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ORIGINAL PAPER

Safety evaluation and antimicrobial properties of *Lactobacillus pentosus* 22C isolated from traditional yogurt

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Abstract Traditional dairy products are the main source of probiotic microorganisms. Hence, this study aimed to evaluate the safety and antimicrobial potential of *Lactobacillus pentosus* 22C, isolated from traditional yogurt from Kermanshah province, Iran. Strain 22C showed no undesirable amino acid decarboxylase and β-hemolytic activities. Virulence associated genes were detected in the isolate. The strain produced an antimicrobial molecule named pentocin 22C, a small peptide with a relative mass between 5 and 10 kDa. Bacterial inhibition was pH-independent, with greater activity at pH 4–6. Purified or semi-purified antimicrobial agents can be used as bio-preservatives, and the producing strain can be used as a starter culture to improve food safety. Therefore, a biosafety assessment should be conducted. *L. pentosus* 22C, isolated from a traditional dairy product, has antimicrobial ability, although its safety must be evaluated.

Keywords Antimicrobial · *Lactobacillus pentosus* · Safety · Starter culture · Traditional

Introduction

Lactic acid bacteria (LAB) represent a significant proportion of probiotic organisms, which are defined as live microorganisms that confer health benefits to the host when administered adequately [1]. The positive health effects of LAB, including the effects on the immune system and interactions with the gastrointestinal tract microbiota and intestinal mucus layer, have been extensively reviewed [2, 3]. Most studies on beneficial LAB include the isolation of microorganisms for the selection and characterization of new probiotics [4].

Two of the most important criteria for selecting probiotic candidates are the following: a determination of antimicrobial activity and evaluation of the safety of candidate strains based on antibiotics susceptibility and virulence potential [5].

The ability to inhibit other bacteria may allow the probiotic to inhabit a niche; and increase its ability to competitively inhibit other gastrointestinal microbes and pathogenic bacteria [6]. Moreover, antimicrobial activity may control fermentation and increase the shelf life of dairy products [7]. Purified antimicrobial compounds can be used as additives, but this is not always attractive to the food industry, because they may require labelling as a preservative and regulatory approval [8]. In situ production of antimicrobial compounds using a producer starter culture is regarded as a more commercially attractive strategy because it does not require regulatory approval. However, the safety of these strains must be evaluated [9].

According to the FAO/WHO, candidate commercial probiotics must complete a safety assessment [10]. A thorough strain-specific evaluation of possible pathogenicity and antibiotic susceptibility is a priority to assess the risk associated with their intentional use in the food chain [11]. LAB are generally regarded as safe, because of their long history of use in various fermented products [12]. However, recently, there has been growing concern regarding the health risks from certain strains [13]. Fear and uncertainty pose important challenges in the food industry. Therefore, more research is needed to

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Table 1 Oligonucleotide primers used for detection of virulence genes

Genes	Sequences (5′–3′)	Product size (bp)
gelE	F: ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	419
efaA$_{fm}$	F: AACAGATCCGCATGAATA R: CATTTCATCATCTGATAGTA	735
efaA$_{fs}$	F: GACAGACCCTCACGAATA R: AGTTCATCATGCTGTAGTA	705
Ace	F: AAAGTAGAATTAGATCCACAC R: TCTATCACATTCGGTTGCG	350
Esp$_{fs}$	F: TTGCTAATGCTAGTCCACGACC R: GCGTCAACACTTGCATTGCCGAA	933
cylM	F: CTGATGGAAAGAAGATAGTAT R: TGAGTTGGTCTGATTACATTT	742
cylA	F: TGGATGATAGTGATAGGAAGT R: TCTACAGTAAATCTTTCGTCA	517
cylB	F: ATTCCTACCTATGTTCTGTTA R: AATAAACTCTTCTTTTCCAAC	843

ensure the safety and reliability of strains isolated from traditional sources [9].

Lactobacillus pentosus is a potent producer of antimicrobial compounds. However, the limited spectrum of their activity and its sensitivity to pH and high temperature indicate the need to identify additional molecules with antimicrobial activity over a wide range of pH and high temperature [14, 15]. These compounds can be used effectively for food fermentation and food-borne pathogen control [16].

The present work was carried out in order to investigate the in vitro safety assessment and the antimicrobial potential of *L. pentosus* 22C isolated from Iranian traditional yogurt.

Materials and methods

Bacterial strains and growth media

Lactobacillus pentosus 22C was grown in de Man, Rogosa, and Sharpe (MRS) broth (Scharlau Chemie, Barcelona, Spain) for the production of antimicrobial compound. *Micrococcus luteus* (PTCC[1] 1110), Bacillus cereus (PTCC 1015), *Listeria monocytogenes* (PTCC 1306), *Staphylococcus aureus* (PTCC 1112), and *Escherichia coli* (PTCC 1330), used as indicator strains, were grown in brain–heart infusion broth (Biolife, Milan, Italy).

[1] Persian Type Culture Collection.

Isolation and identification of strain

Lactobacillus pentosus 22C was previously isolated from traditional yogurt in Kermanshah, Iran. The conventional methodology for biochemical classification and molecular techniques (16S ribosomal DNA sequences and a *rec*A PCR species-specific test) were used for strain identification.

A 1523-bp fragment of the 16S rRNA gene was amplified by PCR with the 27F forward primer (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1541R reverse primer (5′-AAG GAG GTG ATC CAG CCG CA-3′). A BLAST analyses revealed that the 16S rRNA gene sequence of strain 22C was most similar to those in the *Lactobacillus plantarum* group. Differentiation of *L. plantarum* group species was performed by amplification of the *rec*A gene. PCR was performed using the following primers: paraF (5′-GTC ACA GGC ATT ACG AAA AC-3′), pentF (5′-CAG TGG CGC GGT TGA TAT C-3′), planF (5′-CCG TTT ATG CGG AAC ACC TA-3′) and pREV (5′-TCG GGA TTA CCA AAC ATC AC-3′) [17].

Safety evaluation

Screening for the presence of virulence factors

PCR was used to detect genes encoding potential virulence factors in *L. pentosus* 22C. The following primer pairs were used for detection: gelE(F)/gelE(R) for gelatinase, *efaA*$_{fm}$(F)/*efaA*$_{fm}$(R) and *efaA*$_{fs}$(F)/*efaA*$_{fs}$(R) for cell wall adhesion protein, *ace*(F)/*ace*(R) for collagen adhesion protein, *Esp*$_{fs}$(F)/*Esp*$_{fs}$(R) for cell wall-associated protein, and *cylM*(*F*)/*cylM*(*R*), *cylA*(*F*)/*cylA*(*R*) and *cylB*(*F*)/*cylB*(*R*) for cytolysin. Oligonucleotide primers were obtained from Sinaclone Ltd. (Tehran, Iran) [18]. The primers are listed in Table 1. *Enterococccus faecalis* ATCC 29212 was used as a positive control strain. PCRs were performed using bacterial DNA (extracted using a DNA extraction kit) (Sinaclone Ltd., Tehran, Iran) in 20-µl reaction mixtures with 100 ng of extracted DNA, 1.25 µM of each primer, 0.2 mM of each dNTP, 2 µL of 10× buffer, 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Sinaclone Ltd., Tehran, Iran). Samples were subjected to an initial cycle of denaturation (95 °C for 5 min), followed by 35 cycles of denaturation (94 °C for 1 min), annealing (48–60 °C for 1 min) and elongation (72 °C for 1 min), ending with a final extension step at 72 °C for 10 min in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). PCR products were detected after electrophoresis for 30 min at 70 V on 1.5% (w/v) agarose gels by visualization under UV light with a Gel Doc 1000 documentation system (Bio-Rad, Madrid, Spain).

Production of gelatinase

Gelatinase activity of the isolate was investigated on Todd-Hewitt agar containing heart infusion (3.1 g L^{-1}), yeast enriched peptone (10 g L^{-1}), glucose (2 g L^{-1}), casein (10 g L^{-1}) sodium chloride (2 g L^{-1}), disodium phosphate (0.4 g L^{-1}), sodium carbonate (2.5 g L^{-1}), Tween 80 (1 mL) and gelatin (30 g L^{-1}, final pH 7.8). Plates were incubated overnight at 37 °C and then placed for 5 h at 4 °C. Gelatinase hydrolysis was determined by screening the plates for turbid areas around colonies. All products were purchased from Merck, Germany.

Production of hemolysin

Hemolysin production was analyzed on blood agar (Biolife, Milan, Italy) containing 5% sheep's blood. The presence of β- or α-hemolysis was determined by the formation of clear or green zones, respectively, around the colonies.

Biogenic amine production

Amino acid decarboxylase was detected according to the method of Bover-Cid and Holzapfel [19]. *L. pentosus* 22C was sub-cultured twice in MRS broth containing 0.1% tyrosine, histidine, ornithine, and lysine (all from Sigma). The isolate was then spotted on decarboxylase medium plates with each amino acid and bromocresol purple as a pH indicator and then incubated at 37 °C in aerobic conditions for 4 days. Decarboxylase medium plates without amino acids were used as a control. Plates were considered positive for decarboxylase activity when purple color was observed around the colonies. Tyrosine decarboxylase was considered present when a precipitated halo disappeared around the colonies.

Determination of antibiotic susceptibility

The antibiotic susceptibility of *L. pentosus* 22C was determined by overlaying antibiotic-containing disks (Padtanteb, Iran) on Mueller–Hinton Agar (Oxoid, Basingstoke, Hampshire, UK) plates that were previously seeded with *L. pentosus* 22C corresponding to a McFarland standard of 0.5. The antibiotics tested were ampicillin (AMP, 10 µg), kanamycin (K, 30 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GMC, 10 µg), penicillin (P, 10 µg), polymyxin B (300 µg), rifampin (5 µg), tetracycline (TE, 30 µg), and vancomycin (V, 30 µg). Following a 24-h incubation at 37 °C, diameters of inhibition zones were measured.

Antimicrobial activity

Antimicrobial activity bioassay

Antimicrobial activity was determined by the turbidometric method [20] and agar well diffusion assay [21]. The turbidometric method was adapted to evaluate inhibitory activity in liquid broth. The percentage of inhibition was calculated by Eq. 1 [22]. *L. pentosus* 22C was grown at 37 °C in MRS broth at a pH of 6.4 for 24 h. Cell-free culture supernatant (CFS) was obtained by medium centrifugation (Universal 320R, Hettich Co., UK) (7000×*g* for 20 min at 4 °C). The supernatant was neutralized by 5 N NaOH to pH 6.5 and filter sterilized through a 0.22-µm filter membrane. Each sample was concentrated by freeze-drying. Bioactivity was determined by the turbidity of the cell suspension at 600 nm on a spectrophotometer-type ELISA plate reader (BioTek, Power Wave XS, KC Junior program) [23].

The indicator inoculate was adjusted to a McFarland standard of 0.5. The inhibitory percentage was calculated using the following equation [22]:

$$I(\%) = OD_{600}\text{control} - OD_{600}\text{sample}/OD_{600}\text{control} \times 100. \quad (1)$$

Characterization of the antimicrobial compound

The CFS was incubated with trypsin (Fluka, St. Louis, Missouri, USA), pepsin (Sigma, St. Louis, Missouri, USA), α-chymotrypsin, and catalase (both Sigma) at final concentration of 1 mg mL^{-1} and incubated at 37 °C for 2 h. Then, the enzymes were inactivated by boiling the samples for 5 min. The inhibitory activity was evaluated within the pH range of 2–10, and the heat stability of the antimicrobial peptide was determined by boiling (100 °C) of CFS for 5, 10, and 15 min.

Production and molecular weight estimation of pentocin 22C

Fifty milliliters of MRS broth was inoculated with 1.0% (v/v) of the overnight culture of *L. pentosus* 22C and incubated at 37 °C. The optical density at 600 nm (OD_{600}), antimicrobial activity, and pH values were measured at specific intervals over 48 h. The antimicrobial compound was partially purified by a two-step protocol [24]. Briefly, it involved ammonium sulfate precipitation and ultra-filtration. The crude samples were precipitated with ammonium sulfate (Merck, Darmstadt, Germany) (0, 30, 40, 50, 60, 70 and 80% saturation). Dialysis through a membrane with a 2-kDa cut-off (Sigma-Aldrich, 2000 Dalton NMWco, Munich, Germany) was used to eliminate salt. To determine the fraction responsible for antimicrobial activity, the samples

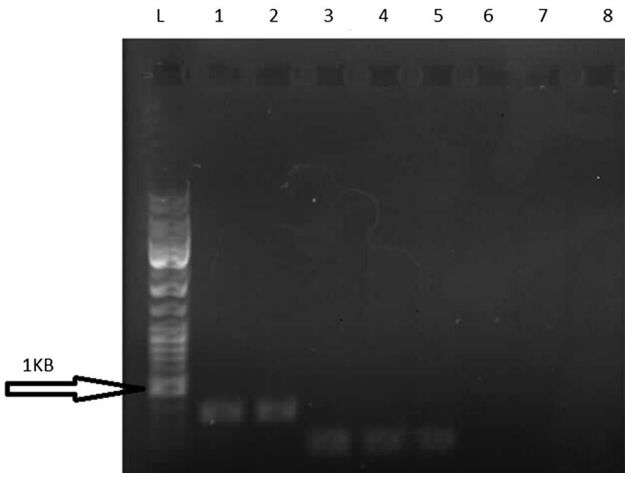

Fig. 1 The PCR-amplification of specie-specific sequences of *recA*. *L* 1 kb DNA ladder. *Lanes 1* and *2* positive control (*L. plantarum* PTCC 1058, annealing 54 and 56 °C). *Lanes 3–5* PCR amplification product from strain 22C (pentF primer, annealing 54, 56 and 58 °C). *Lanes 6* and *7* PCR amplification product from strain 22C (plantF primer, annealing 54 and 56 °C). *Lane 8* PCR amplification product from strain 22C (paraF primer, annealing 54 °C)

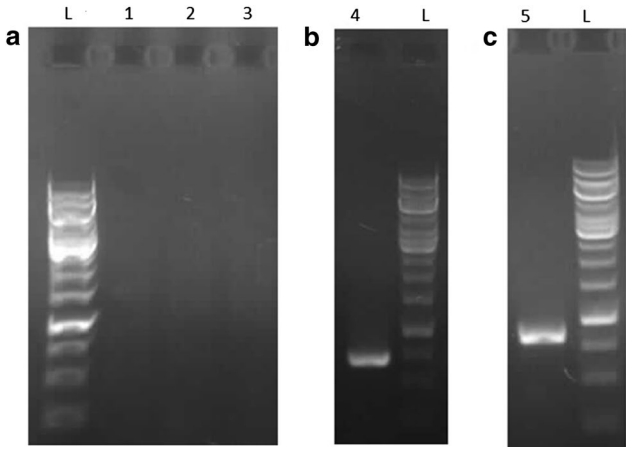

Fig. 2 Amplification products obtained from the virulence determinants. The primers directed against **a** *1* 933 bp fragment of the Esp_{fs}, *2* 735 bp fragment of the $efaA_{fm}$, *3* 419 bp fragment of the gelE, **b** *4* 705 bp fragment of the $efaA_{fs}$ and **c** *5* 742 bp fragment of the *cylM*. *L* 1 kb DNA ladder

were then ultra-filtered with 10- and 5-kDa molecular weight cut-offs. The fractions (greater than 10 kDa, between 5 and 10 kDa, and less than 5 kDa) were collected, freeze-dried, and checked for inhibitory activity.

Results and discussion

Isolation and identification of *L. pentosus* 22C

Strain 22C was isolated from traditional yogurt. The 16S rDNA sequence and carbohydrate fermentation pattern (data not shown) of the strain was typical of those of *L. plantarum* group. The phenotypic and genotypic characteristics of *L. pentosus, L. paraplantarum* and *L. plantarum* (*L. plantarum* group) are similar. The 16S ribosomal sequences of these species showed a very high level of homology [25]. The recA gene has been demonstrated to be a suitable phylogenetic marker for differentiation of species within this group [17]. The *recA* protein is responsible for homologous DNA recombination, SOS induction, and DNA damage-induced mutagenesis. The size of the amplicons are 318, 218, and 107 bp for *L. plantarum, L. pentosus*, and *L. paraplantarum*, respectively. The size of the *recA* amplicon was approximately 200 bp in strain 22C, which indicated that it was a *L. pentosus* strain (Fig. 1).

Safety evaluation of strain: genotypic and phenotypic tests

Lactobacillus pentosus has a long history of use for human nutrition [26]. The results of the present study showed that although most LAB strains are safe, virulence factors in bacteria isolated from traditional foods can be common. Virulence factors have contributed greatly to increasing the risk of infection with some bacteria [27]. *L. pentosus* 22C was found to harbor two virulence factors: *cylA* and *cylM* (Fig. 2c). Nevertheless, *L. pentosus* 22C did not show β-hemolytic activity. Lack of hemolytic activity could be due to absence of *cylB*, whose product is associated with cytolysin transportation [28]. The absence of β-hemolytic activities in LAB is an indicator of safety [29]. Cell wall adhesion genes ($efaA_{fs}$ and $efaA_{fm}$) are the predominant virulence genes detected in enterococci [30], and the $efaA_{fs}$ gene has been shown to play a pathological role in animal models [18]. *L. pentosus* 22C was found to possess the $efaA_{fs}$ determinant, similar to those in *Enterococcus* spp. (Fig. 2b). However, $efaA_{fs}$ has not yet been reported in lactobacilli. Microbial surface components recognizing adhesive matrix molecules have homologous domains in the S-layer-like protein YadA of Yersinia enterocolitica, collagen-binding protein CbsA of *Lactobacillus crispatus*, collagen-binding protein of *E. faecalis* (ace) and the collagen-binding adhesions CNA of S. aureus [31]. The presence of ace on the surface of *L. pentosus* 22C was investigated. The strain did not possess $efaA_{fm}$, *cylB*, or Esp_{fs} (Fig. 2a). Furthermore, the lack of gelatinase activity agrees with the absence of *gelE* in *L. pentosus* 22C. No detectable ornithine, lysine, tyrosine, or histidine decarboxylase activities were observed in phenotypic tests. Some strains of

Table 2 Antibiotic susceptibility profile of *L. pentosus* 22C

Antibiotics	Inhibition zone (mm)	Susceptibility
Ampicillin	32	S
Ciprofloxacin	18	S
Gentamicin	7	R
Kanamycin	15	S
Penicillin	28	S
Tetracycline	23	S
Vancomycin	17	S

Table 3 Inhibitory spectrum of *L. pentosus* 22C against indicator strains

Indicator strains	Antimicrobial activity	Diameter of inhibition zone (mm)
M. luteus	+	28
B. cereus	+	14
L. monocytogenes	+	11
S. aureus	+	15
E. coli	+	11

lactobacilli have been reported to produce harmful biogenic amines as a result of amino acid decarboxylase activity [32]. Further in vivo and in vitro studies are needed to clarify the potential health benefits and virulence risk factors of bacteria isolated from traditional foods, which may contain virulence factors despite their long history of use.

Antibiotic resistance

The absence of virulence factors alone does not guarantee the safety of lactobacilli. Antibiotic resistance can lead to an increased risk of infection [27]. The results of disk diffusion tests revealed that the strain was highly resistant to GMC. Previous studies have shown that several Lactobacillus strains are intrinsically resistant to GMC and to other aminoglycosides, probably due to cell wall impermeability, which raises no concerns about the potential for horizontal transfer of resistance factors to other bacteria [26]. Some researchers have identified links between a high incidence of resistance to GMC among lactobacilli and the use of this antibiotic on farms. However, highly GMC-resistant LAB strains that were used as a starter culture for the production of fermented sausages were reported [33]. *L. pentosus* 22C was found to be susceptible to most antibiotics, namely CIP, AMP, TE, P, K, and V (Table 2). Other studies have demonstrated the high rate of resistance to V among lactobacilli [34], and several genes associated with TE and erythromycin resistant have been reported in food-grade LAB. The increasing numbers of reports of antibiotic-resistant LAB are indicative of a major global health threat. There is an urgent need to prevent the transmission and spread of antibiotics resistance genes in LAB [35].

Antimicrobial activity

Determination of the inhibitory spectrum

A potential antimicrobial compound called pentocin 22C was identified in *L. pentosus* 22C. It showed antagonistic activity against gram-positive bacteria namely *M. luteus*, B. cereus, *L. monocytogenes* and *S. aureus*, and also against a gram-negative bacterium *E. coli*. These results are summarized in Table 3. The most sensitive of these to pentocin 22C was *M. luteus*. Inhibitory ability is important in the selection of LAB to be used in the food industry [11]. Pentocin 22C inhibited gram-positive and gram-negative bacteria. Generally, gram-negative bacteria are resistant to inhibitory compounds [36]. However, there are times when only gram-negative bacteria are needed to be inhibited [37]. To our knowledge, only pentocin ST12BZ [38] and pentocin CH2 [39], both produced by *L. pentosus* strains, are able to exert inhibitory activity against gram negative bacteria. In addition, the ability of pentocin 22C to control *S. aureus* and *L. monocytogenes* is important because of the enterotoxins produced and food poisoning by *S. aureus* and because of listeriosis, which is caused by *L. monocytogenes* [40].

Fermented dairy products provide a special environmental niche that is suitable for bacterial growth and antimicrobial compound production [41]. The release of active antimicrobial substances in fermented dairy products can contribute to dairy product preservation [42]. However, the compounds that inhibit pathogens, at the amount needed to inhibit them, and the producer culture should be safe for consumption.

Stability of pentocin 22C

The antimicrobial activity of pentocin 22C was found to be stable at pH levels from 2.0 to 10, and a high level of inhibitory activity was observed at pH 6.0 and 8.0. This property makes pentocin 22C suitable for use with alkaline-fermented foods and beverages. Antimicrobial activity was not decreased by heat treatment at 100 °C for 10, 20, and 30 min, but pentocin 22C activity was completely destroyed by treatment with trypsin. Activity remained after treatment with catalase, pepsin, and α-chymotrypsin. Stability in different conditions is a feature that makes antimicrobial compounds more attractive for various applications [43]. Thermal stability opens the possibility for an antimicrobial agent to be used as a bio-preservative in the dairy industry [44].

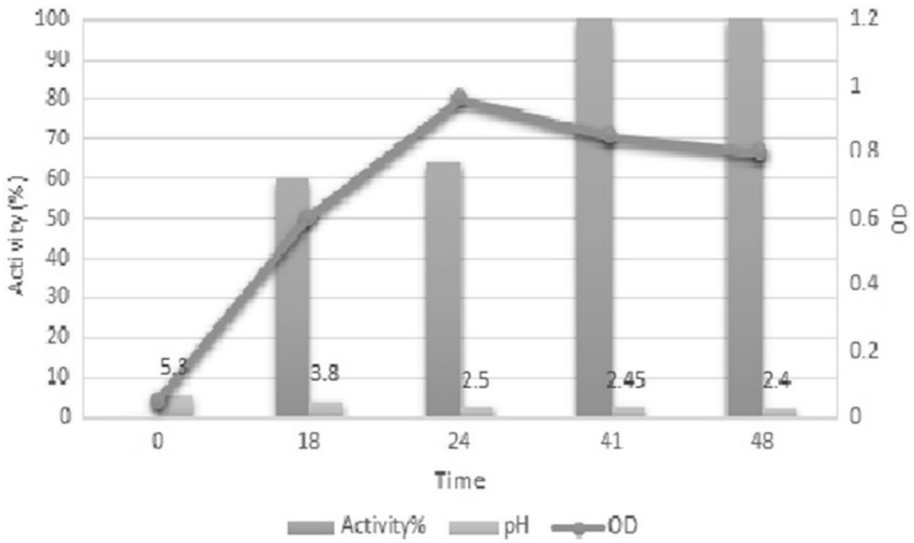

Fig. 3 Kinetics growth curves and antimicrobial activity of pentocin 22C. The growth curve study was done by absorbance measuring at 600 nm spectrophotometrically. The turbidometric method was used to check inhibitory activity at each time intervals

Antimicrobial agents are considered safe, but their application in the food industry is currently regulated because of concerns about the cancer risk of chemical preservatives [45]. Some bio-preservation techniques have been validated for use. These involve the introduction of strains with antimicrobial activity as protective cultures and the direct addition of purified or partially purified peptides as natural food additives to reduce or eliminate undesirable bacteria [46]. Inhibitory activity of purified peptides under thermal and pH stress conditions is an attractive feature for a bio-preservative [47].

Production and estimation of the molecular weight of pentocin 22C

Microbial growth, medium pH, and antimicrobial compound production were investigated over 48 h of growth at 37 °C (Fig. 3). During the study period, the OD of *L. pentosus* 22C increased from 0.02 to 0.8 (OD_{600}). A slight decrease in pH was observed with growth, and the pH of the medium dropped down to 2.4. A growth-dependent inhibitory effect was observed in the early logarithmic phase (18 h) and reached its maximum (100%) in the stationary phase (41 h). Inhibitory activity remained unchanged during the stationary phase. Antimicrobial activity was first detected after 18 h of incubation at 37 °C, which is similar to that found for pentocin TV35b produced by *L. pentosus* TV35b [15]. Furthermore, the molecular weight of the compound was estimated using a two-step purification procedure. An ammonium sulfate precipitation and ultra-filtration assay enabled partial purification of the active compound. An antimicrobial activity assay of the ultra-filtration fractions (greater than 10 kDa, between 5 and 10 kDa, and less than 5 kDa) indicated that the molecular weight of pentocin 22C is between 5 and 10 kDa.

Conclusions

In conclusion, virulence factors and antibiotic resistance were found in *L. pentosus* 22C isolated from traditional food. Hence, the safety of this strain is not clear. Strains with antimicrobial activity have the potential to be applied as starter cultures, co-cultures, or bio-preservative cultures to enhance food safety. Therefore, it is important to evaluate the safety of strains isolated from various products to determine their potential application to the food industry. The ingestion of a large numbers of resistant bacteria in dairy products can lead to significant health problems.

Moreover, antimicrobial compounds, purified or semi purified peptides, can be used as food preservatives. *L. pentosus* 22C produces an antimicrobial heat- and pH-resistant compound. The peptide inhibited not only gram-positive bacteria, but also *E. coli*. This purified peptide may has the potential to be used as a food preservative.

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