CHAPTER 6

INHIBITION OF FOOD SPOILAGE ISOLATE *CANDIDA TROPICALIS* BSS7 EXPRESSING VIRULENCE FACTORS IN A FRUIT JUICE MODEL USING *LACTOBACILLUS*- DERIVED EXTRACELLULAR METABOLITES

Inhibition of food spoilage isolate *Candida tropicalis* BSS7 expressing virulence factors in a fruit juice model using *Lactobacillus*- derived extracellular metabolites

6.1. Abstract

The contribution of yeasts and moulds in food- borne diseases has so far been underestimated. The present study deals with a *Lactobacillus*- mediated approach for the inhibition of food spoilage isolate *Candida tropicalis* BSS7 showing potential virulent properties like biofilm and germ tube formation. Cell-free extract of *Lactobacillus paracasei* D6 exhibited minimum biofilm inhibitory concentration of 0.438 mg/ml against *C. tropicalis* BSS7, which was found to be sufficient to inhibit its germ tube formation. The adhesion of *C. tropicalis* BSS7 to the epithelial Caco-2 cell line was also significantly reduced by the antifungal metabolites. GC-MS analysis of the exometabolites revealed the abundance of 6 different compounds with potential antifungal properties. An optimal combination of inoculum 3.67 log CFU/ml, 1.75 mg/ml antifungal metabolite and 1.46 min of heat treatment led to the maximum inhibition of *C. tropicalis* in fruit juice. This is the first report of inhibition of virulence factors produced by *Candida* using antifungal metabolites of *Lactobacillus* in an *in vitro* model of intestinal epithelia. Moreover, the model for synergistic effect of *Lactobacillus*- derived antifungal metabolites and temperature treatment on *Candida* inhibition also gives an idea about an alternative strategy for the prevention of food spoilage.

6.2. Introduction

Food spoilage is detrimental to the food industry that significantly affects the cost and availability of food [1]. *Candida tropicalis* is responsible for the spoilage of fruit juices [2] and acts as a biofouling agent in fruit juice processing plants [3].

Different species of *Candida* have been investigated for their biofilm formation abilities on various biotic and abiotic surfaces. Both biofilm and germ tube formation are very important virulence factors expressed by different *Candida* sp. isolated from clinical specimens. However, *Candida* sp. isolated from spoiled foodstuffs has never been investigated for the abovementioned factors. There are few reports where it was established that there is a homology between food- borne yeasts

and their clinical counterparts in terms of biochemical profile and there is a chance of high-frequency plasmid DNA transfer between pathogenic and food- borne yeasts while grown together [4, 5]. *Candida* showing putative virulent properties like biofilm and germ tube formation can evade phagocytosis and promote invasion of host epithelial cells [6].

The use of chemotherapeutic agents has been monitored since last two decades, but due to the varying epidemiology of *Candida* infections and the development of antifungal resistance, replacement of conventional antifungal drugs with alternative therapeutic agents like lactic acid bacteria has become a necessity in the present day scenario. Lactic acid bacteria grow in nutrient-rich environments and are reported to produce antifungal metabolites [7, 8]. They can be considered as bioprotective cultures since they produce antifungal compounds which inhibit various fungi in a synergistic manner without changing organoleptic properties of a food [9]. Moreover, lactic acid bacteria achieved the Generally Regarded as Safe (GRAS) status and are included in the qualified presumption of safety (QPS) list [10].

Different fermented bamboo shoot (FBS) products are consumed by the ethnic people living in the biodiversity- rich North-East region of India including Arunachal Pradesh which provide an optimum climate for the growth of many edible bamboo species. *Ekung* is such a product which is prepared by the *Nyshi* community of Arunachal Pradesh that employs their unique methods of fermentation [11]. The presence of LAB in FBS was previously reported [12]. However, health- beneficial effects of LAB present in these products are not yet completely understood. The present study deals with the anticandidal effects of *Lactobacillus paracasei* D6 and its exometabolites. This is the first report of inhibition of virulence factors produced by *Candida* using antifungal metabolites of *Lactobacillus* in an *in vitro* model of intestinal epithelia.

6.3. Material and methods

6.3.1. Isolation of microbial strains and culture conditions for different strains used

The strain BSS7 was isolated from spoiled commercial orange juice and was routinely grown in potato dextrose agar (PDA) at 28°C.

Fermented bamboo shoots (*ekung)* were collected aseptically in sterile containers and stored at 4°C. 1g of sample was blended with sterile 0.85% (w/v) saline solution using mortar and pestle under aseptic conditions and 10 fold serial dilution was performed. Different dilutions were spread on de Man, Rogosa and Sharpe (MRS) agar (Himedia Labs, Mumbai) and incubated at 37°C under anaerobic conditions for 24 h. Gram positive and catalase negative strains [13] were selected for further evaluation.

Candida albicans MTCC 3017, which was used as a biofilm- positive strain, was grown in PDA at 28°C. It was procured from The Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh.

6.3.2. Molecular identification

The isolate D6 was identified by 16S rDNA gene sequencing followed by phylogenetic tree construction. Universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used for the amplification of 16S rRNA gene sequence [14].

For the identification of the strain BSS7 Sequencing of ITS1-5.8S rDNA intergenic region was done using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [15]. Sequencing of D1/D2 region of 26S rRNA was also done using the primers: NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTC AAGACGG-3′) [16].

Amplification of the 16S rRNA gene was done using a previously reported protocol developed in Applied and Industrial Microbiology Laboratory, Tezpur University [17]. Amplification parameters for the ITS1-5.8S rDNA intergenic region and the D1/D2 region consisted of an initial denaturation step of 3 min at 95ºC, followed by 30 cycles of 95ºC for 30 sec, primer annealing for 30 sec at 58ºC, elongation for 1 min at 72ºC, and final extension of 10 min at 72ºC for 1 cycle. The amplified PCR products were purified and subjected to automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Rotkreuz, Switzerland). The sequence was analyzed using BLAST algorithm (http:// www.ncbi.nlm.nih.gov/blast) and was submitted to the NCBI GenBank (http:// www.ncbi.nlm.nih.gov/genbank).

The phylogenetic tree was constructed by neighbor- joining (NJ) method using MEGA 5.05 software [18].

6.3.3 Extraction of antifungal metabolites (AFMs) from *Lactobacillus paracasei* D6 spent broth

A method reported by Rizzo [19] was used to extract antifungal metabolites (AFMs) from cell free supernatant of D6 with some modifications. Cells were separated from the spent broth by centrifugation at 3000 g for 15 min, and filter sterilized (0.45-µm pore size; Millipore). 10 ml of the spent broth culture filtrate was taken and 4 g of NaCl and 2 ml of 50% H_2SO_4 were added. This solution was then extracted with 5 ml of diethyl ether and concentrated. Uninoculated MRS broth was used as a control.

6.3.4. Antifungal assays

For the determination of antifungal activity of the strain D6 agar overlay method reported by Schnürer and Magnusson [20] was employed. D6 was streaked on to MRS agar plate as two 3 cm long lines and incubated at 37ºC for 3 days. The plate was overlaid with potato dextrose agar (PDA) containing 10^4 *candida* cells per ml and incubated at 30ºC.

Minimum inhibitory concentration (MIC) values for the AFMs were determined according to a poison food technique described by Sridhar et al. [21] with some modifications. AFMs were mixed with molten Czapek Dox Agar (CDA) at various concentrations and *Candida* was spotted onto the agar surface. Minimum concentration of AFM that resulted in no visible mould growth after 72 h of incubation would be considered as MIC. In a different set of experiment, susceptibility towards amphotericin B, a standard antifungal drug was also evaluated using the same protocol.

6.3.5. Antibiofilm assay and inhibition of germ tube formation

Bacterial cells were diluted with Phosphate buffered saline (PBS, pH 7.4) to 10^7 CFU/ml and an aliquot of 20 µl was transferred to the wells of a 96 well microtiter plate containing 180 µl of potato dextrose broth (PDB) and allowed to grow for 48 h at 28ºC. Different concentrations of AFMs (µg/ml) were previously added to PDB and PDB without AFM was used as control. Following incubation, wells were rinsed three times with sterile de-ionized water and the plates were air-dried for 45

min, and then each well was stained with 200 µl of 0.1% (w/v) crystal violet for 30 min [22]. Crystal violate bound cells were solubilized by 100% (v/v) ethanol and absorbance was taken at 570 nm. Bifilm inhibition was calculated as

Biofilm inhibition= $[(O.D._{.control} - O.D._{.test})/O.D._{.control}]\times 100$

Minimum biofilm inhibitory concentration (BIC_{50}) was defined by the minimum concentration that resulted in 50% inhibition of biofilm formation [23].

For the visualization of germ tube formation *Candida* strains were grown in spider medium (1% nutrient broth, 0.4% potassium phosphate, and 2% mannitol) containing 10% (w/v) fetal bovine serum (FBS) with or without the presence of BIC_{50} of AFM on 24 well microtiter plates. Plates were incubated at 37ºC for 36 h [24]. Morphological transition of rounded cells of *Candida* into germ tube bearing structures was observed under light microscope at 40X magnification.

6.3.6. Caco-2 cell culture and inhibition of *Candida* adhesion to Caco-2 cell line by AFM

The human colon adenocarcenoma (Caco-2) cell line was procured from National Centre for Cell Science (NCCS), Pune and grown in minimum essential medium (MEM, St. Louis, MO, USA) supplemented with 10% (w/v) fetal bovine serum (Invitrogen, Gibco), 1% (v/v) nonessential amino acids (Invitrogen, Gibco) and 50 µg/ml gentamicin (Invitrogen, Gibco). The cells were routinely grown at 37˚C in a humified atmosphere of CO_2 (5%) until confluent growth was obtained.

A method reported by Murzyn et al. [25] was employed for the inhibition of *Candida* adhesion to Caco-2 cell with some modifications. Briefly, Caco-2 cells were seeded on a 96 well tissue culture plate at a concentration of $2.0 – 2.5 \times 10^4$ cells per well. Following confluence, cells were washed with PBS and replenished with fresh MEM medium supplemented with 2% FBS (without antibiotics). Freshly grown *Candida* cells were harvested by centrifugation and washed with PBS. For the adhesion assay, experimental set up consisted of (1) Pure Caco-2 cell, (2) Caco-2 cells + *C. tropicalis* BSS7 (control), (3) Caco-2 cells+ *C. albicans* MTCC 3017 (control), (4) Caco-2 cells + *C. tropicalis* BSS7 + BIC_{50} of AFM (test) and (5) Caco-2 cells + *C. albicans* MTCC 3017 + BIC_{50} AFM (test) in separate wells. All wells contained *Candida* cells at a concentration of 2.0×10^6 CFU/ml, except for the wells containing

pure Caco-2 cells which were not inoculated with *Candida*. Adhesion experiment was performed for 3 h at 37 ºC followed by washing with PBS to remove the unattached cells and fixing with 4% (w/v) *p*- formaldehyde. Cells were visualized under light microscope (EVOS FL cell imaging system, ThermoFisher) under 20X. For the quantitative assessment of adhesion, cells were stained with 0.5% (w/v) crystal violet and absorbance was taken at 595 nm.

6.3.7. Identification of AFMs

Extracted AFMs were subjected to gas chromatography - mass spectrometry (GC–MS) analysis [19] using The Agilent 7890A Gas Chromatograph and 240-MS Ion Trap with Column Length 30 m, Diameter 0.320 mm, Film 0.25 µm and temperature range: 60 - 325 ºC. The gas chromatograph oven was temperature programmed and maintained at 60 ºC for 2 min, followed by 115 ºC with a rate of 15 ºC per min and held for 15 min. All the major metabolites were identified by comparing with mass spectra obtained from commercial library.

6.3.8 Inhibition of *Candida* in fruit juice

Apple and oranges were purchased from local market (Napaam, Tezpur) and juices were made separately in mixture grinder before mixing them in 1:1 ratio. In order to find out the optimum conditions for *Candida* inhibition an experiment was designed using various parameters previously reported by Bukvicki et al. [26]. Box- Behnken model in RSM (Design- Expert version 6.0.2, Stat-Ease Inc., Minneapolis, MN, USA) was applied for designing the experiment with 3 independent variables and 3 levels, i.e. *Candida* inoculums (2, 3 and 4 log CFU/ml), MIC, ½ MIC and ¼ MIC of FAM and thermal treatment (0.5, 1.0 and 1.5 min) for 70 $^{\circ}$C. Highest, intermediate and lowest levels were denoted by the codes 1, 0 and -1 respectively. Data obtained was fitted in to a quadratic polynomial equation as given below:

$$Y= b_0 + b_1A + b_2B + b_3C + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{12}AB + b_{23}BC + b_{13}AC \qquad (1)$$

Where *Y* is the predicted response (inhibition), b_0 is a constant, b_1, b_2 and b_3 are linear regression coefficients, $b_{11,}$ b_{22} and b33 are squared coefficients, b_{12}, b_{23} and b_{13} are interaction coefficients and A, B and C are independent variables (inoculum,

concentration and treatment time respectively). The relationship between the independent variables and the response was observed by response surface graphs.

6.4 Results and discussions

6.4.1 Isolation and identification of strains

The virulence patterns shown by the pathogens have profoundly changed during the past few decades. For instance, the non- *albicans Candida* spp. once considered as non- pathogenic contaminating agents have emerged as potential pathogens [27]. *Candida tropicalis* is a widespread yeast species and there is a dearth of knowledge regarding the virulence attributes of *Candida* sp. isolated from non- clinical sources. In our study, the identity of the strain *C. tropicalis* BSS7 was confirmed using 26S rDNA sequencing which is considered as the most consensus approach for the identification of yeasts [28]. Based on the colour formation on chromogenic medium (Fig. 6.1A), the strain BSS7 was presumptively identified as *Candida tropicalis*. Molecular typing of the strain is shown in the Fig. 6.1B, 6.1C and 6.1D. In this study, sequencing of both the ITS1-5.8S-ITS2 region and D1/D2 region of 26S rDNA reveals that the sequences have maximum identity to *Candida tropicalis*. After submission to NCBI GenBank, the sequences received accession numbers KT387284 and KT387283 respectively.

The gram positive and catalase negative strain D6 was identified by 16S rDNA sequencing and the sequence was used to construct the phylogenetic tree (Fig. 6.2). The BLAST analysis showed 99% similarity with the 16S rDNA sequence of *Lactobacillus paracasei* strain RU4-1 16S. This sequence received an accession number KJ867173.

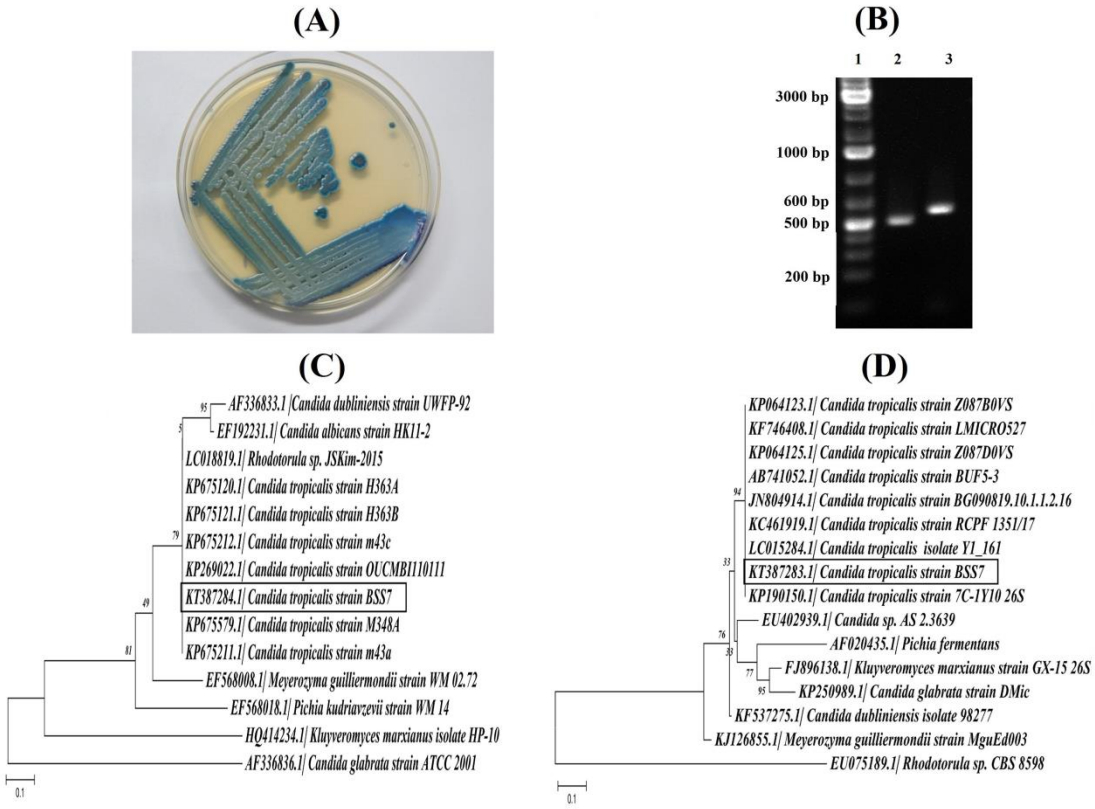

Fig. 6.1. Identification of the strain *C. tropicalis* BSS7. (A) Colony morphology in Hichrome *Candida* differential agar. (B) Molecular typing using ITS (internal transcribed spacer)-primer (Lane 2) and 26S rRNA gene D1/D2 primer (Lane 3); Lane 1 indicates GeneRuler 1 Kb Plus DNA ladder (Fermentas). Phylogenetic tree showing the position of BSS7 with closely related species based upon (C) 5.8S ITS rRNA and (D) D1/D2 26S rRNA sequences. Bootstrap values (1,000 replicates) are indicated at branch nodes.

6.4.2. Antifungal assays

The use of probiotics and probiotic- derived products as antibiofouling agents has gained immense attention in food industries. Many lactic acid bacteria can inhibit the growth of yeasts and moulds due to the production of acids, volatile fatty acids, cyclic dipeptides, diacetyl, phenyllactic acid etc. [20]. Antifungal activity on agar overlay plates is shown in the Fig. 6.3A and 6.3B for C. *tropicalis* BSS7 and *C. albicans* MTCC 3017 respectively. Antifungal activity was confirmed by the production of halos around streaks. The strain *L. paracasei* D6 which is producing antimicrobial substances led to the inhibition of *Candida* strains around the streaks. The minimum inhibitory concentration (MIC) of the AFM was found to be 1.75 mg/ml for *C. tropicalis* BSS7 and 3.5 mg/ml for *C. albicans* MTCC 3017 respectively (Fig. 6.3C and 6.3D). Both C. *tropicalis* BSS7 and *C. albicans* MTCC 3017 were also found to

be susceptible to the antifungal drug amphotericin B and MIC values were found to be 0.195 µg/ml and 0.390 µg/ml respectively.

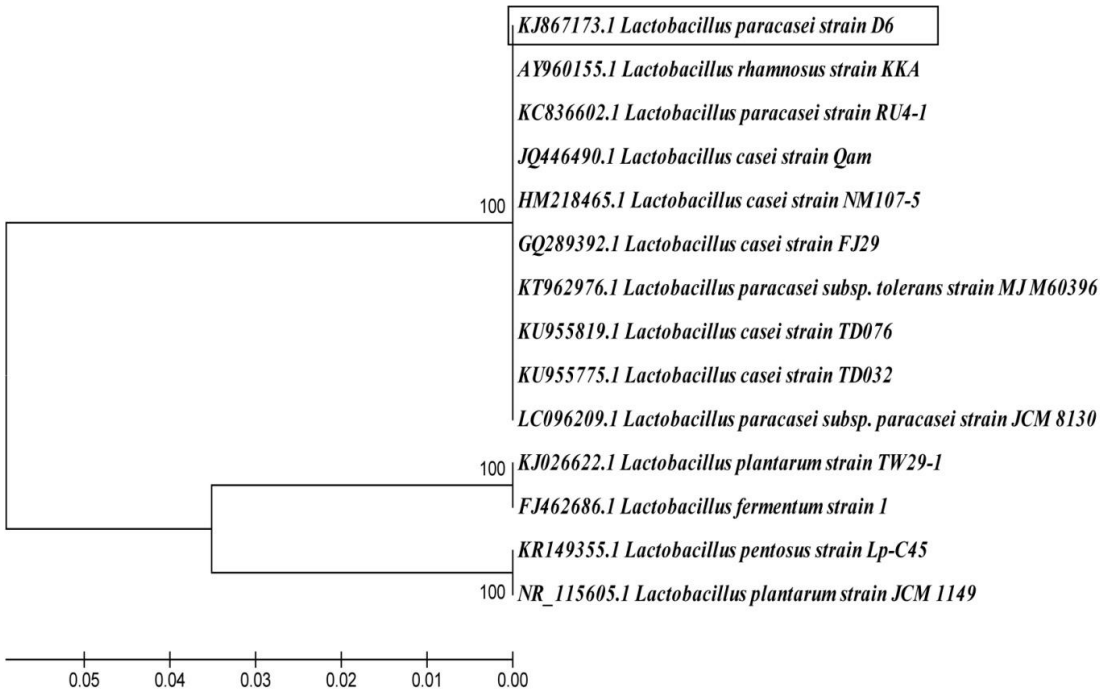

Fig. 6.2. Phylogenetic tree showing the position of *L. paracasei* D6 with closely related species based upon 16S rDNA sequence. Bootstrap values (1,000 replicates) are indicated at branch nodes.

The MIC values of AFMs were comparable to the MIC values reported by Nyanzi et al. [7]. These MIC values were found to be less than that of 3-phenyllactic acid, an antifungal compound of lactic acid bacteria origin [29]. On the other hand, the MIC values of the most abundant compound lactic acid were found to be much higher (data not shown). This suggests synergistic anticandidal activities of different antifungal metabolites present in our extract.

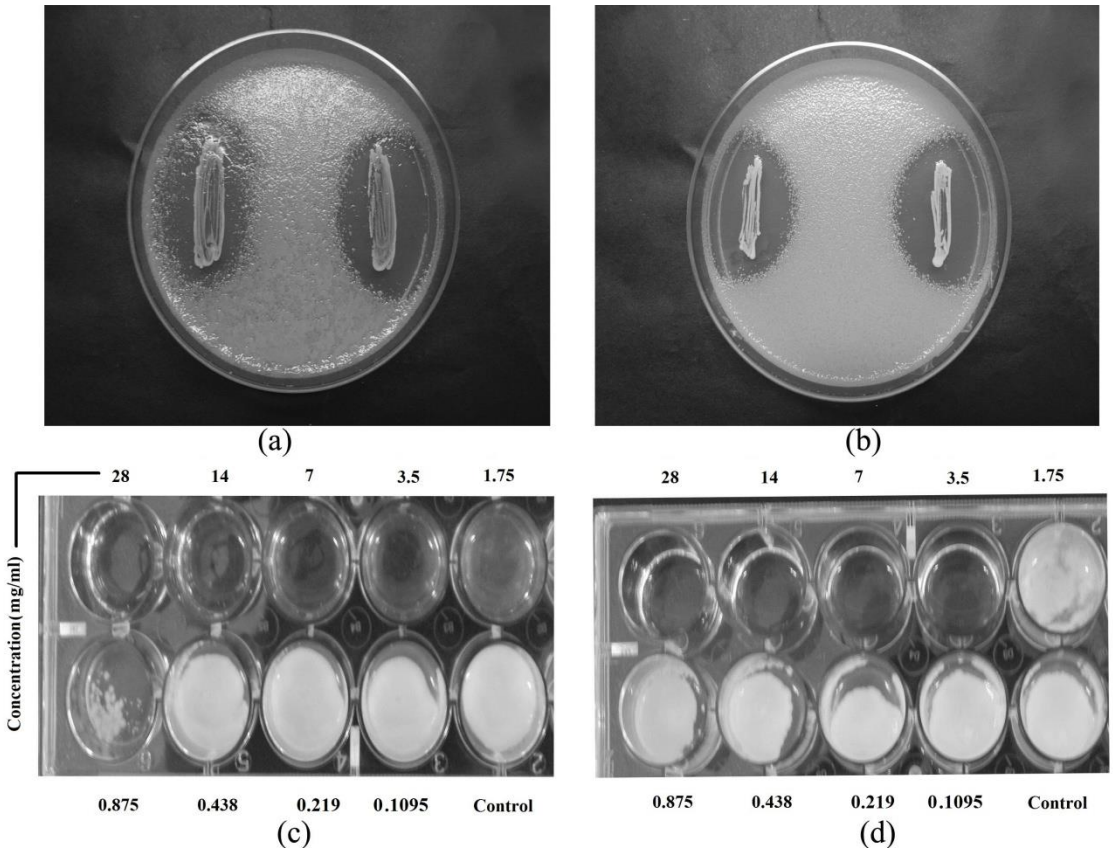

Fig. 6.3. Antifungal activities of *L. paracasei* D6 against (A) *C. tropicalis* BSS7 and (B) *C. albicans* MTCC 3017 visualized as halos in agar overlay method. MIC values of *Lactobacillus* metabolites against (C) *C. tropicalis* BSS7 and (D) *C. albicans* MTCC 3017 respectively.

6.4.3. Antibiofilm assay and visualization of hyphal transition inhibition

The transition of *Candida* cells from yeast like morphology to germ tube and hypha bearing structure is essential for biofilm formation because the hyphal cell wall contains proteins which aid in adhesion to different surfaces and ultimately biofilm formation [30]. Therefore it can be inferred that biofilm inhibition mechanism predominantly lies in yeast- hyphal transition inhibition. The minimum concentration for biofilm inhibition (BIC_{50}) of AFM against *C. tropicalis* and *C. albicans* were calculated as 0.438 mg/ml and 0.875 mg/ml respectively as shown in the Fig. 6.4A. These concentrations were found to be lower than the minimum inhibitory concentrations (MIC) for both the species. The exometabolites of lactic acid bacteria are shown to exhibit anticandidal effects that inhibit the adhesion of *Candida albicans* [31]. In our experiment, qualitative observation under light microscope (20X) also confirmed that BIC_{50} concentrations loosen biofilm cells resulting in dispersed growth

(Fig. 6.4.D and 6.4.E) as compared to the control (Fig. 6.4B and 6.4C). BIC_{50} concentration of AFM was found to be sufficient for the germ tube formation in *Candida* as shown in the Fig. 6.5. Inhibition of *Candida* germ tube development by compounds isolated from cell free supernatant of lactic acid bacteria has been previously reported [32, 33].

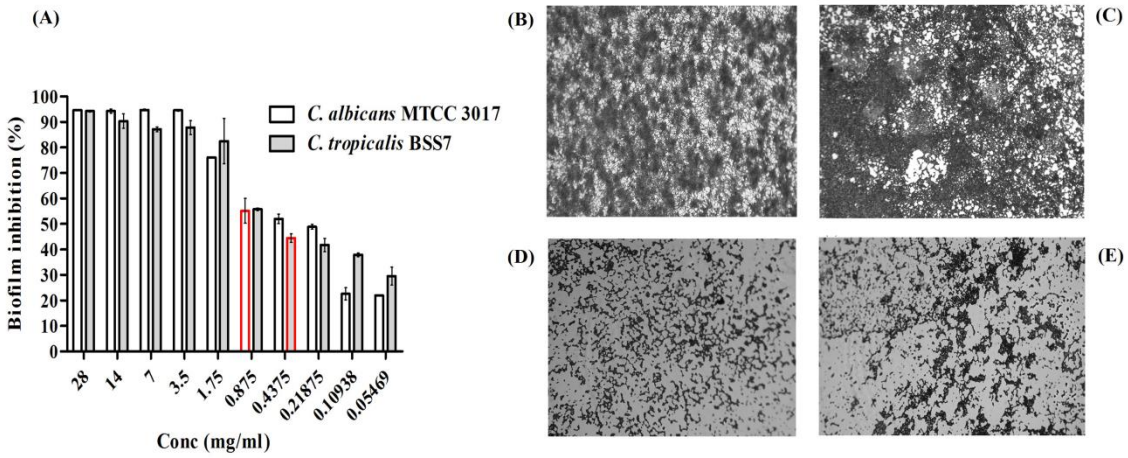

Fig. 6.4. Biofilm inhibition assay. (A) Biofilm inhibition by various concentrations of *Lactobacillus* metabolites; bars with red borders indicate BIC_{50}. Biofilm formation by *C. tropicalis* BSS7 on microtitre plates (B) without treatment and (D) treatment with BIC_{50}. Biofilm formation by *C. albicans* MTCC 3017 (C) without treatment and (E) treatment with BIC_{50} as observed under light microscope (20X).

6.4.4 Inhibition of *Candida* adhesion to Caco-2 cell line

Dalle et al. [34] reported that the propagation of *Candida albicans* was largely due to the translocation of fungal cells from the gut to the bloodstream and initial propagation is mediated by the adhesion of *Candida* cells to the enterocytes. Moreover, the alkaline pH prevalent in the small intestine may act as a mediator for the colonization of *Candida,* since environmental pH influences the process of biofilm formation and colonization [35].Therefore, adhesion inhibition of *Candida* to epithelial cell is the first step towards minimizing *Candida*- related infections. Fig. 6.6 illustrates *Candida* adhesion to Caco-2 cells in presence or absence of AFM. It was found that crystal violet absorption by pure Caco-2 cells was significantly lower (P <0.05) than the control wells containing *Candida*- infected Caco-2 cells (Fig. 6.6.F). This confirms adhesion of *Candida* cells to the epithelial cell line [25]. Adhesion capacities of BSS7 and *C. albicans* were not significantly different. However in the

test wells, there was significant decrease in adhesion compared to the respective control groups suggesting possible anti- adhesive properties of AFM which inhibited the adhesion of both *Candida* strains.

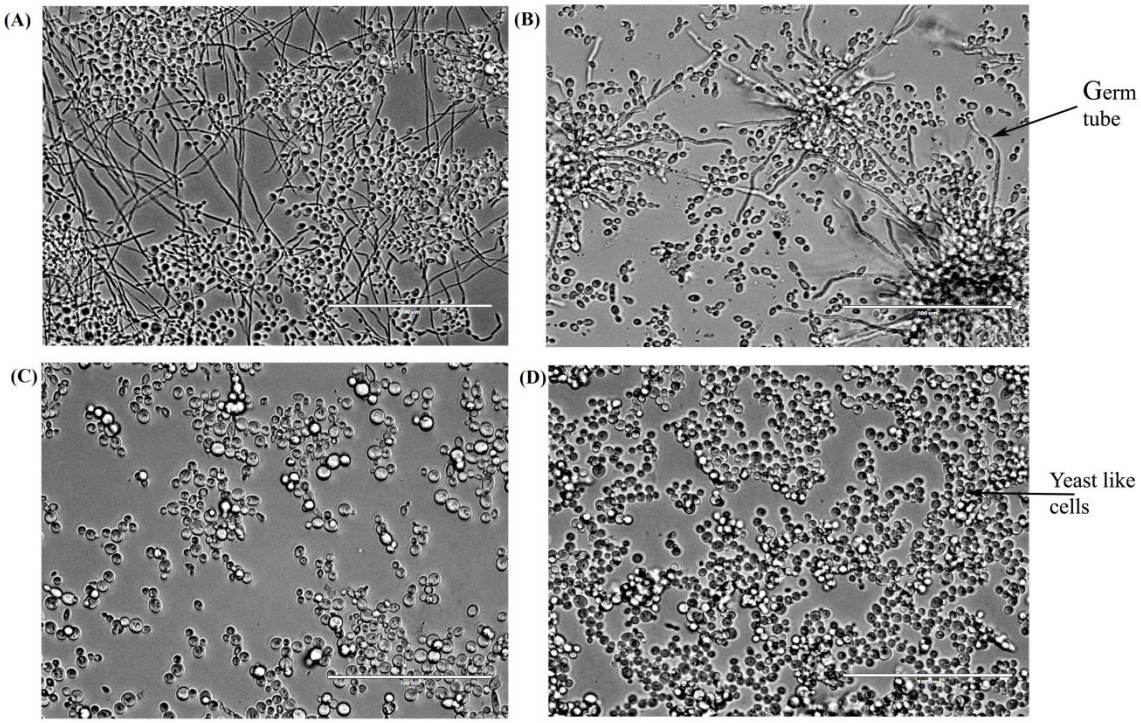

Fig. 6.5. Germ tube inhibition assay. (A) *C. tropicalis* BSS7 control, (B) Candida *albicans* MTCC 3017 control, (C) *C. tropicalis* BSS7 treated with BIC_{50} and (D) *C. albicans* MTCC 3017 treated with BIC_{50}, as observed under light microscope (40X).

6.4.5 Identification of AFMs

GC-MS analysis revealed the presence of fatty acids having chain lengths ranging from 3 to 18 carbons. The compounds identified were small molecules with molecular masses ranging from 90.08 282.468 g mol^{-1} to 282.468 g mol^{-1}. Total 6 compounds could be identified; the major constituents being lactic acid (28.66% at 4.89 min), followed by α-Hydroxyisocaproic acid (12.18% at 6.66 min), benzoic acid (11.85% at 7.01 min), 6-Octadecenoic acid (9.17% 15.03 min), Benzeneacetic acid (7.59% at 7.82 min) and 2-hydroxy, 3-methyl butanoic acid (6.47% at 5.79 min) as shown in the Fig. 6.7. lactic acid bacteria produces lactic acid as the major fermentation product after breakdown of six- carbon sugars such as glucose, sucrose, lactose etc. Lactic acid, which is present in many fermented foods such as yogurt, cheese, kimchi etc., can inhibit the growth of yeast and moulds by decreasing the pH of the growth media.

[36]. α- Hydroxyisocaproic acid (2- Hydroxyisocaproic acid) is a nutritional additive that has anticandidal activities [37]. Salts of benzoic acid are also used as food preservatives. 6-Octadecenoic acid or petroselinic acid is an omega-12 fatty acid essential for human health. Benzeneacetic acid or phenylacetic acid is found to possess activities against various yeasts and moulds [38]. Therefore, the AFM of *L. paracasei* D6 was found to contain compounds having antifungal properties with a safe history of use as food additives.

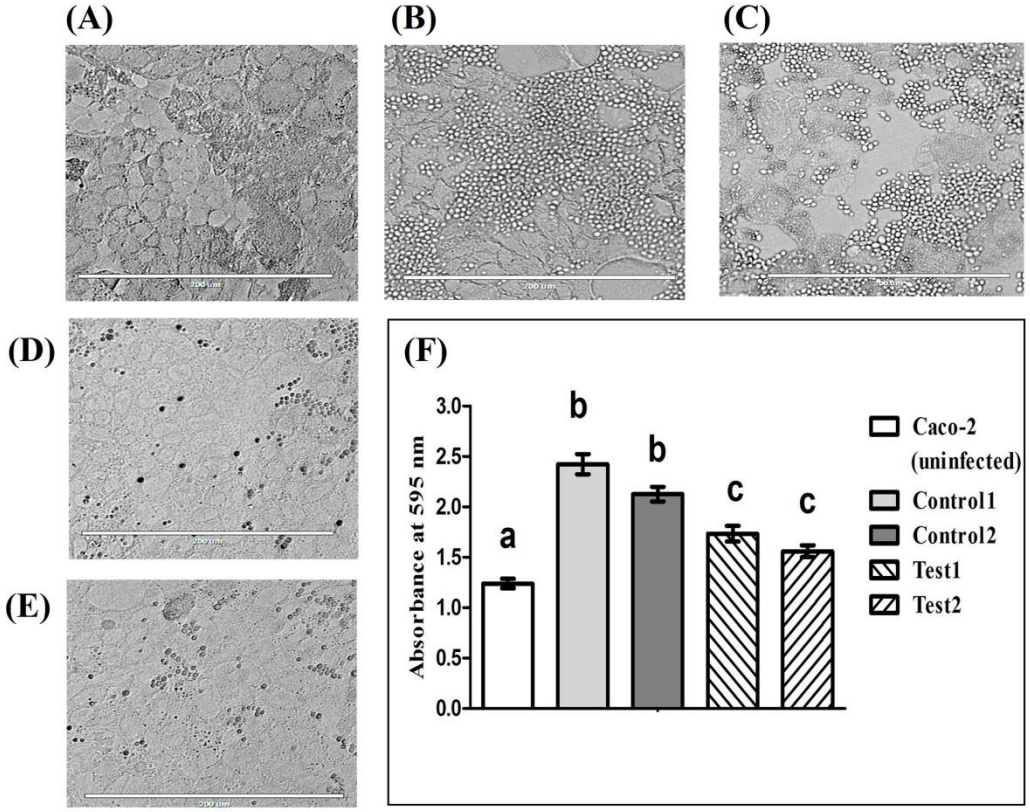

Fig. 6.6. *Candida* adhesion inhibition assay. (A) Uninfected Caco-2 cell, (B) Caco-2 cells + BSS7 (control 1), (C) Caco-2 cells+ MTCC 3017 (control 2), (D) Caco-2 cells + BSS7 + BIC_{50} of AFM (test1), (E) Caco-2 cells + MTCC 3017 + BIC_{50} AFM (test 2) and (F) Spectrophotometric adhesion inhibition assay; different letters signify statistical difference (P <0.05), estimated by Turkey's Multiple comparison test, GraphPad Prism, Ver 5.0.

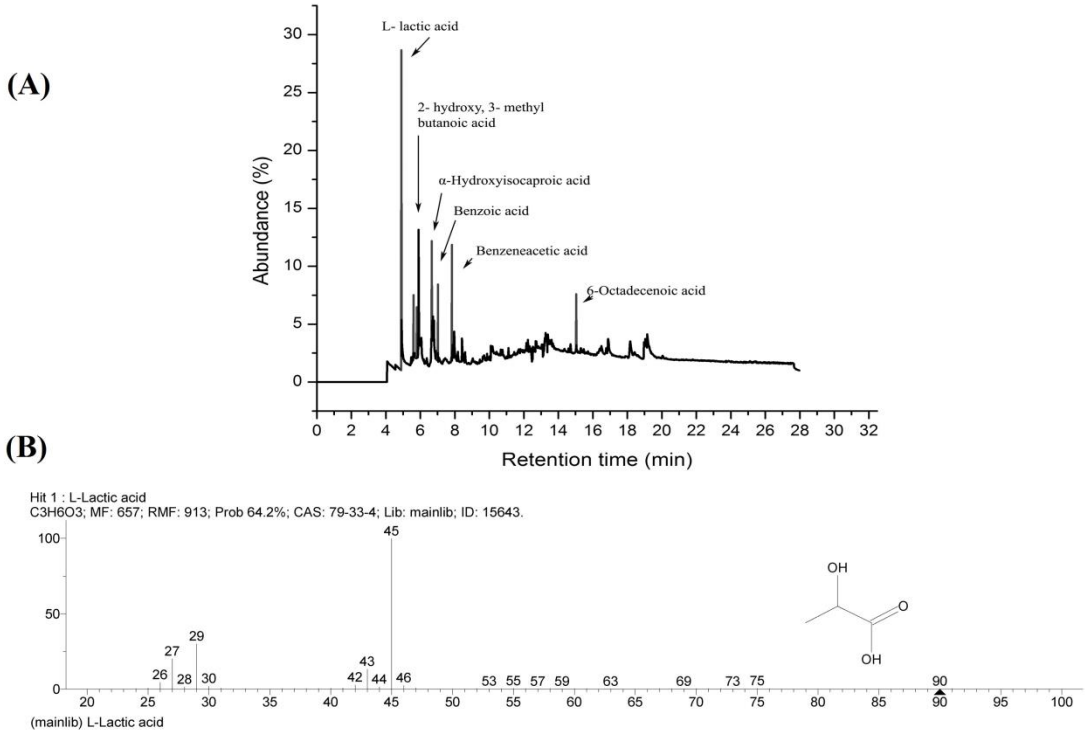

Fig. 6.7. Identification of antifungal metabolites. (A): Relative abundance of compounds in antifungal metabolites of *L. paracasei D6* identified by GC-MS. (B): Mass spectra of the most abundant compound.

6.4.6. Inhibition of *Candida* in fruit juice:

The application of minimal of food processing operations in the preparation of fruit juices has been recently focused due to consumer's increased concern about health. Processes like pasteurization are combined with the use of natural products that can not only reduce the use of hazardous chemical preservatives but also drastically eliminate the yeast population that has the capacity to survive harsh conditions like low pH and mild pasteurization [39]. In the present study we examined the synergistic effect of mild heating conditions with different concentrations of FAM obtained from the probiotic *L. paracasei* D6 that has anticandidal effect. For determining the optimum conditions of three different factors on the inhibition of the two types of *Candida* species, 17 different runs were carried out separately based on the model generated by Design- Expert (Table 6.1).

Table 6.1. Predicted and experimental values of *C. tropicalis* inhibition

Run	**Factor 1**	**Factor 2**	**Factor 3**	**Response**	
	A:Inoculum	B:Concentration	C:Treatment time	*C. tropicalis* inhibition (Experimental)	*C. tropicalis* inhibition (Predicted)
	log CFU/ml	mg/ml	Min	log CFU/ml	Log CFU/ml
1	1	0	1	1.35 ± 0.046	1.35
2	0	0	0	1.38 ± 0.046	1.34
3	-1	0	1	0.79 ± 0.014	1.83
4	1	1	0	1.47 ± 0.070	2.05
5	0	1	1	1.86 ± 0.049	1.4
6	0	0	0	1.58 ± 0.024	1.33
7	0	0	0	1.57 ±0.009	1.83
8	0	0	0	1.53 ± 0.026	2.12
9	-1	0	-1	1.35 ± 0.066	0.77
10	0	0	0	1.29 ± 0.123	1.51
11	0	-1	1	1.61 ± 0.049	1.53
12	1	0	-1	2.16 ± 0.177	1.97
13	-1	1	0	1.64 ± 0.060	1.59
14	0	-1	-1	1.95 ± 0.010	1.59
15	0	1	-1	1.82 ± 0.032	1.59
16	-1	-1	0	1.57 ± 0.074	1.59
17	1	-1	0	2.03 ± 0.035	1.59

ANOVA was used for data analysis. The ANOVA results were given in the table 6.2 which confirms that the inhibition model was significant. The quadratic equations for inhibition are as follows:

$$\text{Inhibition } (\textit{C. tropicalis}) = 1.59 + 0.054A + 0.3B + 0.31C + 0.14A^2 - 0.083B^2 - 0.059C^2 + 0.058AB + 0.091AC - 0.076BC \quad (2)$$

After observing from the *P* values from the table 6.2 it was confirmed in case of *C. tropicalis* inhibition, linear effects of inoculum, concentration and treatment time, quadratic effects of inoculum and concentration and interactions among inoculum and time and concentration and time were significant. Thus, a similar trend of inhibition was observed as reported by Bukvicki et al. [26] who showed synergistic effect of different factors for the inhibition of *S. cereviciae* in fruit juice by liverwort

extracts. For numerical optimization desired goal for the responses were set as maximum and importance was set as 5. The desirability ramp (Fig. 6.8B) shows that inoculum 3.75 log CFU/ml, concentration 4.89 mg/ml, treatment time 1.47 min resulted in maximum inhibition (2.2 log CFU/ml) of *Candida tropicalis* in fruit juice.

Table 6.2. ANNOVA table for the quadratic model of inhibition

	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	1.6718733	9	0.1857637	50.267184	< 0.0001*
A	0.0229783	1	0.0229783	6.2178749	0.0414*
B	0.7035945	1	0.7035945	190.39089	< 0.0001*
C	0.7535316	1	0.7535316	203.90372	< 0.0001*
A^2	0.0842521	1	0.0842521	22.7984	0.0020*
B^2	0.0293437	1	0.0293437	7.9403194	0.0259*
C^2	0.0147408	1	0.0147408	3.988824	0.086
AB	0.0135141	1	0.0135141	3.6568708	0.0974
AC	0.0330785	1	0.0330785	8.9509619	0.0202*
BC	0.0232563	1	0.0232563	6.2930819	0.0405*

* indicates significant difference ($P < 0.05$)

6.7. Conclusion

Food safety is a major concern of this present era due to the emergence of pathogenic microorganisms with unprecedented virulent properties and antimicrobial resistance. This work stresses on possible virulent nature of the food isolate *C. tropicalis* BSS7 that mimics the biofilm forming strain *C. albicans* MTCC 3017. Exometabolites produced by *L. paracasei* D6 that contained different fatty acid metabolites were able to inhibit the growth, biofilm and germ tube formation of both the strains which gives an insight in to an alternative strategy for anticandidal therapy.

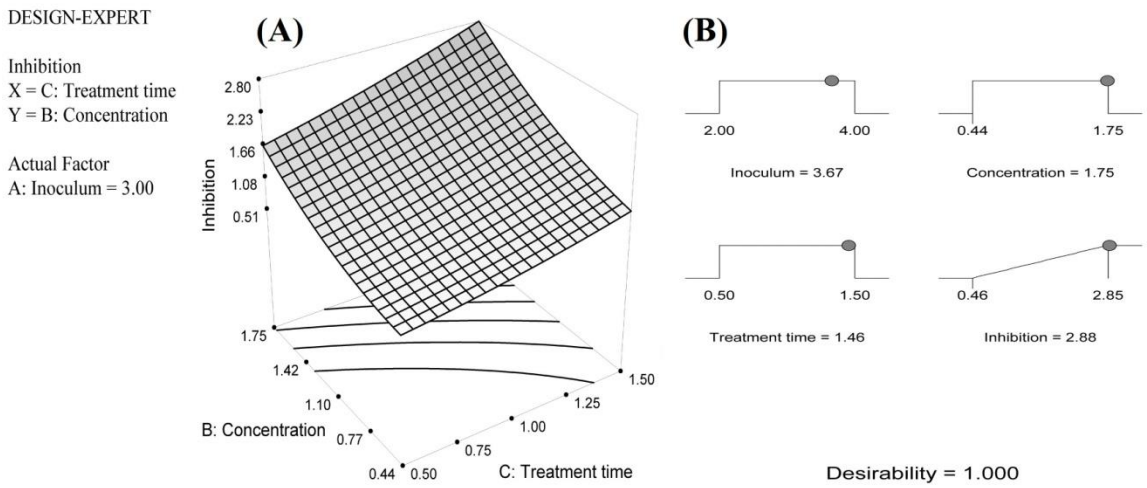

Fig. 6.8. Optimization of different parameters for the inhibition of *C. tropicalis* BSS7 in fruit juice. (A) DESIGN- EXPERT Plot showing effect of different concentrations of antifungal metabolites and treatment time for the inhibition of *C. tropicalis* BSS7 in fruit juice. (B) Multiresponse optimization showing desirability ramps for numerical optimization of parameters for the inhibition of *C. tropicalis* BSS7.

6.8. Bibliography

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