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Research article

Media Optimization and Partial Purification of Anti-Candida Compound of Probiotics Cultures

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purification

Abstract

Fungal infections in humans are mostly due to *Candida albicans*, an opportunistic commensal yeast that causes systemic and invasive candidiasis. Conventional treatment of candidiasis using antifungal compounds such as polyenes and azoles can cause various side effects. Besides, treatment becomes difficult as the organism acquires resistance. Probiotics with anti-candida activity have been projected as a safe and alternative solutions to treat candidiasis. Probiotic *Bacillus* isolates previously reported showed wonderful activity against pathogenic *Candida* species. This study reports on media optimization and partial purification of anti-candida compounds from probiotic *Bacillus* isolates MW3, MW9, MW19, and MW27. Maximum growth and anti-candida activity (2.9 cm inhibition zone) by the isolate culture was observed when media had been optimized with dextrose, casein and peptone. The media composition numbers 18, 4 and 19 showed the best growth for MW3, MW9, MW19 and MW27. Fractions obtained by solvent precipitation using acetone and methanol showed activity against the pathogen, and the fractions were further purified by ultra-filtration. After ultra-filtration, it was found that the size of the active compound was between 10 to 30 kDa. The compound lost its activity after treatment with proteinase K.

1. Introduction

Candida albicans is the most prevalent cause of fungal diseases [1, 2] which range from non-life-threatening oral thrush to life-threatening invasive diseases like candidemia. Globally, 75% of women have experienced *C. albicans* infection at some point in their lifetime, and 40-50% of these

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have recurrent infections [3]. These infections are mostly caused by dysbiosis in the gastrointestinal (GI) tract. Apart from an increase in the pathogenic count in the GI tract, dysbiosis reduces host immunity, which favors *C. albicans* evasion and infection. Polyenes, azoles, allylamines, morpholines, antimetabolites, and echinocandins are antifungal medications that have been conventionally used to treat *C. albicans* infections [4]. However, common medications like clotrimazole, miconazole, and fluconazole interfere with normal metabolic processes, cause allergic reactions, and are toxic to the liver and kidneys [5]. It has been well established that a balanced proportion of probiotics in the intestinal tract plays a vital role in preventing pathogens as well as maintaining the proper immunity of the host.

Probiotics are live microorganisms, which can be administered in adequate amounts to confer health benefits to the host [6, 7]. Different businesses, such as dairy/functional foods/dietary supplements and pharma, have formulations with various probiotic strains that offer specific human health advantages [8, 9]. *Lactobacillus* and *Bifidobacterium* are well-known probiotic microorganisms that have been linked to a variety of health benefits. In addition to these two genera, *Enterococcus*, *Streptococcus*, and *Bacillus* species have been described as probiotics with potential health benefits [10, 11]. *Bacillus* species, in particular, have been observed to resist the intense digestive action of bile salts due to their spore-producing ability [7, 11]. *Bacillus* spp. are preferred on an industrial scale because they are stable during numerous industrial processes. This species has a significant commercial value due to its ability to secrete vast amounts of extracellular compounds [12], as well as its antibacterial, anti-oxidant, and immunomodulatory properties [13-15]. Due to the increase in anti-fungus resistance, it is critical for developing therapeutic or prophylactic alternatives that have a distinct mechanism of action capable of inhibiting fungal infections without harming the host organism. Hence a probiotic *Bacillus* with anti-fungal activity offers an alternate option for candidiasis prevention and therapy. The composition of the medium has a strong impact on the bacteria's ability to create diverse extracellular components. This is accomplished by including the appropriate carbon and nitrogen sources at manageable concentrations. Carbon is essential for growth as well as the creation of primary and secondary metabolites. The rate of carbon metabolization is directly proportional to the rate of biomass creation and metabolite production [16]. While nitrogen is responsible for protein synthesis, it also serves as an energy source in the absence of carbon and is important in a variety of metabolic processes [16, 17].

Bacillus species have been found to produce a wide spectrum of antifungal and antibacterial proteins. *Bacillus* is non-pathogenic bacteria that can non-ribosomally generate a variety of short antibiotic peptides (<2000 kDa) with antifungal properties. *Bacillus* sp. can even generate a wide spectrum of lipopeptide antibiotics, which have been widely used in medicine and agriculture [18]. Production of antimicrobial peptides (AMPs) by *Bacillus* strains has been progressively studied in recent years, with several peptides produced by this group of bacteria discovered to be suitable for a variety of uses [19]. *Bacillus* has always had an advantage over *Lactobacillus* due to its ability to produce resistant spores, its creation of various antifungal compounds, particularly lipoproteins or bacteriocins, and the ability to manufacture vast numbers of proteins [20]. Anti-microbial peptides (AMPs) are naturally the first line of defense between the host and the environment since they have the potential to inhibit a wide range of microorganisms [21]. *Bacillus* species produce AMPs that have wide antimicrobial efficacy against harmful bacteria.

As a result, this study presents media optimization for the maximum anti-candida activity of previously identified probiotic anti-candida *Bacillus* cultures designated as MW3, MW9, MW19, and MW27 [22-24]. The partial purification and chemical characterization of an anti-candida molecule are also reported in this study.

2. Materials and Methods

2.1 Culture Collection

Bacillus isolates designated as MW3, MW9, MW19, (*Bacillus tequilensis*) and MW27 (*Bacillus subtilis*), previously isolated from Indian traditional fermented batter of Meduwada, were used for further analysis of their anti-candida components [22-24]. These cultures were grown on Sabouraud dextrose agar (SDA) slants and stored in a refrigerator for further use. *Candida albicans* (NCIM-3557) culture was obtained from NCIM and cultivated in SDA slants before being stored for future use.

2.2 Inoculum development

The inoculum was prepared by adding 24-h old cell suspension into a 100 ml conical flask containing 50 ml of sterile inoculum medium Sabouraud dextrose broth, (SDB). At 37ºC, the flask was kept under shaking at 120 rpm on a rotary shaker.

2.3 Media optimization experiments

The inoculum was centrifuged after 24 h, and the cell pellet was suspended in 100 mL of different production media to get 1 OD at 600 nm. The production medium contained only dextrose as a carbon source, and the nitrogen sources were the peptic digests of animal tissue and pancreatic digest of casein [25]. To optimize biomass output, several permutations and combinations of medium components were tested. As a result, three different concentrations of media components (Table 1) were added while the other two components remained unchanged, and the pH was adjusted to 6.5. The best combination was chosen based on the maximum bacterial growth measured as optical density at 600 nm after 24 h of incubation at 37ºC.

2.4 MIC

A 96-well plate was used to find out the MIC (minimum inhibitory concentration) of the supernatant after growing the culture in the optimized medium. The protein concentration of different culture supernatant was determined by the Lowry method and compared with standard BSA (Bovine Serum Albumin) [18]. The supernatant was then diluted to adjust the protein concentration from 256 µg/µL to 4 µg/µL by the double dilution method and was tested against *Candida albicans*. The experiment was carried out by first adding 100 µL of *Candida* suspension (0.5 OD at 600 nm) in SDB medium, followed by 100 µL culture supernatants produced in the optimized media. The plates were incubated at 37ºC for 3 h before being measured for optical density at 600 nm with an ELISA plate reader and compared to the positive (fluconazole 1 mg/mL) and negative controls (*Candida* without any inhibitor).

2.5 Anti-candida activity

The isolates cultivated in an optimized medium were tested for antimicrobial activity against *Candida albicans* NCIM 3557. The pathogen was spread on SDA plates (100 µL, 0.5 OD at 600 nm) and incubated for 10 min at 37ºC. An agar borer was used to create wells in agar and 20 µL of 24- h old grown culture supernatant was added to the wells before incubating at 37ºC for 24 h [26, 27].

Table 1. Composition of media optimization

Sr. No.	Peptic digestof animaltissue (g/L)	Pancreaticdigest of casein (g/L)	Dextrose(g/L)	Optical Density at 600 nm			
				MW3	MW9	MW19	MW27
1	2.5	2.5	10	0.042	1.188	1.1	1.155
2	2.5	2.5	20	0.056	0.673	0.608	0.831
3	2.5	2.5	30	0.052	0.04	0.025	0.934
4	2.5	5	10	0.029	1.278	1.134	0.859
5	2.5	5	20	0.016	0.498	0.381	0.579
6	2.5	5	30	0.027	0.082	0.066	0.147
7	2.5	7.5	10	0.016	1.169	0.852	1.218
8	2.5	7.5	20	0.098	0.425	0.141	0.741
9	2.5	7.5	30	0.078	0.014	0.12	0.691
10	5	2.5	10	0.047	1.073	1.145	1.117
11	5	2.5	20	0.028	0.067	0.582	0.869
12	5	2.5	30	0.048	0.019	0.034	0.416
13	5	5	10	0.098	1.11	1.135	1.204
14	5	5	20	0.028	0.561	0.161	0.853
15	5	5	30	0.015	0.165	0.064	0.379
16	5	7.5	10	0.015	1.063	1.07	1.215
17	5	7.5	20	0.037	0.122	0.241	0.28
18	5	7.5	30	0.451	0.119	0.144	0.576
19	7.5	2.5	10	0.008	1.07	1.159	1.24
20	7.5	2.5	20	0.007	0.545	0.111	0.928
21	7.5	2.5	30	0.045	0.03	0.088	0.563
22	7.5	5	10	0.016	0.973	0.858	1.007
23	7.5	5	20	0.046	0.301	0.119	0.235
24	7.5	5	30	0.324	0.059	0.248	0.216
25	7.5	7.5	10	0.289	0.831	0.78	1.176
26	7.5	7.5	20	0.242	0.361	0.109	0.821
27	7.5	7.5	30	0.264	0.017	0.114	0.491

2.6 Partial purification of anti-candida compound

The anti-candida compound from the supernatant was precipitated using three methods; i) equal proportion (10%) v/v of TCA and ice-cold acetone were mixed thoroughly with culture supernatant and incubated overnight at -20°C. ii) The culture supernatant was mixed thoroughly with 4 Vol of methanol followed by the addition of chloroform (1 Vol). After adding 3 Vol of distilled water, the mixture was then allowed to settle for 2-3 min before centrifuging at 6000 rpm for 2 m. The aqueous top layer was removed, and 4 Vol of methanol was added before centrifuging the mixture at 6000 rpm for 2 min. The precipitate formed were collected after removing the supernatant. iii) The culture supernatant was slowly mixed with an equal volume of acetone. The mixture was incubated at -20°C for 12 h and the pellets were collected by centrifugation at 7500 rpm at 4°C for 15 min. The pellets collected using the above methods were then air dried and washed twice with 1M Tris base buffer (pH 7). The pellets were re-suspended in a solution of 1M Tris base buffer (pH 7) and used for anti-candida activity by agar well diffusion method.

2.7 Effect of proteolytic enzyme and temperature on anti-candida activity

To check the proteinaceous nature of the anti-candida molecules, the supernatant was treated with an equal volume of proteinase K in phosphate buffer (pH 7) (1mg/mL) and incubated at 37°C, and the mixture was incubated for 2 h. Similarly, each supernatant was heated for 3 min at 80ºC to check the effect of temperature on the anti-candida activity. All samples were used for checking the anti-candida activity using the agar well diffusion method [28].

2.8 Ultra-filtration

The Sigma-Aldrich Amicon ultracentrifugal filter units, Ultra-15-MWCO-30kDa, 10kDa, and 4kDa, were used for ultrafiltration. *Bacillus* cultures were cultivated in an optimized medium for 24 h. The culture was then centrifuged twice at 10000 rpm for 10 min each time until the supernatant became cell-free. Each supernatant was then passed through 0.22 µm filter using a syringe filter. The filtrate was collected and ultra-centrifuged at 10000 rpm for 30 min using Ultra-15-MWCO-30 kDa. The filtrate of Ultra-15-MWCO-30 kDa was filtered with an Ultra-15-MWCO-30 kDa 10 kDa filter, and the resulting filtrate was then filtered using an Ultra-15-MWCO-4 kDa filter. Both the filtrates and retentates were tested for anti-candida activity using the agar well diffusion method.

2.9 Germ tube inhibition assay

Germ tube inhibition was performed according to Palande *et al*. [22]. *Candida* culture (100 µL) suspended in SDB, supplemented with 0.2% human serum was added to 96-well flat-bottom plates followed by the addition of 100 µL of culture supernatant or partially purified compound (acetone precipitated). The plate was incubated at 37°C for 4 h. *Candida* culture with saline was used as a negative control. After the incubation period, the medium in the wells were discarded, and each well was washed with 70% ethanol followed by 25% SDS and twice with distilled water. Crystal violet (0.1%) 200 µL was added to each well and allowed to react for 10 min. After the crystal violet treatment, the wells were washed twice with D/W (distilled water) and then with 0.25% SDS. The remaining crystal violet was eluted with 200 µL of a mixture of isopropanol with 0.04 N HCl and 50 µL of 0.25% SDS. Using an ELISA plate reader, the absorbance was measured at 590 nm.

2.10 SDS-PAGE

Different fractions collected in the ultra-filtration step and the cell-free supernatants were subjected to SDS- PAGE, with 12% and 5% acrylamide as the resolving gel and stacking gel, respectively. With the current flow of 120 V after the run, the gel was exposed to Coomassie staining for visualization.

3. Results and Discussion

3.1 Media optimization for anti-candida activity

The influence of nitrogen and carbon sources on the growth rate of cell production was evaluated using different production mediums [28]. The results revealed that the eupeptic digest of animal tissue, pancreatic digest of casein, and dextrose were good sources for growing *Bacillus* species. A total of 27 combinations were prepared for the experiment (as shown in Table 1) to check the growth of *Bacillus* species. Each culture produced a different pattern of growth in different media. It was

observed that except for MW3, all cultures showed the highest growth when the dextrose concentration was at a minimum (10 g/L). Hence, we can conclude that glucose concentration plays a major role in controlling the growth of these three cultures. Carbon is a major constituent in the culture media. Optimum concentration of glucose is essential for optimum growth, thus an excess carbon source can lead to a different mode of growth in the microorganisms. A similar condition was observed by Stamenković-Stojanović *et al*. [29] with *Bacillus subtilis* culture. However, with respect to the peptic digest of animal tissue (peptone) and pancreatic digest of casein, cultures showed a different pattern. For MW 3, the maximum growth was observed when the peptone and pancreatic digest of casein were highest (7.5 g/L), and for MW 9, the concentrations that gave maximum growth were 2.5 and 5 g/L. MW 19 and 27 showed maximum growth with 7.5g/L peptone and 2.5 g/L pancreatic digests of casein. Media optimization is important to check for the enhancement of the production of biomolecules responsible for inhibiting pathogenic bacteria [30]. Accordingly, the maximum growth for MW3 was observed in formulation 18, and for MW9, it was seen in formulation 4. However, MW19 and MW27 showed maximum growth in formulation 19.

3.2 MIC and anti-candida activity

The MIC values of each supernatant were measured by comparing the optical density (OD at 600 nm) of *Candida* culture exposed to different dilutions of culture supernatant after growing them in the optimized medium. The OD was measured at 0 h and 3 h of incubation at 37ºC using an ELISA plate reader at 600 nm. The MIC of different cultures is shown in Table 2. The supernatant (S) and the *Bacillus* culture were tested against *Candida* cultures. The results showed that the MW3, MW9, MW19, and MW27 cultures were able to inhibit *C. albicans* NCIM 3557 with inhibition zones of 0.6 cm, 0.8 cm, 1.0 cm, and 1.5 cm, respectively (Figure 1). The lowest concentration of an antimicrobial compound that inhibits the visible growth of a microorganism is an important step to avoid the development of antibiotic resistance [31].

3.3 Effect of proteolytic enzyme

The supernatants of the MW27, MW3, and MW9 cultures completely lost their anti-candida activity when they were treated with proteolytic enzyme, while the supernatant of the MW19 culture showed a reduction in anti-candida activity from a 1.5 cm zone of inhibition to 0.6 cm. This indicates that the activity of MW27, MW3, and MW9 may be due to protein-based inhibitors and MW19 may include both protein and other types of molecules (Figure 2). Moreover, supernatants MW19 and MW9 did not lose activity even after treatment with high temperature, indicating the heat stability of the anti-candida molecule. Antimicrobial proteins produced by the cultures are excellent candidates to improve the safety of probiotics. Some bacteriocin-like compounds are sensitive to proteolytic enzymes but resistant to heat at 80ºC [32, 33] (Figure 2).

Table 2. MIC of *Bacillus* cultures on selected media formulations

Culture	MIC (Concentration of proteins, µg/µL)
MW3	64
MW9	32
MW19	64
MW27	128

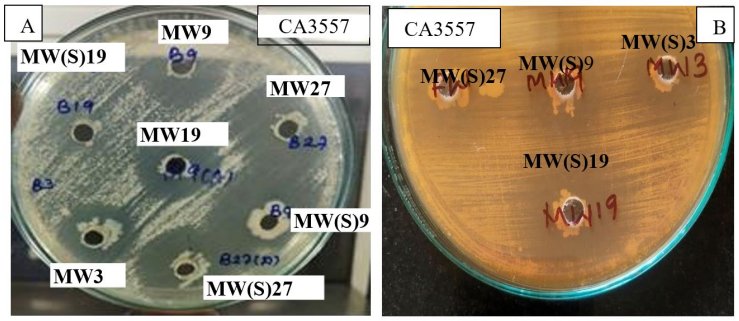

Figure 1. Inhibition of *C. albicans* NCIM 3557 by *Bacillus* cultures
(A) MW3, MW9, MW19, and MW27 showed zones of inhibition of 0.5 cm, 0.6 cm, 0.7 cm, and 1.2 cm against *C. albicans* NCIM 3557. (A and B) Negative control supernatant, (S): MW(S)9, MW(S)19, and MW(S)27 showing zones of inhibition of 0.8 cm, 0.2 cm, and 0.9 cm against *C. albicans* NCIM 3557

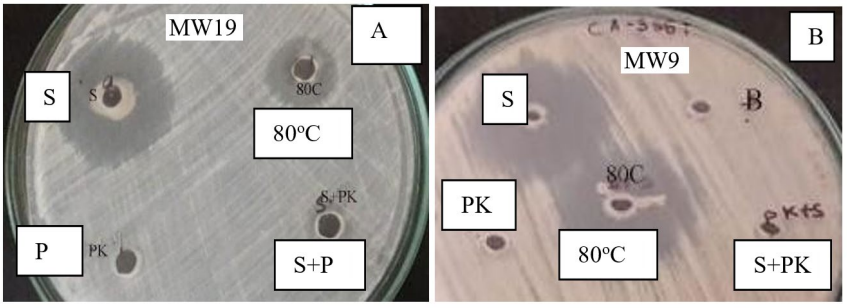

Figure 2. Inhibition of *C. albicans* NCIM 3557 by supernatant of MW19 and MW9 before and after treatment, (A) *Bacillus* MW19 and (B) MW9 with the effect proteinase K and temperature against *C. albicans* NCIM 3557
(S: Supernatant, PK: proteinase K, S+P: Supernatant with proteinase K)

3.4 Solvent precipitation method

MW19 supernatant precipitated with different solvents, acetone, methanol, and a mixture of methanol with chloroform precipitates showed activity against *Candida* species. Methanol precipitate showed 0.6 cm inhibition zone, the mixture of methanol with chloroform precipitate showed 0.3 cm and acetone precipitate showed 0.5 cm zone of inhibition (Figure 3).

3.5 Partial purification of protein by ultrafiltration method

The ultra-filtration results showed that the filtrates of 30 kDa and 10 kDa retentate has anti-candida activity. The zone of inhibition was 0.7 cm for the 30 kDa retentate and 0.5 cm for the 10 kDa retentate. There was no activity found in the 10 kDa filtrate and 4 kDa filtrate, as well as in the retentates. This indicates that active molecule was retained between the 30 to the 10 kDa fractions (Figure 4).

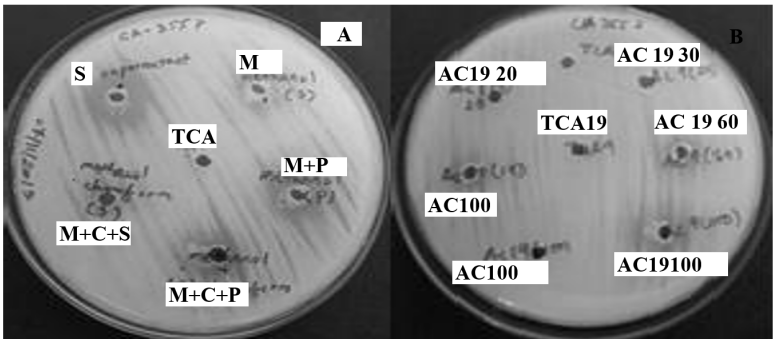

Figure 3. Zone of inhibition of *C. albicans* NCIM 3557 by various fractions of solvent precipitation
(A) MW19S: Supernatant, M: Methanol, MW19 M+P: Methanol precipitated MW19M+C+P: Methanol chloroform precipitate, MW19M+C: Methanol and TCA
(B) AC19 20: Acetone (20%) with precipitated of 19, AC19 30: Acetone (30%) with precipitated of 19, AC19 60: Acetone (60%) with precipitated of 19, AC19 100: Acetone (100%) with precipitated of 19, AC100: Acetone (100%)

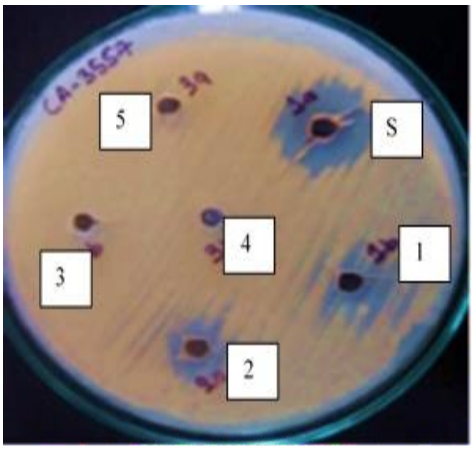

Figure 4. Zone of inhibition of *C. albicans* NCIM 3557 by different ultrafiltration fraction
S: supernatant, 1: 30 kDa retentate, 2: 10 kDa retentate, 3: 10 kDa filtrate, 4: 4 kDa retentate, and 5: 4 kDa filtrate

3.6 Germ tube inhibition

Germ tube formation is a very important stage in which the *Candida* culture is in the virulence stage, and so it is important to inhibit the formation of germ tubes in *C. albicans*. Germ tube inhibition assays were carried out with bacterial suspension precipitates and extracted compounds. The bacterial suspensions of MW9, MW19, MW27, MW3, and the acetone precipitates, methanol precipitates, extracted compounds, and supernatants were capable of maximum inhibition of germ tube formation (Table 3).

Table 3 Germ tube inhibition percentage

MW3	MW9	MW19	MW27	Extracted compound		Acetone	Methanol	Clotrimazole	Ethanol
				<10kDa	<30kDa				
97%	99%	99%	99%	89%	80%	77%	77%	30%	0%

3.7 SDS PAGE

By visual inspection of the patterns of proteins, all the examined partially filtered compound bands were observed. Although the different fractions have shown anti-candida activity, the SDS PAGE (Figure 5) showed that the anti-candida molecule may be between 30 to 10 kDa size. Further studies are required to understand the actual molecular weight of the anti-candida molecule.

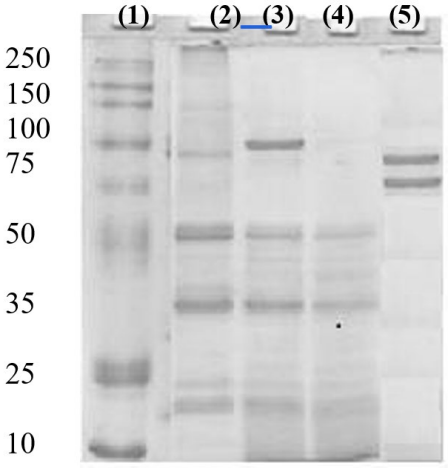

Figure 5. SDS PAGE of cell free supernatant of *Bacillus* culture after partial purification Well (1) ladder: contains the perfect protein marker from 10 kDa -250 kDa, Well (2) 30 kDa retentate, Well (3) 30 kDa filtrate, Well (4) 10 kDa retentate, and Well (5) un-inoculated media

4. Conclusions

The main contribution of this study was the design of cost-effective production media that could be explored for use by industries for the production of probiotic *Bacillus* culture with anti-candida activity. The growth media containing lower carbon content showed high viable counts and were suitable for culturing *Bacillus* isolates. The media composition number 18 showed the best growth for MW3, while composition number 4 showed the best growth for MW 9. Composition number 19 showed the best growth for MW19 and MW27. A lower sugar concentration in the medium produced a substantial increase in cell concentration. Bacterial growth in the selected media was similar to or better than in MRS and SDB. Moreover, the active ingredient was partially purified using solvent precipitation and ultra-filtration methods after growing the culture in the optimized medium. The activity of the compound produced by MW19 was stable even after proteinase K and high-

temperature treatment. Even the partially purified compound showed anti-candida activity and could inhibit the formation of germ tubes in *Candida albicans.* Ultra-filtration results indicated that the active compound was between 30 to 10 kDa in size. However, further studies are required to more precisely calculate the molecular weight of the active molecule.

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