Chemically Defined Medium for Optimization of Proteolytic Activity of *Lactobacillus bulgaricus* 761N

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***Abstract*-Subcellular fractions of *Lactobacillus bulgaricus* 761N were investigated for their proteolytic activity that results in production of bioactive peptides in the cell-free supernatant. It was observed that the extracellular extract of *L. bulgaricus* 761N possessed the highest proteolytic activity of about 63.1 U/ml/min. Consequently, the extracellular fraction was chosen for assessing the proteolytic activity after each step of the optimization process was carried out. The factors of the optimization process involve the incubation period, temperature, pH, salinity (expressed by NaCl concentration), casein content, trace element concentration. The trace elements include cobalt chloride, copper sulphate, calcium chloride and ferrous sulphate. The basal medium used was skim milk agar. The other constituents of the chemically synthesized medium, that would optimize the proteolytic activity that in turn elevate the bioactive peptides production, are determined upon the results of the optimization process. The preferred conditions for maximal activity occurrence was after 48h incubation period, at pH 7, 37 ^{o}C, brackish environment at 5gl^{-1} NaCl content and 5gl^{-1} casein content. $CaCl_2$ and $CoCl_2$ were found to enhance the proteolytic activity at 1mM, while $CuSO_4$ and $FeSO_4$ were found to be needed in fewer amounts at 0.5mM and 0.1mM, respectively. The antibacterial activity of these bioactive peptides produced in the cell free supernatant was investigated against some human pathogenic bacteria. It was found that it impose a high antibacterial capacity on tested pathogens reaching 89% inhibition.**

Keywords- Lactobacillus Bulgaricus 761N; Proteolytic Activity; Chemically Synthesized Medium; Optimization Process; Antibacterial Activity

I. INTRODUCTION

Bioactive peptides coincide with bacteriocins as bacteriocins were defined by Klaenhammer [1], as bioactive peptides with a bactericidal mode of action towards other Gram-positive bacteria. A number of bioactive peptides have been identified in milk proteins, such as casein and whey proteins, where they are present in an encrypted form, stored as propeptides or mature C-terminal peptides that are only released upon proteolysis [2, 3]. The first antimicrobial peptides of casein origin were identified by Hill et al. [4], who isolated antibacterial glycopeptides, known as casecidins.

Lactobacilli, being among the probiotic bacteria, are the live microbial feed supplement which benefited the host when administered in a certain number, thus they are purposely used for their immunomodulatory, antilipidemic, antitoxin, antimicrobial and anti-allergic properties [5]. Moreover, the proteolytic system of lactobacilli is complex and is composed of proteinases and exopeptidases with different subcellular locations [6]. No extracellular proteinase was detected during the growth of *L. bulgaricus* in a rich peptide medium [7]. The partial purification of cell wall enzymes released by washing bacteria in the exponential growth phase with buffer revealed the presence of at least three proteinases which haven't been characterized further [8]. The exopeptidase system has been studied and several enzymes have been purified page layout and partially characterized [8-12]. Ezzat and co-workers [8, 10, 11] have reported that *L. bulgaricus* produces two aminopeptidases (APs; AP I and AP II) and two dipeptidases (I and II) but are devoid of the carboxypeptidase activity that is found in another thermophilic lactobacillus, *L. lactis* [13].

Other studies reported that lactobacilli possess endopeptidase system, for instance, *L. delbrueckii* subsp. *bulgaricus* B14 possesses endopeptidase [14] besides possessing a dipeptidase [15], and a tripeptidase [16]. Another study has found that some lactobacilli as *L. brevis* NRRL B-1836, *L. casei* ATCC 11-443 and many other strains under study possessed aminopeptidases that shown a higher activity in the intracellular extract rather than the extracellular extract [17]. This wide spectrum of peptidases either exo- or endopeptidases could be applied in their purified form for the production of a broad array of bioactive peptides that have antimicrobial capacity in a plentiful and adequate amounts. Therefore, the factors affecting these enzymes activities were extensively studied involving type of substrate, growth rate and stage, temperature, pH, and NaCl concentration [18]. Substrate availability represents an important factor, for example, presence of casein in culture media encourages cell-wall-bound proteinases of LAB [19, 20] and subsequent peptidases [21, 22] to catalyse casein catabolism.

Later, it was observed that peptidolytic and aminotransferase enzymes involved in amino acid release and catabolism were

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formed constitutively by cheese lactobacilli concentration [18]. Their activities in controlled batch and continuous cultures of *Lactobacillus rhamnosus* F3 were affected by the rate and stage of growth and declined, under aerobic and anaerobic conditions, in the stationary growth phase. Enzyme formation was repressed by glucose and activity in cell lysates was affected by the nitrogen source [18]. Activity was retained when cell integrity was maintained at pH 5.0–6.5 and at salinities of 5 g 100 mL^{-1}. Aminotransferase activity in lysates of two *L. paracasei* strains was maximal at, or close to, pH 6.0 and 30 ℃ but was detectable under simulated cheese conditions [18]. Therefore, it was a necessity to carry out optimization process to enhance the enzymatic activity which is mainly the proteolytic activity to obtain the bioactive peptides that could impose an antibacterial capacity against a wide range of human pathogenic bacteria. Hence, a chemically defined medium, depending upon definite optimization criteria under simulated conditions of the natural habitat of some probiotic bacteria constituting a part of the human intestinal microflora, could be a proper method to obtain a significant proteolytic activity and consequently a broad spectrum antibacterial capability based on the short peptides mediator.

II. MATERIALS AND METHODS

A. Isolation and Identification of L. Bulgaricus 761N

L. bulgaricus 761N was isolated from yogurt sample and identified up to PCR level at the laboratory of microbial biochemistry of dairy microorganisms (LMB), Alexandria University, Egypt.

B. Target Pathogens Preparation

Pathogens used involves *Staphylococcus aureus, Pseudomonas aeruginosa, Burkholderia cepacia, Salmonella spp., Escherichia coli, and Shigella spp.* were subcultured on their specific media involving mannitol salt agar, Pseudomonas citrimide agar, brilliant green agar, Eosin methylene blue agar, and triple sugar iron. All cultures are incubated for 24-48 h at 37˚C then kept at 2-8˚C for not more than 1 month. Prior use of prepared pathogens kept at 2-8˚C, they were enriched on tryptic soya broth.

C. Subcellular Fractionation of Cell Lysate

(i) Total cellular extracts: Total cellular extracts were obtained as described by Atlan et al. [6]. (ii) Cell wall and cytoplasmic extracts: Proteins were extracted from the bacterial cell wall of *Lactobacilli* using a modification of the procedure previously developed to release APII from *Lactobacillus delbrueckii* subsp. *bulgaricus* [6]. After 4 h growth in 100 ml MRS or milk medium (A_{600} = 0.8-1.0), cells were harvested by centrifugation (10000 g, 10 min, 6˚C), washed in 25 mM-KH,PO, 10 mM-$MgC1_2$, (PH 5.8) and resuspended in 10 ml of the same buffer supplemented with 0.6 M-sucrose and treated with 0.1 or 1 mg ml^{-1} (for cells grown in MRS or milk medium, respectively) of lysozyme from chicken egg white (Sigma) for 30 min at 25 or 37˚C (for cells grown in MRS or milk medium, respectively) with gentle shaking. Bacterial suspensions were centrifuged (10000g, 10 min, 6˚C) and the supernatants are referred to as lysozyme fluids. Lysozyme-treated bacteria were osmotically shocked by suspending them in 10ml cold distilled water and maintained on ice for 10 min. Shocked cells were centrifuged (10000 g, 10 min, 6˚C) and these supernatants are referred to as osmotic fluids. The bacterial pellets were resuspended in 10 ml cold distilled water and disrupted with a pulse sonicator at 6˚C for 30min. The supernatants obtained after centrifugation of the broken cells (25 000 g**,** 20 min, 6˚C) are referred to as soluble cytoplasmic fluids.

D. Protease Activity Assessment

The protease assay was done according to the method of Kunitz [23] with some modifications. The enzyme extract (1 ml) of bacterial cell wall fractions was incubated with 1.0ml of 1% casein substrate prepared using 0.1M phosphate buffer at pH 6.9 ±0.1 for 20 minutes at 35 ℃. 1 ml of trypsin was incubated with 1.0ml of 1% casein substrate for 20 minutes at 35 ℃, used as a positive control. The reaction was arrested by addition of 3.0ml of 5% Trichloro Acetic acid (TCA). The TCA soluble fragments and total protein of the enzyme extract was estimated by the procedure of Lowry et al. [24].

E. Antibacterial Assay

Antibacterial assay was evaluated by microplate reader assay method according to Bechert et al. [25] with some modifications. Aliquot of 100 µl of preinoculated pathogens (10^6 CFU/µl) in LB broth was transferred to each well of 96 well plates, the same volume (100 µl) of extracellular lactic acid bacterial extracts added to each well in replica. The plates were incubated under microaerophilic conditions at 37 ℃ for 24 h. After incubation, the absorbance of the plates was determined using automated ELIZA microplate reader adjusted at 620 nm. The inhibition percentage of lactic acid bacterial extracts was calculated according to the following equation (Inhibition percentage= (A-A1/A0) ×100), Where, A: the absorbance of the treatment group, A1: the absorbance of the blank, A0: the absorbance of the control group. Standardization of the microbial count of the control group was carried out using McFarland turbidity standard procedure [26].

F. Determination of the Optimal Incubation Period

The optimal incubation period was determined through incubation of the primary media (skim milk broth) inoculated by the isolate and reference culture microorganisms (lactobacilli) at different incubation periods of 24h- and 48h-incubation. Then

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total proteolytic activity was determined.

G. Determination of Optimal Temperature

The influence of temperature on lysate activity was determined by incubating the inoculated primary media at temperatures 35 ℃, 37 ℃ and 45 ℃. Then the total proteolytic activity was measured.

H. Determination of the Optimal pH

The effect of pH on lysate activity was determined at pH 5, 6 and 7. The pH stability was ensured by use of 100 mM sodium phosphate buffer. Total proteolytic activity was then measured to determine optimal pH.

I. Determination of Optimal Salinity

The optimal salinity was expressed in optimal sodium chloride content and was measured at $5gl^{-1}$, $7.5gl^{-1}$ and $10gl^{-1}$ sodium chloride. The inoculated media including different concentrations of sodium chloride were incubated and total proteolytic activity was measured to determine the optimal salinity.

J. Determination of Optimal Casein Content

The optimal casein content was determined by introducing different concentrations of casein to the inoculated media. Casein concentrations used were zero, 5, and $10gl^{-1}$. The inoculated media with different casein concentrations were then incubated and the total proteolytic activity was then measured to determine the optimal casein content.

K. Determination of Trace Elements Optimal Concentration

Stock solutions of $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$ were prepared with distilled water in different concentrations. $CaCl_2$ and $CoCl_2$ were added to the inoculated media at concentrations 0.1, 1 and 10mM, while $CuSO_4$ and $FeSO_4$ were added to the inoculated media at concentrations 0.1, 0.5 and 1 mM. The inoculated media with different concentrations of metal ions were then incubated at 37 ℃ and the total proteolytic activity was measured to determine the effect of metal ions and the optimal metal ions' concentration enhancing lysate activity.

L. Measurement of Total Proteolytic Activity of Cell Free Extracts (CFE)

1) Preparation of CFE:

Inoculated media after the incubation period were centrifuged at 20,000 xg for 20min at 4 ℃. The supernatant obtained was filtrated through Millipore filters of 0.22 μm diameter, the obtained filtrate was used as CFE.

2) Total Proteolytic Activity Measurement:

The proteolytic activity of CFE towards β-casein peptides was followed by the release of free amino acids, whose amino groups were estimated by the Cadmium-Ninhydrin reaction, according to a modification of the method of Doi et al. [27]. Results were reported as free amino acids (mM Gly equivalents) Klein et al. [28].

M. Statistical Analysis

Statistical analysis of data is represented by the mean of triplicate groups ± standard deviation. One way analysis of variance (ANOVA) and two way analysis of variance were used according to (Bishop, 1983). Probability test (P) was carried out to show the significance degree, $p \leq 0.0001$ highly significant, $P \leq 0.005$ significant and $P > 0.005$ non significant. LSD (at $\alpha = 0.05$) is the least significant differences.

III. RESULTS AND DISCUSSION

A. Protease Activity Results of Subcellular Fractions

The proteolytic system of lactobacilli is complex and is composed of proteinases and exopeptidases with different subcellular locations [6]. It was found that the extracellular fraction for all strains under study possessed the highest proteolytic activity as shown in Table 1. This is in agreement with Kok and Venema [20], who reported that the cell-wall-bound proteinases of lactic acid bacteria perform the first steps in the hydrolysis of casein. Seo et al. [30], however, observed that the first step in casein degradation is mediated by proteases in the cell membrane. Further degradation into smaller peptides and amino acids that can pass through cell membrane is achieved by peptidases of lactic acid bacteria [31, 32]. The findings of Kabadjova-Hristova, et al. [33] approved that Lactobacillus (L. kefir DR22x) had cell-wall-bound proteinases with an extracellular location.

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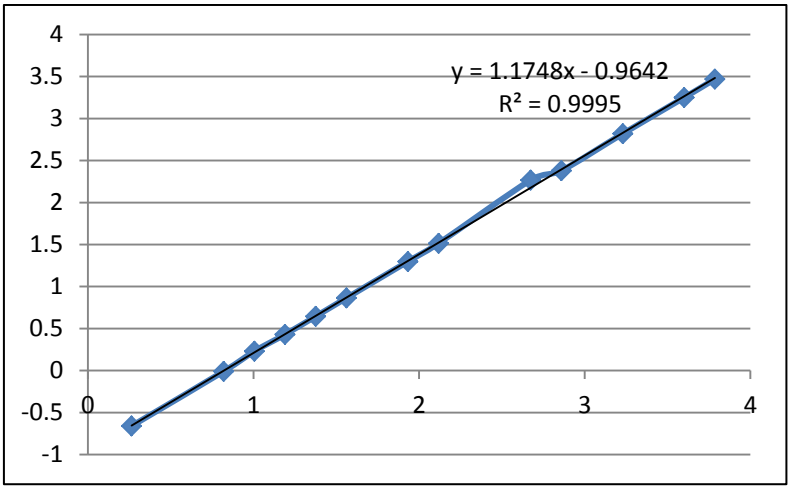
TABLE 1 761N PROTEASE ACTIVITY OF SUBCELLULAR FRACTIONS OF *L. bulgaricus* 761N (MEAN±SD)

Subcellular fractions	Protease activity (U/ml/min)	F value	LSD
Intracellular fraction*	39.5[c]		
Intercellular fraction**	42.1[b]	1183.4	1.2879
Extracellular fraction***	63.1[a]		

*cytoplasmic fluids, ** osmotic fluids, *** lysozyme fluids
Equation: y = 0.001x + 0.0859.
Where y = Test – Control, x = Proteolytic activity (U/ml/min).
a, b, and c: symbols to express significant difference in the statistical analysis (i.e. similar symbols means no significant difference while different symbols means that these data are significantly different).

B. Optimization of Lysate Activity of L. bulgaricus 761N

A standard curve was constructed for conversion of the detected absorbance to the equivalent concentration of glycine in mM in order to measure the proteolytic activity achieved by the lysate of *L. bulgaricus* 761N as mM glycine equivalent (Fig. 1).



*Absorbance was measured at 490 nm.
Slope equation: y = 1.1748x - 0.9642.
Y: Absorbance, X: Concentration of glycine in mM.

Fig. 1 Concentrations of glycine (mM) and their equivalent absorbance

C. Incubation Period

It was observed that the proteolytic activity increases with the increase in the incubation period as it was found that proteolytic activity after 48h of incubation was higher than that after 24h as shown in Table 2. Literature of Tufail et al. [35], revealed through their study that incubation period of 48 h for *L. bulgaricus* isolated from yogurt results in maximum production of bacteriocin resulted from the higher proteolytic activity at this condition mentioned for *L. bulgaricus*. Other literature elicited that the proteolytic activity increases with increase in incubation time till 36h of incubation then the proteolytic activity begins to decline [36] that disagree with the current study and the previous study by Tufail et al. [35]. That difference may be owing to species difference or isolation origin as it was investigated by Kabadjova-Hristova et al. [33]. Kabadjova-Hristova et al. [33], have found that according to the literature of Pereira et al. [37], the proteinases of *L. curvatus* and *L. homohiochii* showed maximum activity in the early exponential phase, that findings disagreed with their investigations and informed that the disagreement is owing to the origin of the strain used which was isolated from kefir grains that presented too complex media.

TABLE 2 LYSATE ACTIVITY OF *L. bulgaricus* 761N EXTRACELLULAR EXTRACT

Incubation period	Concentration (mM Glycine equivalent)
24 hours	1.076±0.002[a]
48 hours	1.116±0.005[b]
F value	166.37
LSD	0.0087

D. Temperature

The lysate activity reached the maximum value at 35 ℃ and went down gradually from 37 ℃ to 45 ℃ as shown in Table 3,

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i.e. The temperature levels have a significant influence ($P < 0.0002$) on the lysate activity. Generally, but within limited range, the decrease in temperature was found to be accompanied with an increase in the proteolytic activity. Kabadjova-Hristova, et al. [33], and El-Ghaish et al. [38], have reported that the highest proteolytic activity was at 37 ℃ and that raising the temperature up to 42 ℃ slightly affected the proteolytic activity. The current study recorded that the optimum temperature was at 35 ℃, and a slight decrease in proteolytic activity with increasing temperature to 37 ℃ and then 42 ℃. That optimum temperature agreed with the previous work by Tufail et al. [35]. We can conclude that, the difference in the optimum temperature is due to the difference in the species.

TABLE 3 LYSATE ACTIVITY OF *L. bulgaricus* 761N EXTRACELLULAR EXTRACT AT DIFFERENT TEMPERATURES

Temperature	Concentration (mM Glycine equivalent)
35˚C	1.511 ± 0.055^{a}
37˚C	1.253 ± 0.06^{b}
45˚C	1.132 ± 0.014^{c}
F value	166.37
LSD	0.0087

E. pH

Generally, the optimum pH for that activity was ranging from slightly acidic to slightly alkaline that means around the neutrality. Going more towards the strong acidity or alkalinity, has adverse effect on the proteolytic activity. In the current study, the optimum pH was pH 7.0 (Table 4) and decreased towards the acidity. These findings agreed with Fira, [39], Kabadjova-Hristova [33], and El-Ghaish et al. [38], with some variations but with the same trend. The variations might be due to the difference in bacterial species. Those results disagreed with some other studies such as De Giori et al. [40], where he reported that the optimum pH was ranging from pH 5.2 to 5.6. Also, Hutkins and Nannen [41], has found that *Lactobacillus casei* showed high proteolytic activity at pH 4.8 to 5.2 that disagree with our study observations. Tufail et al. [35], found that his strains of *L. bulgaricus* were sensitive to alkaline pH while resistant to acidic pH. Other literature indicated that the assossiative growth affects the proteolytic activity owing to corresponding alteration of pH. It was found that mixed cultures of *Streptococcus thermophilus* and *L. bulgaricus* cultures in one combination was less proteolytic than the corresponding *L. bulgaricus* strain in pure culture that was combined with lowering in pH [42]. Lowering in pH and correspondingly lowering in proteolytic activity agree with our study observations.

TABLE 4 LYSATE ACTIVITY OF *L. bulgaricus* 761N EXTRACELLULAR EXTRACT AT DIFFERENT PH VALUES

pH	Concentration (mM Glycine equivalent)
5	0.915 ± 0.002^{a}
6	1.022 ± 0.037^{b}
7	1.144 ± 0.029^{c}
F value	53.8
LSD	0.054

F. Salinity

Salinity was found to affect the proteolytic activity, since the increase in sodium chloride concentration was observed to be followed with a decrease in the proteolytic activity. The current study shows that the highest proteolytic activity was at $5gl^{-1}$ NaCl followed by $7.5gl^{-1}$ NaCl, while the minimum activity was at 10 gl^{-1} NaCl as in Table 5. De Vuyst and Leroy [43], has reported that the presence of elevated amounts of sodium chloride usually decreases bacteriocin production that is produced as a result of the proteolytic activity of lactic acid bacteria. This agrees with our current research findings. At higher NaCl concentrations, specific bacteriocin production decreased further [44]. In contrast, Uguen et al. [45], reported an increased lacticin 481 production when the osmolarity of the growth medium increased due to added NaCl. Also, for plantaricin S, the highest production was observed at a sodium chloride concentration of 2.5% (wt/vol) as reported by Leal-Sa ńchez et al. [46]. On the other hand, the production of sakacin K by *L. sakei* CTC 494 was negatively affected by added NaCl [47], as was the case for the antilisterial carnobacteriocin B2 produced by *Carnobacterium piscicola* A9b [48]. Although enterococci are more salt resistant, production of the enterocins A and B by *Enterococcus faecium* CTC 492 was also inhibited in the presence of NaCl [49].

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TABLE 5 LYSATE ACTIVITY OF *L. bulgaricus* 761N EXTRACELLULAR AT DIFFERENT NACL CONCENTRATIONS (G/L)

NaCl concentration (g/l)	Concentration (mM Glycine equivalent)
5	1.13 ± 0.003^{a}
7.5	1.052 ± 0.003^{b}
10	1.009 ± 0.004^{c}
F value	1012.75
LSD	0.0067

G. Casein Content

The lysate activity increased with increase of casein protein content till 5 gl^{-1} and the least activity was at 10 gl^{-1} as shown in Table 6. The three concentrations of casein content have a highly significant different influence on the lysate activity ($P < 0.0001$). Lim [50] has found that bacteriocin production of *L. plantarum* KC21 was detected at 0.25% and 0.5% casein that was active against *S. aureus* ATCC 6538, while bacteriocin production was not detected in 1% casein. The findings of Lim approve this research results, as the lysate activity that resulted in bacteriocin production increased with the increase in casein content till 5 g/l i.e. 0.5% casein and then decreased at 10 g/l (1%) casein. Difference in bacteriocin production at 1% casein between *L. bulgaricus* 761N and *L. plantarum* KC21 may be due to difference in species origin. Other literatures have reported that casein content affects extensively the proteolytic activity. The proteolytic activity of the intestinal bacterium *Bacteroides fragilis* NCDO 2217 was investigated for its relationship to the casein content of the culture media [51].

TABLE 6 LYSATE ACTIVITY OF *L. bulgaricus* 761N EXTRACELLULAR EXTRACT AT DIFFERENT CASEIN CONTENTS (G/L)

Casein content (g/l)	Concentration (mM Glycine equivalent)
Zero	1.111 ± 0^{a}
5	1.242 ± 0.012^{b}
10	1.213 ± 0.003^{c}
F value	277.12
LSD	0.0143

H. Trace Elements Concentrations

Trace elements were mentioned in many literatures to play an important role as essential micronutrients for LAB. Chervaux et al. [52], have conducted a physiological study on *Lactobacillus delbrueckii* subsp. *bulgaricus* strains in a chemically defined medium and have found that elimination of manganese or iron did not affect growth as long as calcium and micronutrients was present, which led to keeping trace elements other than the amount of iron, which was observed to be potentially toxic. That agreed with the current study observations, where it was found that iron should be added at concentration 0.1mM rather than 0.5mM and 1mM. On the contrary, Grobben et al. [53], have reported that *L. delbrueckii* subsp. *bulgaricus* grew well when the trace elements involving ferrous chloride, Cobalt chloride, zinc chloride, nickel chloride and copper chloride were omitted individually or all at once. The current study showed that cobalt chloride and copper sulphate were essential for the candidate strain leading to optimal activity at 1mM and 0.5mM, respectively. Chervaux et al. [52], have found that calcium chloride was important for efficient growth of *L. delbrueckii* subsp. *bulgaricus* as is the case in our study. Therefore, skim milk being used as a convenient culture media for lactobacilli is owing to the highly calcium bioavailability as mentioned by Adolfsson et al. [54]. Other forms of dairy products involving yogurt, cheese, etc. enhance the modulation of LAB. Because of the lower pH of yogurt compared with that of milk, calcium and magnesium are present in yogurt mostly in their ionic forms [54]. These ionic forms of trace elements were suggested to bind to the bioactive peptides resulting from the proteolytic activity of lactobacilli.

1) Calcium Chloride ($CaCl_2$)

The lysate activity increased with the increase of calcium chloride concentration till 1mM and was the least at 10mM of calcium chloride. 0.1mM and 10mM $CaCl_2$ have no significantly different effect ($P > 0.005$) on the lysate activity, while 1.0mM $CaCl_2$ is highly significant ($P < 0.002$) as shown in Table 7.

TABLE 7 LYSATE ACTIVITY OF *L. bulgaricus* 761N EXTRACELLULAR EXTRACT AT DIFFERENT $CACL_2$, AND $COCL_2$ CONCENTRATIONS (MM)

Trace element concentration (mM)	Concentration (mM Glycine equivalent) for $CaCl_2$	Concentration (mM Glycine equivalent) for $CoCl_2$
0.1	1.065 ± 0.009^{b}	1.136 ± 0.009^{a}
1.0	1.102 ± 0.003^{a}	1.151 ± 0.013^{a}
10	1.058 ± 0.004^{b}	1.098 ± 0.014^{b}
F value	44.73	15.75
LSD	0.0121	0.024

2) Copper Sulphate (CuSO4)

The lysate activity increased with the increase of copper sulphate calcium chloride concentration till 0.5mM and was the

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least at 1mM of copper sulphate. 0.1mM and 1.0mM $CuSO_4$ have no significantly different effect ($P > 0.005$) on the lysate activity, while 0.5mM $CuSO_4$ is significant ($P < 0.0222$) as shown in Table 8.

TABLE 8 LYSATE ACTIVITY OF *L. bulgaricus* 761N EXTRACELLULAR EXTRACT AT DIFFERENT $CUSO_4$, AND $FESO_4$ CONCENTRATIONS (MM)

Trace element concentration (mM)	Concentration (mM Glycine equivalent) for $CuSO_4$	Concentration (mM Glycine equivalent) for $FeSO_4$
0.1	1.064 ± 0.02^b	1.125 ± 0.005^a
0.5	1.104 ± 0.01^a	1.123 ± 0.002^a
1.0	1.072 ± 0.007^b	1.021 ± 0.029^b
F value	7.68	38.13
LSD	0.0268	0.0335

3) Cobalt Chloride (CoCl2)

The lysate activity increased at 1mM of cobalt chloride and was the least at10mM of cobalt chloride. 0.1mM and 1.0mM $CoCl_2$ have no significantly different effect ($P > 0.005$) on the lysate activity, while both formerly-mentioned concentrations are significantly different from 10mM $CoCl_2$ ($P < 0.0041$) as shown in Table 7.

4) Ferrous Sulphate (FeSO4)

The lysate activity decreased with the increase of the concentration of ferrous sulphate. The lysate activity was the highest at 0.1mM of ferrous sulphate and decreased slightly at 0.5mM, while the activity was the least at 1mM of ferrous sulphate. 0.1mM and 0.5 mM $FeSO_4$ have no significantly different effect ($P > 0.005$) on the lysate activity, while both formerly-mentioned concentrations are significantly different from 1.0mM $FeSO_4$ ($P < 0.004$) as shown in Table 8.

Clare and Swaisgood [55], have observed that caseinophosphopeptides (CPP), refering to casein-derived phosphorylated peptides, contain single and multiple phosphoryl residues, and these phosphopeptides are released by enzymatic hydrolysis of αs1-, αs2-, β- and k-caseins both *in vitro* and *in vivo*. Due to the high content of negative charges, these peptides efficiently bind divalent cations and, therefore, may act as biocarriers for trace elements such as Fe, Mn, Cu and Se. CPPs generally refer to peptides generated after enzymatic treatment [56, 57] which approve the previous suggestions. Binding of trace elements to peptides generated from protease activity of lactobacilli may support the hypothesis that addition of trace elements in definite concentrations, acting as a cofactor, enhances the activity of protease enzymes to produce the bioactive peptides that would in turn bind to the elevated amounts of trace elements.

I. The Chemically Defined Medium for Optimization of the Proteolytic Activity of L. bulgaricus

The medium constituents, shown in Table 9, were determined depending upon the optimization factors investigated in this study.

TABLE 9 CONSTITUENTS OF THE CHEMICALLY DEFINED MEDIUM

	Approximate formula (g/L)
Skim milk	10
Casein hydrolysate	5
Sodium chloride	5
Calcium chloride	0.111
Copper sulphate	0.125
Ferrous sulphate	0.015
Cobalt chloride	0.13
Sodium dihydrogen phosphate	15.6
Disodium hydrogen phosphate	17.8

J. Antibacterial Activity Assessment (Expressed in Inhibition Percent Using Microplate Reader Assay Method)

A standard curve has been constructed to calculate the microbial count of bacteria as shown in Table 10. It converted any observed turbidity resulting from microbial growth to concentration of microorganisms, i.e. microbial count.

TABLE 10 CONCENTRATIONS OF MCFARLAND EQUIVALENCE TURBIDITY STANDARDS AND THEIR EQUIVALENT ABSORBANCES

Concentration (cfu/ml)	Absorbance at 600 nm
1.5×10^8	0.10117
3×10^8	0.24292
6×10^8	0.4407
9×10^8	0.64304
12×10^8	0.81687

Slope equation: $y = 7E\text{-}10x + 0.0244$.
Y: Absorbance, X: Concentration of McFarland Equivalence Turbidity Standards (cfu/ml).

The antibacterial activity of *L. bulgaricus* 761N extracellular extract against different pathogenic bacteria under study was obviously illustrated in Table 10. It indicates that the inhibition percent of pathogenic bacteria increases with the increase of the concentration of the extracellular extract of *L. bulgaricus* 761N. The inhibition percent ranged from 0% at 50%

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concentration of the extracellular extract against *Salmonella* spp. to 89% at 100% concentration of the extracellular extract against *Shigella* spp (Table 11).

TABLE 11 ANTIBACTERIAL ASSAY FOR *L. bulgaricus* 761N EXTRACELLULAR EXTRACT TESTED AGAINST DIFFERENT PATHOGENIC BACTERIA

Pathogens	*S.aureus*	*Salmonella*	*P.aeruginosa*	*E.coli*	*Shigella spp.*	*B. cepacia*
Treatment Concentration	Inhibition percentage					
100%	82.90 ±0.03 [a]	66.98 ±0.02 [a]	72.29 ±0.02 [a]	72.24 ±0.04 [a]	89.00 ±0.05 [a]	79.76 ±0.06 [a]
90%	81.74 ±0.03 [b]	65.84 ±0.04 [a]	66.69 ±0.01 [b]	68.57 ±0.02 [b]	86.40 ±0.02 [b]	79.16 ±0.04 [b]
80%	80.48 ±0.04 [c]	29.50 ±0.02 [b]	30.18 ±0.03 [c]	54.12 ±0.02 [c]	71.35 ±0.03 [c]	76.31 ±0.01 [c]
70%	76.39 ±0.01 [d]	22.07 ±0.02 [c]	24.42 ±0.02 [d]	51.27 ±0.03 [d]	69.85 ±0.04 [d]	72.52 ±0.03 [d]
60%	76.18 ±0.02 [d]	13.13 ±0.03 [d]	22.37 ±0.02 [e]	49.31 ±0.01 [e]	68.56 ±0.01 [e]	64.60 ±0.03 [e]
50%	75.55 ±0.05 [e]	0 [e]	8.39 ±0.03 [f]	39.02 ±0.02 [f]	66.52 ±0.02 [f]	51.08 ±0.08 [f]
F value	218331	374458.3	490577.4	739294	382936	232799
LSD	0.22	0.15	0.04	0.04	0.06	0.08
P value	*	**	< 0.0001	< 0.0001	< 0.0001	< 0.0001

*****: No significant difference was observed for the extracellular extract of *L. bulgaricus* 761N at concentrations 60% and 70% against *Staphylococcus aureus* ($P > 0.005$), i.e. in the case of using the crude extract as a treatment against *S. aureus* at treatment concentration 60% instead of 70%, it would be better owing to gaining a less side effects, for example cytotoxicity, and roughly the same influence as that of the latter treatment concentration.
******: No significant difference was observed for the extracellular extract of Lactobacillus bulgaricus 761N at concentrations 90% and 100% against Salmonella spp. ($P > 0.005$), i.e. using the crude treatment against *Salmonella* spp. at a concentration of 90% is better than using it at 100% concentration achieving less negative effects, and roughly the same influence as that of the latter concentration.

The antibacterial activity of extracellular extract referring to bacteriocins was mentioned in many literatures. Earlier reports [58, 59, 60] have shown that some bacteriocins produced by Gram-positive bacteria have a broad spectrum of activity. However, it was generally observed that bacteriocin from the producer organism had no inhibitory effect on the organism producing it. Tufail et al. [35], have observed that culture supernatants that were obtained from the sixty out of hundred isolates of *Lactobacillus* spp. involving *L. bulgaricus* isolated from yogurt exhibited varying degrees of inhibitory activity against strains of *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 10536, *Salmonella typhi* ATCC 19430, *S. aureus* ATCC 6538, and *Vibrio cholerae* ATCC 25870. This is in agreement with the current research findings that observed a significant antibacterial capability against a range of pathogenic bacteria involving *E. coli*, *S. aureus*, *Pseudomonas aeroginosa*, *Salmonella* sp, *Burkholderia cepacia*, and *Shigella* sp. These results are also in agreement with previous work carried out by Erdourul and Erbulur [61], but on different strain types, in which supernatants obtained from *L. casei* and *L. bulgaricus* exhibited varying degrees of inhibitory activity against strains of *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *P. aeroginosa* ATCC 9027, *B. subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 18833, *Salmonella typhimurium* ATCC 13311, and *Enterobacter cloacae* ATCC 13047. The probiotic potential of these bacteria is also vastly investigated [62, 63, 64, 65].

Akpinar et al. [66] have shown that *L. bulgaricus* produces a bacteriocin called Bulgarican that is inhibitory towards both Gram-positive and Gram-negative bacteria. Some inhibitory compounds against *Staphylococcus* and *Clostridium* species have also been found [67]. Ocanˇa et al. [68] have observed using transmission electron microscopy that bacteriocin obtained from *L. salivarius* subsp. *salivarius* CRL 1328 causes vesiculization of protoplasm, formation of pores, and complete disintegration of cells of *Enterococcus faecalis*. Meydani and Ha [69] has reported that the inhibitory mechanisms of LAB against disease-causing bacteria are due primarily to two metabolites of lactic acid fermentation involving organic acid [70, 71] and bacteriocin [72]. Thus, bacteriocins that have been mentioned in many literatures to be of mainly a protein ontogeny, found in the extracellular extract, have a wide range antibacterial capacity against many pathogenic bacteria including both gram positive and gram negative bacteria according to our study observations.

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