* and *E. coli* against *Salmonella*.

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