INTRODUCTION

Cheese whey (a byproduct of cheese processing industries) has been efficiently exploited for the production of single cell protein (SCP) over the years as cheese whey has an immense nutritional value (Ayoola et al., 2008; Carvalho et al., 2013). It contains 4.5-5% (w/v) of lactose, 0.6-0.8% (w/v) of soluble proteins, 0.4-0.5% (w/v) of lipids and 8-10% (w/v) of mineral salts of the dried extract. Efficient utilization of cheese whey for SCP conversion reduces the biochemical oxygen demand (BOD) by 75% and thus decreases the disposal problem (Eyster, 1950; Prazeres et al., 2013). Mostly lactose-consuming organisms, such as Kluyveromyces spp. and Lactobacillus spp. have been tested against a wide range of essential oils where ethanol 70% v/v served as control (Nel et al., 2006; Souza et al., 2008; Waema et al., 2009). Another significant approach to inhibit C. krusei was using NaCl; however it depends on the sensitivity of the organism and the concentration of NaCl used. The sensitive strain of C. krusei undergoes cell death at 2 M concentration of NaCl (Agual and Lucas, 2000).

However, certain food spoilers (contaminant) e.g. Candida krusei still survive under extreme conditions (pH 3.0 and 45 °C) (Guo and Bhattacharjee, 2006). The opportunistic Candida species exist as commensal in healthy individuals (Heard and Fleet, 1988). During the production of SCP, C. krusei emerges as a contaminant while it grows along with K. marxianus and this is a concern for food safety. C. krusei is known as a food contaminant and an opportunistic pathogen (Siso, 1996; Hornback et al., 2006; Maneesri and Maneesri, 2007; Kim and Lee, 2012). However, C. krusei is reported to be present in many dairy and fermented food products, but yet does not come under generally recognized as safe (GRAS) microorganism (Walker and Dijck, 2006; Walker et al., 2008).

Therefore, the eradication of C. krusei is essential from food products to meet safety regulations. Certain chemical and biochemical approaches were reported to employ for selective inhibition of C. krusei. The chemical (NaCl, H2O2) and the biological inhibitors (medicinal plants, such as Lupinus angustifolius, Syzygium aromaticum (clove) oil; nisin and Willipiss saturnus and synergistic effect of W. saturnus and H2O2 to inhibit C. krusei have been reported (Ayoola et al., 2008; Dingman, 2008; Adeniyi et al., 2010; Da Silva et al., 2011). It was reported that H2O2 inhibited C. krusei (Morgulis et al., 1926). Apparently, C. krusei has also been tested against a wide range of essential oils where ethanol 70% v/v served as control (Nel et al., 2006; Souza et al., 2008; Waema et al., 2009). Another significant approach to inhibit C. krusei was using NaCl; however it depends on the sensitivity of the organism and the concentration of NaCl used. The sensitive strain of C. krusei undergoes cell death at 2 M concentration of NaCl (Agual and Lucas, 2000).

Yeasts such as Aspergillus niger, W. saturnus (major yeast from yogurt) have the capacity to produce killer proteins (Fang et al., 2002; Brock, 2008). The mycoxotin/killer proteins produced by W. saturnus have a broad spectrum of inhibitory activity at wide range of pH and temperature (Buzzini et al., 2004). These could be used as the versatile anti-spoilage agents for food and feed production (Kao et al., 1999; Liu et al., 2006). Another killer protein is nisin, which is used for food preservation and is produced by Lactobacillus spp. or lactic acid bacteria (LAB) (Guwy et al., 1999). Nisin is used to stabilize food products and is often added to the cheese for inhibiting toxin production by Clostridium botulinum. It was also reported to inhibit C. krusei efficiently (Lowes et al., 2000; Russell and Jarvis, 2001).

In certain industrial fermentation processes, stress of pH shock was encountered to inhibit certain food spoiler yeasts (Siso, 1996; Pinheiro et al., 2002). At pH 2, C. krusei did not grow well (Lowes et al., 2000). In a mixed culture, when the presence of other yeast strains was also reported, effective utilization of any inhibitor (i.e. chemical or biochemical inhibitor against C. krusei) depends entirely upon whether the other type of yeast was also inhibited by the specific inhibitor being used. Another effective way of inhibition of pathogens is by the usage of metal nanoparticles (NPs) or biomolecule based nanoparticles (Dingman, 2008). It has already been observed that silver NPs can kill pathogens...
at very low concentrations and biomolecule based nanoparticles do not exert any toxic effects on human cells. Apart from that, silver NPs do not cause any microbial resistance and also there is no specific site of action for inhibition of the microbial cells (Patacek et al., 2009). Hence, the aim of the present study was to evaluate different inhibition methods to inhibit C. krusei alone as well as in a mixed culture system without affecting the growth of K. marxianus.

MATERIALS AND METHODS

Chemicals

Analytical grade chemicals were used in the experiments. NaCl (QueLab Lab Inc., Montréal, Canada), H2O2 (Laboratoire Mat, Québec), yeast Extract (Fisher Scientific, USA), malt extract (Oxoid Ltd., Basingstoke, England), meat peptone (Organotechnie SA., La Courneuve, France), glucose, ethyl alcohol 95% (Fisher Scientific, USA), agar (Quebec Lab Inc., Montréal, Canada), cheese whey (Agropur, Canada), and AgNO3 (Fisher Scientific, Ottawa).

Microorganisms

K. marxianus strain used in the study was isolated and characterized from the SCP production plant using cheese whey as substrate. C. krusei strain was also isolated and identified as a contaminant during SCP production employing cheese whey. W. saturnus strain DBVPG 4561 was obtained from the Industrial Yeasts Collection DBVPG of Perugia (Italy). Strains were sub-cultured on YEPD yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L agar slants and stored at 4 °C for further use.

Inhibition studies for C. krusei

Chemical Methods

Inhibition by NaCl

Pre-culture broth of K. marxianus of 2.0x10^9 CFU/mL and C. krusei of 4.0x10^9 CFU/mL were prepared in 100 mL YEPD media in 500 mL Erlenmeyer flasks. The medium pH was adjusted to 3.5 and sterilized at 121 °C for 15 min. After sterilization, 1.5 M and 2 M NaCl were added in different sets of flasks. C. krusei is a non-lactose assimilating organism, while K. marxianus is a lactose assimilating organism. Therefore, sterilized YEPD media were inoculated with 30 μL (from stock culture) of K. marxianus and 50 μL of C. krusei. Inoculated flasks were incubated at 40 °C for 24 h. Samples were taken at regular intervals for the analysis of total cell count. Total cell concentration was measured using standard agar plate technique (Logothetis et al., 2007; Goretti et al., 2009; Kosseva et al., 2009).

Inhibition by H2O2

A pre-culture was prepared for C. krusei and K. marxianus as above. After that, culture media of cheese whey powder 4.5% (w/v) and urea 0.22% (w/v) were prepared and pasteurized at 80 °C for 20 min. The pasteurized cheese whey culture media at different pH (3.5, 4.5, 5.5, and 6.0) was added to different 500 mL flasks and inoculated with 1% (v/v) inoculum of C. krusei and K. marxianus. Different concentrations of H2O2 (100, 200, 300 and 400 ppm) were added to these flasks. After inoculation, flasks were incubated at 28 °C and 40 °C in an orbital incubator shaker for 24 h. Samples were drawn at regular intervals to analyze the total cell count.

Simultaneously, two different set of experiments were conducted, where in the primary set of experiments the H2O2 concentration were varied (0, 300, 400, 500, 600 and 800 ppm) and applied directly on the fermentor broth containing K. marxianus and C. krusei, which was collected from commercial continuous SCP production plant. 100 mL of fermented broth of K. marxianus (3.0x10^9 CFU/mL) severely contaminated with C. krusei (1.8x10^9 CFU/mL) was taken in 500 mL 2 sterilized flasks. Flasks were incubated at pH 3.5, 150 ppm and 40 °C in an incubator shaker.

In the secondary set of experiments, variation in H2O2 concentration (2400, 3200 and 4000 ppm) were considered and applied directly to the fermentor broth and flasks were incubated at pH 5.0, 150 ppm and 45 °C in an incubator shaker.

Biochemical Methods

Inhibition by S. aromaticum oil

A set of experiments were conducted in which 0.4% (v/v) of clove oil was added in fermenter broth which contains C. krusei and K. marxianus. The initial cell count of C. krusei and K. marxianus was 5.0x10^8 CFU/mL and 6.0x10^8 CFU/mL, respectively. The flasks were placed in an orbital incubator shaker at 28 °C at 150 rpm for 6 h. Sampling was performed at an interval of 2 h. Samples were analyzed for total cell count using standard agar plate technique.

Inhibition with nisin

The culture of C. krusei and K. marxianus were grown separately in MRS broth at 35 °C for 24 h. Bioassay MRS media with 0.75% of Bacto agar and 1% Tween-20 were prepared. Media were sterilized at 121 °C for 15 min. A solution of nisin (1,000 IU/mL) was prepared by adding 0.025 g of commercial nisin (Sigma-Aldrich, Milwaukee, USA) into 25 mL of sterile solution of 0.02 N HCl. Sterilized media were cooled down to 40 °C and inoculated with 1% (v/v) of the 24 h culture of C. krusei and K. marxianus in two sets (duplicate). Then the bioassay agar (25 mL) was aseptically poured into sterile petri dishes (150 mm) and allowed to solidify for 1 h. On each plate, four or five holes were bored, using a 7 mm outer diameter stainless steel borer with a slight suction. An aliquot (50 μL and 100 μL) of standard nisin solution was placed into a well and the bioassay agar plate was incubated right away at 35 °C for 24 h. The control for each plate was prepared using sterile distilled water in wells. Zone of inhibition was observed in control and test samples.

Inhibition study with W. saturnus

Preparation of W. saturnus culture broth

YEPA (100 mL) was prepared in 500 mL flasks and sterilized at 121 °C for 15 min. The sterilized flasks was inoculated with loopful of W. saturnus and incubated in an orbital incubator shaker at 150 rpm and 28 °C for 48 h. Samples were taken at regular time intervals for total cell count.

Well assay method

Pre-culture of C. krusei was prepared in YEPA as described above using 1% (v/v) inoculum. After 24 h, C. krusei sample was diluted 10^2, 10^3 and 10^4 times in saline solution and different diluted samples were spread plated in YEPA agar plates. After spread plating, wells were made in agar plates using borer and 60 μL of W. saturnus (48 h) culture was added in each well. The plates were incubated in an orbital incubator at 28 °C for 24 h. The plates were visually observed after 24 h.

To differentiate the morphology of C. krusei from W. saturnus, Methyline Blue Citric-Phosphate agar (MB A) plates were prepared and spread plated using C. krusei and W. saturnus. Plates were incubated for 24 h at 28 °C and were visually examined to check the morphology.

Inhibition by W. saturnus

W. saturnus was grown in YEPA and cheese whey medium for 24 h. YEPA and cheese whey powder 4.5% (w/v) with 0.22% (w/v) urea were prepared in two flasks of 2 L capacity each containing 500 mL medium. After sterilization, each flask was inoculated with 2% (v/v) W. saturnus and incubated in an orbital incubator shaker at 150 rpm and 28 °C. The culture was harvested at 48 h. The culture broth was centrifuged at 10 000 x g and the supernatant was lyophilized and the powder was lyophilized. The extracellular proteins specifically contain a particular protein 45.62 kDa protein, which has anti-myotic activity (Buzzini et al., 2004). Simultaneously, another set of flasks containing W. saturnus were grown, where no centrifugation was performed. Henceforth, the cultures were directly taken for lyophilization.

The cultures were prepared by lyophilizing C. krusei, K. marxianus and W. saturnus in YEPA medium for 24 h. One hundred milliliters of fresh cheese whey powder 4.5% (w/v) with urea 0.22% (w/v) solution was added to each five hundred milliliters Erlenmeyer flask (two flasks) and pasteurized at 80 °C for 20 min. After pasteurization, media were aseptically adjusted to different pH (3.5 and 4.5) followed by inoculation with 1% (v/v) mixed culture (C. krusei and K. marxianus).

Inhibition by lyophilized supernatant from W. saturnus

Various concentrations of lyophilized supernatant of W. saturnus were considered (well plate assay method) and the zone was created by the inhibitory effect of the killer protein. The inhibition zones were measured after 24 h of incubation at 30 °C. A linear equation (y = 0.30x-0.36) was sketched out between the diameter of the clear zone (measured in millimeters, x axis) and the logarithm of the quantity of the killer protein (measured in nanograms, y axis). This method was used to determine the killer protein concentration required for the inhibition of C. krusei which is similar to the technique mentioned in (Chen et al., 2000). Lyophilized supernatant prepared in YEPA media was served as the control and lyophilized cheese whey was the experimental product.

Inhibition of C. krusei by synergistic effect of H2O2 and W. saturnus

To study inhibition of C. krusei, different H2O2 concentrations were used along with W. saturnus (entire organism lyophilized supernatant powder, as described
above). 300 ppm of \(\text{H}_2\text{O}_2\) was used along with 1% (v/v) of \(W. \text{sartornus}\) (inoculum from pre-culture) for the inhibition of \(C. \text{kruzei}\) in a mixed culture of \(C. \text{kruzei}\) and \(K. \text{marxinus}\) at pH 6.0 and 28 °C. Similar sets of experiments were conducted with a variation in pH (3.5-4.5) at 28 °C. Two different set of experiments were conducted, where in the primary set of experiments was conducted where lyophilized \(W. \text{sartornus}\) was used by varying the \(\text{H}_2\text{O}_2\) concentration directly on the fermenter broth containing \(K. \text{marxinus}\) and \(C. \text{kruzei}\), collected from commercial continuous SCP production plant. 100 mL of fermented broth of \(K. \text{marxinus}\) (3,1x10^6 CFU/mL) grossly contaminated with \(C. \text{kruzei}\) (1.5x10^5 CFU/mL) was taken in 500 mL 2 sterilized flasks. The lyophilized powder of \(W. \text{sartornus}\) (200 mg/mL) along with different concentrations of \(\text{H}_2\text{O}_2\) (2400 and 4000 ppm) was then added to each flask. Flasks were incubated at pH 5.0, 150 rpm and 40 °C in an incubator shaker. Whereas in secondary set of experiments, about 400 mg/mL of lyophilized supernatant of \(W. \text{sartornus}\) was used along with 2400 ppm of \(\text{H}_2\text{O}_2\) in a mixed culture by adjusting the pH of fermenter broth to 5.0. As the killer protein produced by lyophilized \(W. \text{sartornus}\) is highly effective at pH range of 4.5-10.0 and temperature from 25 to 45 °C (Goretti et al., 2009). Flasks were kept at 150 rpm and 40 °C in an orbital incubator shaker.

**Inhibition of \(C. \text{kruzei}\) by Ag-KT4561 NPs**

Synthesis of nanoparticles (Ag-KT4561) was carried out in the previous study (Bhattacharya et al., 2015). However a bulk preparation of the same has been conducted in this study. During the scale-up process, 20 mL of 0.1 M AgNO\(_3\) solution is continuously stirred along with 18 mL of \(W. \text{sartornus}\) supernatant at 25 °C for 48 h. Ag\(^{+}\) ions were completely reduced at 48 h of stirring. After which the bulk nanoparticle solution was taken for lyophilization and the lyophilized product was tried against \(C. \text{kruzei}\) in 4.5% (w/v) cheese whey and 0.22% (w/v) of urea. From the lyophilized product different concentration of Ag-KT4561 ranging from 10 \(\mu\)M - 1 mM were tried at pH 5.5 and 30 °C in shake flasks. In these experiments, mixed culture of \(C. \text{kruzei}\) (2% (v/v)) and \(K. \text{marxinus}\) (2% (v/v)) were tested for 12 h and total cell concentration (CFU/mL) was measured at 3 h time intervals.

**Analytical methods**

**Cell count**

Total cell count as CFU (colony forming units) was estimated by standard agar plate technique in YEPD agar plates (Nathan et al., 1978). The appropriately diluted samples were plated on agar plates and incubated at 30 °C overnight to form fully developed colonies. The colonies of \(K. \text{marxinus}\), \(C. \text{kruzei}\) and \(W. \text{sartornus}\) were identified based on its morphology by visible examination.

**Protein estimation**

The soluble protein concentration was determined by Lowry’s method (Lowry et al., 1951) using bovine serum albumin as standard.

**UV-Vis Spectroscopy**

The bulk sample of AgNO\(_3\) and \(W. \text{sartornus}\) supernatant were prepared at 48 h and samples were collected at every 6 h to analyze for nanoparticle formation at 300-700 nm in Spectrophotometer (Carry 100 Bio®, Varian USA).

**Statistical method**

For each set, samples were analyzed in triplicates and mean values are taken in account. Further standard deviation of the colonies in log units (Log\(_{10}\) CFU/mL) were calculated for each experimental point in Microsoft excel 2013 external package similar to the method of De Oliveira et al., 2014.

**RESULTS**

**Inhibition by NaCl**

Different concentrations (1.5- 2 M) of NaCl were tested to inhibit the growth of \(C. \text{kruzei}\) in mono-culture and the results were presented in Figure 1. It was clear that NaCl concentration of 2 M showed significant inhibition of \(C. \text{kruzei}\) compared to 1.5 M without having any effect on \(K. \text{marxinus}\). This is due to the fact that \(C. \text{kruzei}\) exhibited salt-stress (Aguiar and Lucas, 2000) and got killed at pH 3.5 and 40 °C. No inhibition of \(C. \text{kruzei}\) was observed when NaCl concentration was less than 2 M at pH 3.5 and 40 °C.

![Graph](image_url)

**Figure 1 Impact of a) 1.5 M NaCl and b) 2.0 M on \(C. \text{kruzei}\) (Ck) and \(K. \text{marxinus}\) (Km) in YEPD medium at pH 3.5 and 40 °C (Shake flask experiments)**

**Inhibition by \(\text{H}_2\text{O}_2\)**

Viability of individual cultures of \(C. \text{kruzei}\), and \(K. \text{marxinus}\) at different concentrations of \(\text{H}_2\text{O}_2\) (100 - 400 ppm) in cheese whey at pH 6.0 and 28 °C was studied (Table 1). It showed that \(C. \text{kruzei}\) was not inhibited at lower concentration of \(\text{H}_2\text{O}_2\). However, inhibition occurred at 300 ppm \(\text{H}_2\text{O}_2\). \(K. \text{marxinus}\) was not inhibited at these concentrations of \(\text{H}_2\text{O}_2\).
Table 1 Impact of various concentrations of H₂O₂ on C. krusei and K. marxianus in cheese whey powder at pH 6.0 and temperature 28 °C (Shake flask)

<table>
<thead>
<tr>
<th>H₂O₂ (ppm)</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>300</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ck</td>
<td>Km</td>
<td>Ck</td>
<td>Ck</td>
<td>Ck</td>
</tr>
<tr>
<td>0</td>
<td>5.73±0.7</td>
<td>4.83±0.1</td>
<td>5.81±0.1</td>
<td>5.81±0.1</td>
<td>5.15±0.3</td>
</tr>
<tr>
<td>12</td>
<td>6.52±0.2</td>
<td>9.33±0.1</td>
<td>6.08±0.3</td>
<td>5.23±0.1</td>
<td>5.40±0.4</td>
</tr>
<tr>
<td>24</td>
<td>8.51±0.2</td>
<td>8.31±0.1</td>
<td>6.12±0.5</td>
<td>5.18±0.5</td>
<td>NG</td>
</tr>
</tbody>
</table>

**Legend:** NG - No Growth Observed, Ck – C. krusei, Km – K. marxianus

The inhibition of C. krusei in mixed cultures (C. krusei and K. marxianus) in cheese whey was studied and the results were presented in Figure 2. The concentration of H₂O₂ used was 300 ppm and 400 ppm (from previous results in Table 1). C. krusei was not inhibited at 300 - 400 ppm of H₂O₂. On the contrary, C. krusei dominated over K. marxianus in a mixed culture at 24 h.

Variations in pH along with 300 ppm H₂O₂ concentration

pH variations were carried out and it was lowered to 3.5 from 6.0 and studies of mixed culture (C. krusei, K. marxianus and W. saturnus 1% and 2% (v/v)) were also carried out maintaining similar parameters in cheese whey. C. krusei was not inhibited at these parameters, whereas growth of K. marxianus and W. saturnus remains unchanged.

A mixed culture study at pH 4.5 and 28 °C in cheese whey powder with 300 ppm H₂O₂ exhibited a partial inhibition of C. krusei (Figure 3a). However when bio-preservative W. saturnus was added at the similar condition, the growth of C. krusei decreased by one log-unit at 12 h (Figure 3b), but the growth accelerated after 12 h. As W. saturnus produces killer protein (KT4561) but the concentration of the killer protein remains low which is insufficient to inhibit C. krusei in a large-scale fermentation. Hence, this parameter could be considered for the inhibition of C. krusei.

C. krusei was efficiently inhibited in mixed cultures (C. krusei and K. marxianus) and (C. krusei, K. marxianus and W. saturnus) at pH 4.0, 28 °C and 400 ppm of H₂O₂. Lower CFU/mL of 1.7x10³ was observed for K. marxianus at pH 4.0 and 28 °C. On the contrary, when C. krusei was grown along with K. marxianus and W. saturnus, K. marxianus was observed at high CFU/mL of 2.4 x 10³ (as compared to C. krusei when grown along with K. marxianus). K. marxianus showed remarkable growth at pH 5.5 and 40 °C rather than at other pH values, hence pH 4.5-5.5 was ideal for K. marxianus.
Higher ranges of H$_2$O$_2$ concentrations

After deducing the optimum amount of H$_2$O$_2$ used for the complete inhibition of C. krusei, similar concentration was applied for industrial scale fermenter broth to eliminate C. krusei without affecting the K. marxianus. So, 300 ppm of H$_2$O$_2$ was the optimum concentration for inhibiting C. krusei in the mixed culture in shake flask experiments. When concentration of H$_2$O$_2$ was increased from 300 to 800 ppm in the lab scale fermenter broth, no significant inhibition of C. krusei was observed at pH 3.5 and 40 °C (Table 2). K. marxianus degraded H$_2$O$_2$ at pH 3.5, making H$_2$O$_2$ ineffective for C. krusei inhibition (Pinheiro et al., 2002). In fermented broth, higher concentration (2400 ppm, 3200 ppm, and 4000 ppm) of H$_2$O$_2$ was considered at pH 5.0 and 45 °C. Study was conducted for 6 h, as H$_2$O$_2$ got degraded into H$_2$O and O$_2$ after 6 h (Table 3). A very high concentration 4000 ppm of H$_2$O$_2$ finally could kill C. krusei completely in the fermented broth. Higher concentration of H$_2$O$_2$ was required due to simultaneous degradation of H$_2$O$_2$ by catalase action of K. marxianus (Pinheiro et al., 2002).

Table 2 Impact of varying concentration of H$_2$O$_2$ on the mixed culture in the fermenter broth at pH 3.5 and 40 °C (Shake flask)

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (ppm)</th>
<th>Individual Organisms (Log$_{10}$ CFU/mL) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Time (h) Ck Km Ck Km Ck Km Ck Km Ck Km Ck Km Ck Km</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.88±.01</td>
</tr>
<tr>
<td>3</td>
<td>7.91±.01</td>
</tr>
<tr>
<td>6</td>
<td>8.18±.04</td>
</tr>
<tr>
<td>9</td>
<td>7.78±.01</td>
</tr>
<tr>
<td>12</td>
<td>7.18±.04</td>
</tr>
<tr>
<td>24</td>
<td>8.40±.02</td>
</tr>
</tbody>
</table>

Legend: Ck– C. krusei, Km – K. marxianus

Table 3 Impact of higher concentrations of H$_2$O$_2$ on the mixed culture in the fermenter broth at pH 5.0 and 45 °C (Shake flask)

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (ppm)</th>
<th>Individual Organisms (Log$_{10}$ CFU/mL) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2400</td>
</tr>
<tr>
<td>Time (h) Ck Km Ck Km Ck Km</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.18±.04</td>
</tr>
<tr>
<td>2</td>
<td>6.31±.01</td>
</tr>
<tr>
<td>4</td>
<td>6.47±.02</td>
</tr>
<tr>
<td>6</td>
<td>6.52±.01</td>
</tr>
</tbody>
</table>

Legend: NG– No Growth, Ck– C. krusei, Km – K. marxianus

Inhibition by S. aromaticum oil

A study of the mixed culture (C. krusei and K. marxianus) at pH 3.5 and 28 °C along with various concentrations of clove oil was performed. It was observed that using clove oil concentration 0.5% (v/v) at pH 3.5 and 28 °C is ideal for C. krusei inhibition without affecting the growth of K. marxianus (1.6x10$^7$ CFU/mL) in a mixed culture. However when concentration of clove oil was brought down to 0.4% (v/v) and was used in the fermented broth, C. krusei was inhibited at 0 h and K. marxianus (1.7x 10$^7$ CFU/mL) growth was unhampered at 6 h (Table 4). Clove oil 0.4% (v/v) at similar set of pH and temperature used above was ideal for C. krusei inhibition in a mixed culture. Candida are associated with infections as they form biofilms, S. aromaticum extracts worked against biofilm formation and thus, inhibit the growth of C. krusei (Kim and Lee, 2012).

Table 4 The inhibition performed by using 0.4% (v/v) of clove oil at pH 3.5, 28°C in fermenter broth (100 mL)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Individual Organisms (Log$_{10}$ CFU/mL) ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ck Km</td>
</tr>
<tr>
<td>0</td>
<td>7.21±.03</td>
</tr>
<tr>
<td>2</td>
<td>NG</td>
</tr>
<tr>
<td>4</td>
<td>NG</td>
</tr>
<tr>
<td>6</td>
<td>NG</td>
</tr>
</tbody>
</table>

Legend: NG– No Growth Observed, Ck– C. krusei, Km – K. marxianus

Inhibition by nisin

After 24 h of incubation the plates were observed and no yeast species were inhibited by nisin.

Inhibition by W. saturnus

A primary test was conducted to investigate the interaction between W. saturnus and C. krusei, along with K. marxianus. From the plate technique, it was concluded that W. saturnus could inhibit C. krusei but not K. marxianus. It is necessary to check whether C. krusei is an inducer for the production of the killer protein in W. saturnus or the latter naturally produces extracellular protein KTA561.

Usage of W. saturnus lyophilized powder

A minimum of 156 µg/mL of lyophilized protein in YEPD media is equivalent to 321.9 µg/mL of lyophilized protein in cheese whey needed for the inhibition of C. krusei (Table 5). W. saturnus did not show any effect below pH 4.5 and it grows well at 25-45 °C. Also, W. saturnus grow well at pH 3.5 but failed to produce killer protein at the same pH.
Table 5 The inhibition zone created by the minimum concentration of the killer protein along with varying concentration from the lyophilized supernatant from W. saturnus

<table>
<thead>
<tr>
<th>Media</th>
<th>Lyophilized supernatant concentration (mg/mL) of W. saturnus</th>
<th>Protein concentration (µg/mL) of killer protein</th>
<th>Inhibition zone formed by killer protein (cm) (Average + Standard Deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheese whey</td>
<td>500</td>
<td>321</td>
<td>1.8±0.03</td>
</tr>
<tr>
<td></td>
<td>700</td>
<td>475</td>
<td>1.5±0.08</td>
</tr>
<tr>
<td>Synthetic media (YEPD)</td>
<td>200</td>
<td>156</td>
<td>1.0±0.01</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>158</td>
<td>1.5±0.02</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>168</td>
<td>2.1±0.03</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>176</td>
<td>2.3±0.02</td>
</tr>
</tbody>
</table>

Inhibition by synergistic effect of H$_2$O$_2$ and lyophilized W. saturnus/supernatant from W. saturnus

4000 ppm of H$_2$O$_2$ could inhibit the growth of C. krusei in the fermented broth (mono-culture) obtained from continuous aerated fermentation (Table 3); and 200 mg/mL (killer protein concentration is 156 mg/mL) was the concentration of lyophilized powder needed for the inhibition of C. krusei (obtained from Table 5). A synergistic effect of H$_2$O$_2$ and lyophilized powder of W. saturnus was studied. The set of experiments conducted at pH 5.0 and 40 °C, where 4000 ppm of H$_2$O$_2$ and 200 mg/mL of lyophilized W. saturnus powder was added. W. saturnus was highly effective in killing C. krusei, but in these set of experiments; such an inhibition did not occur because W. saturnus possesses peroxidase activity, which along with K. marxianus degraded H$_2$O$_2$ at a much faster rate than K. marxianus alone (Buzzini et al., 2004).

2400 ppm of H$_2$O$_2$ was considered along with 200 mg/mL of lyophilized supernatant of W. saturnus grown in cheese whey at pH 5.0 and 40 °C. At 24 h, cell concentration of C. krusei was reduced (Figure 4). By increasing the concentration of lyophilized supernatant of W. saturnus to 300 mg/mL, complete inhibition did not take place in a mixed culture. Simultaneously when 2400 ppm H$_2$O$_2$ and 400 mg/mL of lyophilized supernatant of W. saturnus was applied, H$_2$O$_2$ was degraded by 0-6 h because of catalase-peroxidase enzymatic activity from K. marxianus and W. saturnus, but lyophilized supernatant of W. saturnus showed activity till 24 h. C. krusei (CFU/mL) lowered and showed drastic reduction in cell concentration at 24 h, whereas K. marxianus (1.8x10$^5$ CFU/mL) remained unaffected.

Inhibition by synergistic effect of C. krusei and Ag-KT4561 NPs

Higher concentration of the Ag-KT4561 was observed at 48 h than at 12 h (Figure 5a). Therefore NPs formed at 48 h were considered for this study. A concluding study of the mixed culture (C. krusei and K. marxianus) along with silver-KT4561 nanoparticles (Ag-KT4561NP) at pH 5.5 and 30 °C showed that 350 µM of Ag-KT4561 could efficiently inhibit C. krusei. At concentration of 350 µM (Ag-KT4561), the conjugate consists of 1 ppm of reduced Ag.

Figure 4 Impact of 2400 ppm H$_2$O$_2$ with 200 - 400 mg/mL (156 – 200 µg/mL killer protein) of lyophilized supernatant W. saturnus (Ws) powder on the mixed culture (C. krusei (Ck) and K. marxianus (Km)) in the fermenter broth at pH 5.0 and 40 °C (Shake flask)

Inhibition of C. krusei by Ag-KT4561 NPs

While growth curves of K. marxianus slightly decreases from 8.9x10$^7$ to 2.6x10$^7$ (Figure 5b). The decrease was less than a log-unit and this might be due to the presence of silver in the Ag-NPs. The other concentrations of the Ag-KT4561 used are as less as 10 µM and as maximum as 1 mM. In any food and feed grade products, a very high concentration of biomolecule based nanoparticle may be toxic for consumption but at a lower concentration of 350 µM (with 1 ppm of reduced silver ions), Ag-KT4561 is an efficient bio-preservative. Another effective approach to use biomolecule based nanoparticle is, no pH adjustment and no temperature adjustment is required. Ag$^+$ has anti-microbial effects against a wide range of pathogenic microorganisms and since Ag-KT4561 is a combination of killer protein from W. saturnus which specifically targets C. krusei. A synergistic effect of both (reduced Ag ion and killer protein) can kill C. krusei and K. marxianus remains partially affected (Figure 5b and 6a). A stability test of Ag-KT4561(350 µM) was performed on cheese whey till 12 h for 20 weeks and every time C. krusei was killed after being inoculated at 0 h and K. marxianus showed growth at a maximum of 2.3x10$^3$ CFU/mL. Though K. marxianus growth was affected it did not perish away with the concentration of Ag in Ag-KT4561 (Figure 6b). A tabular representation (Table 6) shows the economics of bio-inhibitor (Ag-KT4561) production in a bulk amount of 20,000L with a minimum of 1 ppm reduced silver ions.
Cl could effectively inhibit $K. marxianus$ at industrial scale, such as both yeasts showed catalase activity. Maximum oxidative stress was observed in case of $K. marxianus$ when various concentrations of Ag-KT4561 were mixed with cheese whey; $\text{AgNO}_3$ reagent (36W input power) for 48 h (~1.728 kwh)

**DISCUSSION**

The results indicated that 2 M NaCl could effectively inhibit $C. krusei$ in a monoculture of $K. marxianus$ at temperature 40 °C, pH 3.5 without affecting the growth of $K. marxianus$. However, $C. krusei$ showed lower NaCl tolerance than any other yeast species e.g. *Saccharomyces* had different sensitivity towards osmotic stress, but $C. krusei$ was inhibited efficiently at 2 M without affecting the growth of $K. marxianus$ (*Lynum and Nauth, 2000; Uchida et al., 2005*). The reported concentration is used to discriminate $K. marxianus$ as it is sensitive up to a concentration of 3 M NaCl. Stress-induced by salt induction results into two different phenomena, primarily, ion toxicity and secondly, osmotic stress. Apparently, other physiological changes can also take place such as: a) efflux of intracellular $\text{H}_2\text{O}$, i.e. total cell volume deduction; b) transient increase in glycolytic intermediates and finally triggering the hyper osmotic glycerol signaling pathway. Specific species, such as *Saccharomyces* and *Klyuyveromyces* can develop systems to counteract to osmotic stress by NaCl. Special features of *Saccharomyces* and *Klyuyveromyces* species are that they produce intracellular trehalose under stress conditions to maintain the membrane integrity and stabilizing the proteins (*Kuhn et al., 2004; Wang and Wu, 2008; Davey, 2011*). However, in large-scale fermenters, it was not possible as it would lead to high utilization of NaCl for the inhibition of $C. krusei$. At industrial scale, such inhibitions performed by utilization of NaCl is difficult because of the market price which is 16-20 USD per Kg; but the volume of NaCl required was more than 500 Kg for 40 000 L industrial reactor (*Goretti et al., 2009; Kosseva et al., 2009*). Therefore, NaCl was not a suitable approach for $C. krusei$ inhibition.

Figure 6 a) A 12 h study of $K. marxianus$ when various concentrations of Ag-KT4561 was mixed with cheese whey; b) A stability test done for 20 weeks representing growth of $K. marxianus$ (KM) at a minimum of 2.1 x 10^{10} and no traces of $C. krusei$

Table 6 Bio preservative (Ag-KT4561 conjugate) production of 20,000 L.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Items Required (amount)</th>
<th>Cost of Production (CAD $)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Silver nitrate (271.6 g) for 40 L d.H$_2$O</td>
<td>242</td>
</tr>
<tr>
<td>2</td>
<td>Culture medium of W. saturnus 360 L</td>
<td>306</td>
</tr>
<tr>
<td>3</td>
<td>Mechanical stirring of AgNO$_3$ reagent (36W Input power) for 48 h (~1.728 kwh)</td>
<td>12.19</td>
</tr>
<tr>
<td>4</td>
<td>Centrifugation (700W) for 15 mins (~0.175 kwh)</td>
<td>1.23</td>
</tr>
<tr>
<td>5</td>
<td>Freeze drying (1200W) for 24 h (~28 kwh)</td>
<td>197</td>
</tr>
<tr>
<td>6</td>
<td>10% cost of man-power</td>
<td>760</td>
</tr>
</tbody>
</table>

$kM = \text{Conc. Ag kt4561}^{-2050} nM$

![Figure 6 a) A 12 h study of $K. marxianus$ when various concentrations of Ag-KT4561 was mixed with cheese whey; b) A stability test done for 20 weeks representing growth of $K. marxianus$ (KM) at a minimum of 2.1 x 10^{10} and no traces of $C. krusei$](image.png)
and 400 mg/mL when grown in a lactose-efficient medium (cheese whey) can inhibit C. kruisi. The purpose of production of lyophilized supernatant from W. saturnus is to justify that W. saturnus produces naturally occurring extracellular killer protein KT4561, which strongly inhibited C. kruisi. The killer protein produced by W. saturnus caused cell membrane damage and an independent energy link in between the cell wall receptor and KT4561 at the region of (1→6)-β-D-glucan complex (Fang et al., 2002).

More efficient inhibition of C. kruisi was possible if W. saturnus would have been grown in a glucose-rich medium where efficient production of the killer protein could have inhibited C. kruisi. This study revealed a real understanding of the different microbial species dealt with and different behavioral patterns with respective to the varied inhibitors used for the inhibition of C. kruisi. The factor to be considered when biochemical approaches such as H$_2$O$_2$ were used is whether it again had any effect on the organic matter present in cheese whey. As over the years, several approaches have been made to protect the food and humans from consuming it against any oxidative damage. Free radicals such as hydroxyl, peroxyl, and superoxide have been bound to release when biochemical methods are used for inhibiting the food pathogens (Erdemoglu et al., 2007).

Killer protein-based nanoparticle showed an effective inhibition for C. kruisi. It was observed that 350 μM of Ag-KT4561 (with 1 ppm of Ag) could bring in effective inhibition of C. kruisi within 3 h. But beyond 350 μM concentration could affect the growth K. marxianus. K. marxianus growth was affected due to the presence of Ag$^+$ but killer protein has no effect on it. Other significant consideration was that no pH or temperature was adjusted, because Ag ion was effective against almost all pathogens. Other benefit of using biomolecule based nanoparticle was that metal nanoparticles were toxic for human or animal consumption but biomolecule based nanoparticles had shown no toxicity so far (Nel et al., 2006; Da Silva et al., 2011).

CONCLUSION

Biomolecule based nanoparticle approach (Ag-KT4561) for inhibition of C. kruisi served to be better method than other chemical and biochemical methods used in this study. Other suitable alternative approach might have been ultrafiltration, however, on an industry scale it was an expensive approach. Therefore, other suitable alternative approach towards C. kruisi (non-Candida albicans spp.) and it even supported green chemistry. Although the composition of cheese whey was known, further verification and prolonged usage of killer protein-based silver nanoparticle to sustain the antimicrobial effect need to be investigated further.

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REFERENCES


AGUIAR, C., LUCAS, C. 2000. Yeasts killer/sensitivity phenotypes and antimicrobial effect need to be investigated further.


