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Microbial conversion of tomato by *Pectobacterium carotovorum* subsp. *carotovorum* 21: a biotechnological approach to control pathogenic *Candida* species

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Abstract

The aim of this study was to evaluate the anticandidal effects of bioconverted products, obtained from the microbial conversion of tomato fruits (Solanum esculentum) by a plant pathogenic bacterium Pectobacterium carotovorum subsp. carotovorum (Pcc21) against clinical and laboratory isolates of Candida species. The bioconverted products (500 μg/disc) exhibited a potential anticandidal effect as diameters of the inhibition zones against Candida albicans KBN06P00565, C. albicans KBN06P00566, C. glabrata KBN06P00368, C. albicans KACC 30062, C. albicans KACC 30004 and C. albicans KCTC 7270 were measured in the range of 11 ± 0.7 to 17 ± 0.1 mm. The minimum inhibitory and minimum fungicidal concentration values of bioconverted products against the tested pathogens were ranged from 125 to 500 and 125 to 1000 μg/ml, respectively. The bioconverted products also exerted a remarkable anticandidal effect on the viable counts of the tested fungal isolates. In addition, a scanning electron microscopic study revealed altered or lysed morphology of C. albicans KACC 30062 at the MIC concentration of the bioconverted products. These results suggest that bioconverted products of tomato by Pcc21 could be used as alternative bio-medicinal products against Candida species.

Keywords: microbial conversion, anticandidal potential, tomato (*Solanum esculentum*), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc21)

Introduction

Since the discovery of the genus *Candida*, it has been shown to be the causative agent of many infections in an increasing range of anatomical sites and clinical settings (12). In normal healthy individuals, the yeast *Candida* is classified as a commensal organism that can colonize both internal and external surfaces. Under these circumstances an equilibrium between the host and the yeast microflora ensures the avirulent, commensal status of this pathogen. Although *Candida albicans* has been implicated in the early stages of AIDS, infections due to other *Candida* species are becoming more widespread in late-stage AIDS (9). *Candida* species produce a wide spectrum of diseases, ranging from superficial mucocutaneous disease to invasive illnesses, such as hepatosplenic candidiasis, *Candida* peritonitis, and systemic candidiasis (15). The management of serious and life-threatening

invasive candidiasis remains severely hampered by the lack of reliable antifungal drugs that allow both fungemia and tissue invasion by *Candida* species.

Before the emergence of the HIV epidemic, oral mycotic infections were treated with polyene antifungals, such as amphotericin B or nystatin, and with azoles, such as clotrimazole or miconazole. The high relapse rate in HIV-positive patients and reported toxic side effects of available synthetic chemical drugs led to the use of azoles as the first line of treatment (5, 7), however, there are not as readily absorbed, hence, their use has been limited. Because of the rising incidence of failures in the treatment of mycoses in the case of severely immunosuppressed patients, as well as increased resistance to commercial antibiotics, there is a need for the development of new therapeutic agents that support the antifungal activity of antimycotics (17).

Microbial conversion technique converts substrate products into a new chemical product with multifarious biological significance of antimicrobial, antimycotic or biomedical potentials. As reported previously, several microbially bioconverted products have been shown to possess potent biological efficacy in various *in vitro* and *in vivo* models (1-3). However, a few reports are available on the antifungal effect of bioconverted products, especially on anticandidal effect of bioconverted products of vegetables against *Candida* species. Nowadays, production of anticandidal agents of biological significance using microbial conversion technology has become a major focus of research (1, 2). Hence, microbially bioconverted products of vegetables by bacterial pathogens are being investigated as possible alternatives or complementary therapeutic agents to treat serious fungal infections caused by *Candida* species.

This is the first report on the microbial conversion of tomato fruits by a plant pathogenic bacterium *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc21). Herein this study described, the bioconverted products were obtained from the microbial conversion of tomato by Pcc21, which were further subjected to determine their anticandidal efficacy of medicinal significance against clinical and laboratory pathogens of *Candida* species.

Materials and Methods

Microorganisms

The bacterium species *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc21) used in this study for the microbial conversion of tomato fruits was obtained from Microbial Safety Division, National Academy of Agricultural Science, Rural Development Administration, Suwon, Republic of Korea. Pcc21 was grown at 28^{o}C aerobically at 150 rpm in Luria-Bertani (LB) medium containing per liter 10 g tryptone, 10 g sodium chloride and 5 g yeast extract.

The clinical isolates of *Candida albicans* KBN06P00565, *C. albicans* KBN06P00566 and *C. glabrata* KBN06P00368 were provided by the National Biobank of Korea, Chonbuk National University Hospital with informed consent under institutional review board-approved protocols. Laboratory isolates of *Candida* species such as *C. albicans* KACC 30062, *C. albicans* KACC 30004 and *C. albicans* KCTC 7270 were obtained from the Korean Agricultural Culture Collection, Suwon, Republic of Korea. Cultures of the test clinical and laboratory isolates of *Candida* species were grown on potato dextrose agar (PDA) media at 28^{o}C and stored at 4^{o}C until use.

Substrate and microbial conversion

For microbial bioconversion, ripen tomato fruits (*Solanum esculentum*) were purchased from a local market of Gyeongsan city, Gyeongbuk, Republic of Korea, which

were surface sterilized with 70% ethanol, and further washed three times with distilled water to remove any superficial microbial load. Adhering water contents were dried to avoid any microbial contamination. Around 300 g of tomato fruit samples were crushed in small pieces using a sterilized knife. For microbial conversion, contamination free substrate tomato samples were inoculated with 30 ml of Pcc21 bacteria at 0.5 OD_{600} grown for 24 h in 500 ml Erlenmeyer flask, and then continuously incubated for 7 days at 200 rpm in a Psycro Therm controlled environment shaker (Model: VS 8480, Vision Scientific Co. Ltd., Republic of Korea) at 28^{o}C. After the conversion of the substrate tomato samples by Pcc21, the supernatant and pellet were separated by centrifugation. The pellet was discarded and the collected supernatant layer was immediately extracted twice with ethyl acetate organic solvent to kill the culture. The bioconverted products of tomato were obtained, and the evaporation of the solvent from the combined extract of bioconverted products was accomplished using a rotary evaporator (EYELA N1000, Japan). All chemicals and solvents used were of analytical grade with over 99.5% purity (Merck, Inc., Germany).

Anticandidal activity assay

A standard agar diffusion method was used for determining the anticandidal potential of bioconverted products of tomato (2). A 100 µl of standardized inoculum containing 10^7 CFU/ml of fungal suspension was loaded uniformly on petri plates with 20 ml of potato-dextrose agar (PDA) medium, and allowed to dry for 5 min. A sterile Whatman No. 1 filter paper disc (6 mm diameter) was impregnated with 10 µl of bioconverted products of tomato, corresponding to 500 µg/disc, dissolved in the same solvent used for the extraction. A control was composed of the supernatant of crushed tomato fruits. Negative controls were prepared using the same solvent employed to dissolve the sample as well as the supernatant of 24 h grown culture of Pcc21. Standard reference antibiotic, amphotericin B (10 µg/disc, Sigma-Aldrich Co., St. Louis, MO, USA) was used as the positive control for the tested isolates of *Candida* species. After incubating the plates at 28ºC for 2 - 3 days, anticandidal activity was evaluated by comparing the diameter of the zones of inhibition against the tested isolates of *Candida* species. For the anticandidal activity assay, the experiment was replicated at least three times.

Minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations

The MIC of the bioconverted products of tomato was evaluated by the two-fold serial dilution method (2). The bioconverted products of tomato dissolved in 5% dimethyl sulphoxide were incorporated into PDB medium to obtain a concentration of 2000 µg/ml, and then serially diluted to achieve 1000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/ml, respectively. A 10 µl standardized suspension of each *Candida* isolate (10^7 CFU/ml approximately) was transferred to each tube. The control tubes with only candidal suspension were incubated at 28^{o}C for 2 - 3 days. MIC expressed in µg/ml was defined as the lowest concentration of the bioconverted products that exhibited no growth of test isolates of *Candida* species by macroscopic evaluation. Further, the concentrations showing complete inhibition of visual growth of fungal isolates were identified, and 50 µl of each diluted culture broth transferred on to the agar plates, and the plates were incubated for specified time and at temperature as mentioned above. The complete absence of growth on the agar surface in the lowest concentration of sample was defined as MFC.

Cell viability assay

For viable count assay, each of the tubes containing re-suspended candidal suspension (approximately 10^7 CFU/ml) was inoculated with 125 µg/ml concentration of the

bioconverted products of tomato in 10 ml PDB broth, and incubated at 28ºC as previously described (1). For viable cell counts, samples were taken out at 0, 20, 40, 60, 80, 100 and 140 min time intervals, and the viable cells were counted as follows: after incubation, one ml of the re-suspended culture was diluted into 9 ml buffer peptone water, there by diluting it 10-fold. A 0.1 ml sample of each treatment was diluted further and spread on the surface of PDA agar plates. Newly formed colonies were counted after 2 - 3 days of incubation at 28ºC. The controls were composed of inoculums without bioconverted products of tomato for each *Candida* isolate with same experimental conditions. Each assay in this experiment was replicated three times.

Scanning electron microscopic (SEM) analysis

A SEM study was performed to determine the anticandidal efficacy of bioconverted products of tomato on the morphology of *C. albicans* KACC 30062 isolate, using the MIC. Controls were prepared without the inoculums of bioconverted products of tomato. Further, to observe the morphological changes, a modified method of SEM was adopted from the Kockro method (10). The fungal sample was washed gently with 0.1M phosphate buffer solution (pH 7.2), and fixed with 2.5% (w/v) glutaraldehyde solution. The specimen was dehydrated using sequential exposure per ethanol concentrations ranging from 50 to 100%, followed by replacing ethanol by tertiary butyl alcohol. After dehydration, the specimen was dried under CO_2. Finally, the specimen was sputter-coated with gold in an ion coater for 2 min, followed by SEM examinations (S-4300; Hitachi, Japan).

Statistical analysis

Each experiment in this study was run in triplicate, and the average values were calculated. The statistical analysis was carried out by employing one way ANOVA ($p < 0.05$) with a SPSS statistical package (version 11.0).

Results

Anticandidal effect of bioconverted products of tomato

The anticandidal effect of the bioconverted products of tomato against the employed clinical and laboratory isolates of *Candida* species was monitored by the presence of diameter of zones of inhibition. The bioconverted products of tomato (500 µg/disc) exhibited potent inhibitory effect against the tested isolates of *Candida* species (table 1). Clinical isolates of *C. albicans* KBN06P00565, *C. albicans* KBN06P00566, *C. albicans* KBN06P00368 along with a laboratory isolate of *C. albicans* KACC 30062 were found to be the most inhibited fungal pathogens by the bioconverted products of tomato, with their respective diameter of zones of inhibition of 15 ± 0.3, 17 ± 0.1, 16 ± 0.5 and 14 ± 0.2 mm. However, the diameters of zones of inhibition of bioconverted products against *C. albicans* KACC 30004 and *C. albicans* KCTC 7270 were found to be 12 ± 0.4 and 11 ± 0.7 mm, respectively (table 1). As negative controls, only solvent and tomato supernatant as well as bacterial supernatant of Pcc21 had no anticandidal effect. It was confirmed in this assay that bioconverted products of tomato significantly inhibited the growth of both clinical and laboratory isolates of *Candida* species (diameter of zones of inhibition: 11 ± 0.7 to 17 ± 0.1 mm) than that of standard antibiotic amphotericin B (diameter of zones of inhibition: 11 ± 0.7 to 16 ± 0.3 mm) (table 1).

Table 1. Anticandidal effect of microbially bioconverted products of tomato by Pcc21

Fungal pathogens	Diameter of zones of inhibition (mm)[x]	
	Bioconverted products	Standard[y]
Candida albicans KBN06P00565	15 + 0.3b[z]	12 + 0.5bc
Candida albicans KBN06P00566	17 + 0.1a	16 + 0.3a
Candida glabrata KBN06P00368	16 + 0.5a	12 + 0.8bc
Candida albicans KACC 30062	14 + 0.2bc	11 + 0.7c
Candida albicans KACC 30004	12 + 0.4c	14 + 0.5b
Candida albicans KCTC 7270	11 + 0.7d	12 + 0.8bc

[x] Diameter of inhibition zones of bioconvereted products of tomato by Pcc21 (tested volume 10 µl, corresponding to 500 µg/disc).
[y] Standard antibiotic amphotericin B (10 µg/disc).
[z] Values followed by the same letter are not significantly different ($P > 0.05$).

MIC and MFC

The bioconverted products of tomato showed potent inhibitory effect as MIC and MFC values against the tested isolates of *Candida* species. The MIC and MFC values of the bioconverted products of tomato against the tested isolates of *Candida* species were found in the range of 125 to 500 and 125 to 1000 µg/ml, respectively (table 2). When standard amphotericin B was tested for the MIC values against the tested isolates of *Candida* species, the MIC values were ranged from 15.62 to 125 µg/ml. It was noted that *C. albicans* KBN06P00566 (clinical isolate) and *C. albicans* KACC 30004 (laboratory isolate) were found less sensitive to amphotericin B as compared to the other isolates of *Candida* species. In this assay, clinical isolates of *C. albicans* KBN06P00566, KBN06P00368, KBN06P00565 and *C. albicans* 30062 (laboratory isolate) were found to be the most susceptible *Candida* species to the bioconverted products of tomato with MIC value ranging from 125 to 250 µg/ml, followed by *C. albicans* KACC 30004 (MIC: 500 µg/ml) and *C. albicans* KACC 7270 (MIC: 500 µg/ml).

Table 2. Determination of MIC and MFC of microbially bioconverted products of tomato by Pcc21

Fungal pathogens	Bioconverted products[a]		Standard[d]
	MIC[b]	MFC[c]	MIC
Candida albicans KBN06P00565	250	250	62.5
Candida albicans KBN06P00566	125	125	125
Candida glabrata KBN06P00368	125	125	62.5
Candida albicans KACC 30062	250	500	15.62
Candida albicans KACC 30004	500	1000	125
Candida albicans KCTC 7270	500	1000	62.5

[a] Microbially bioconverted products of tomato by Pcc21; [b] Minimum inhibitory concentration (values in μg/ml).

[c] Minimum fungicidal concentration (values in μg/ml); [d] Amphotericin B (values in μg/ml).

Effect of bioconverted products on cell viable counts

To evaluate the anticandidal effect of the bioconverted products of tomato on the tested isolates of *Candida* species, a cell viable count assay was performed. Bioconverted products of tomato had a negative effect on the growth of tested fungal isolates of *Candida* species at the used concentrations. At 80 min exposure, near about 70-90% inhibition of the viable cells was observed against both clinical and laboratory isolates of *Candida* species (figure 1). Further, a 100 min exposure time of the bioconverted products of tomato completely inhibited the CFU numbers of one of the tested clinical isolates of *C. albicans* KBN06P00566. However, complete inhibition of cell viable counts of *C. albicans* KBN06P00368, KBN06P00565, *C. albicans* KACC 30004, KACC 30062 and KCTC 7270 was observed at 140 min exposure time of the bioconverted products of tomato, and no formation of colony forming units was observed.

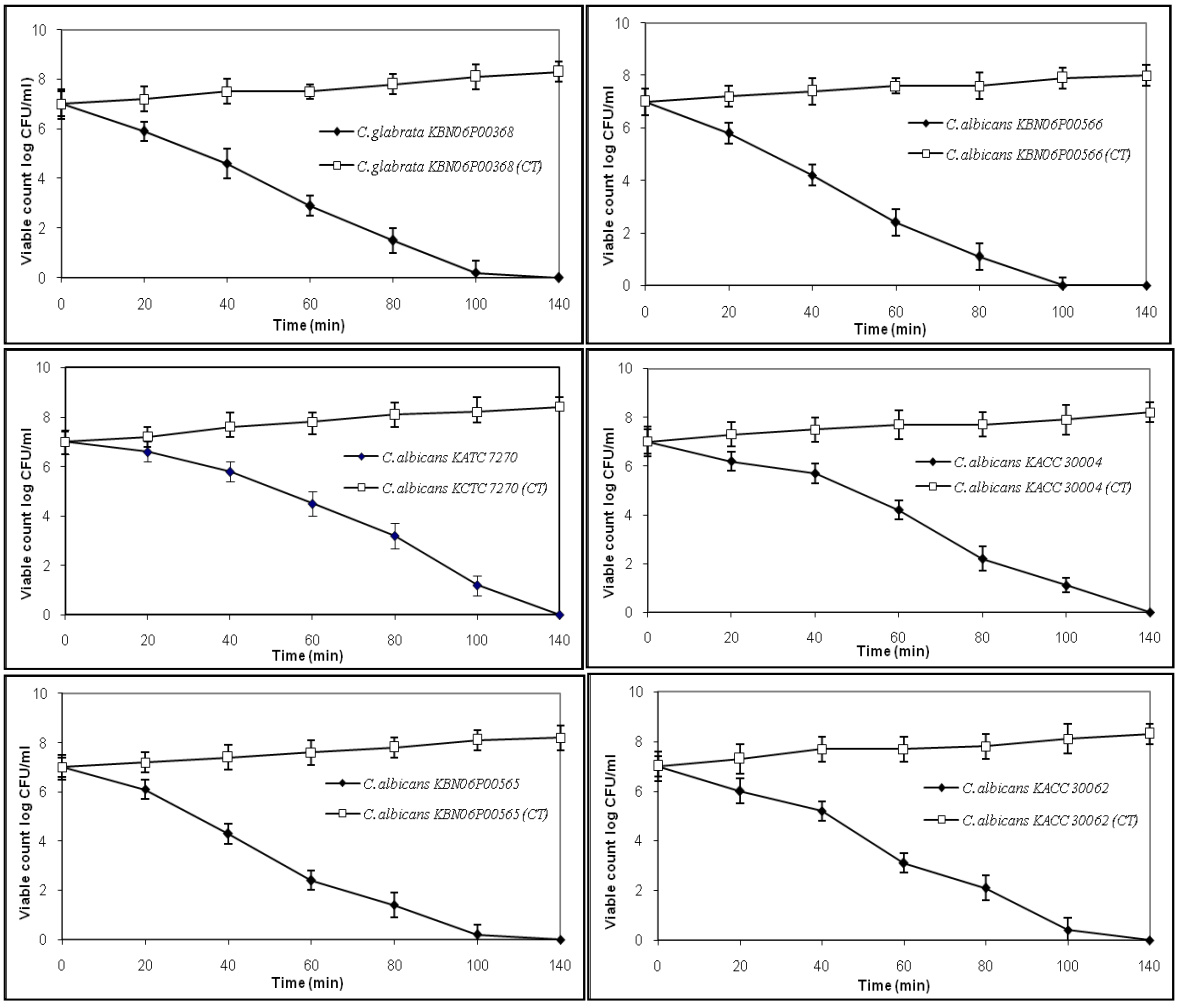

Figure 1. **Anticandidal effect of microbially bioconverted products of tomato by Pcc21 on viable cell numbers of tested fungal *Candida* isolates.**

SEM analysis

The anticandidal effect of the bioconverted products of tomato on the morphology of *C. albicans* KACC 30062 was visualized by the elaborative study of an SEM. The bioconverted products of tomato severely altered the cell morphology of *C. albicans* KACC 30062 as compared to the control group served without any treatment (figure 2). In contrast to

the regular and smooth surface of control *C. albicans* KACC 30062 cells (figure 2a), the cells treated with the bioconverted products of tomato at the MIC concentration (250 μg/ml) had severe detrimental effect on the morphology of *C. albicans* KACC 30062, revealing disruption and swelling of the cells (figure 2b), and formation of dead cells by lysis (figure 2c).

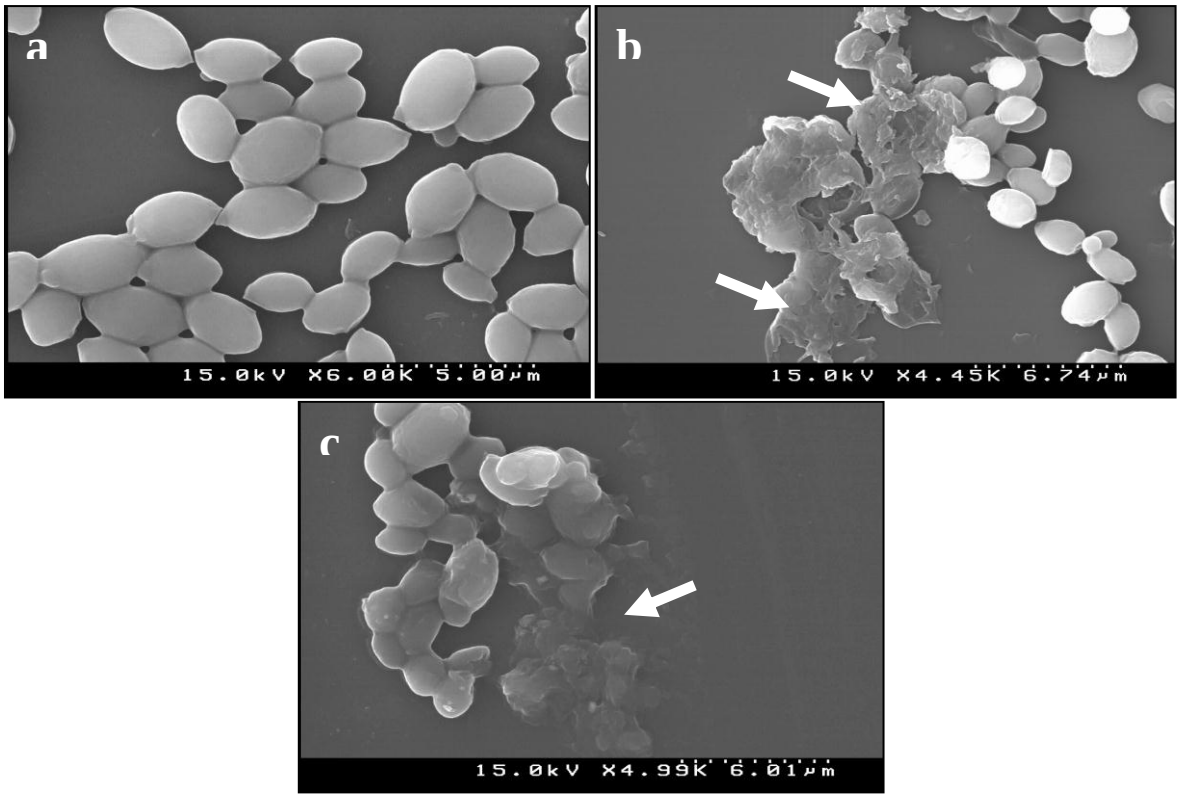

Figure 2. **Scanning microscopy study of microbially bioconverted products of tomato by Pcc21 on the morphology of *C. albicans* KACC 30062.**

a) Control, showing a regular and smooth surface; b) disruption and swelling of the cells; c) lysed or dead cell formation.

Discussion

The results described in this study showed potential anticandidal effects of microbially bioconverted products of tomato fruits, inhibiting the growth of various clinical and laboratory isolates of *Candida* species. As confirmed in this study that bioconverted products of tomato severely inhibited the growth of tested clinical isolates of *Candida* species, these results suggest that bioconverted products of tomato by Pcc21 may act as effective antifungal agents when applied to a clinical *Candida* infection situation. Only the supernatants from tomato and Pcc21, as negative controls had no anticandidal effect against any of the tested clinical or laboratory isolates of *Candida* species (data not shown).

In this study we also described the complex effect of the bioconverted products of tomato on cell viable counts of the tested isolates of *Candida* species. The bioconverted products of tomato exhibited a wide range of anticandidal effect against the tested isolates of *Candida* species. It has been reported that various bioconverted and microbial products were found to display significant inhibitory effects on the viable counts of the fungal pathogens (8, 16). Previous studies suggest that various bioconverted products from different origins can be available for trials to control severe fungal infections caused by various pathogenic and

clinical isolates of *Candida* species. Certain extracts as bioconverted products act in many ways on various types of disease complex, or may be applied in the same way against pathogenic microorganisms as alternative bio-medicinal products. Hence, it might be possible that bioconverted products of tomato can be used as leading factors in a wide range of activities against many pathogenic microbes, where these pathogens have developed resistance against the specific fungicides (13). In addition, the SEM study revealed potential detrimental effect of the bioconverted products of tomato on *C. albicans* KACC 30062 with altered morphology (figure. 2). These altered morphological features might be occurred due to the effect of bioconverted products of tomato on membrane integrity, thereby resulting in the lysis of cell wall followed by the loss of intracellular dense material on the surface of treated cells, as also evident by the previous findings (8, 18). As reported previously, *Candida* species treated with therapeutic antifungal agent accumulated phosphatidylserine with a lower proportion of phosphatidylcholine. Phosphatidylcholine is synthesized from phosphatidylserine, hence, accumulation of phosphatidylserine was suggested to the interference of the enzymes, catalyzing the biosynthetic pathway. Changes in membrane fluidity usually occur due to alterations in the membrane lipid composition (14, 19), which are suspected to be a compensatory mechanism to counter the lipid disordering effects of the treatment agent. Therefore, relevance of bioconverted products of tomato as an antifungal treatment to the accumulation of phosphatidylserine is clearly required in further investigation.

In fact, information on the anticandidal effects of the bioconverted products of tomato is scant, and these results show, for the first time that bioconverted products of tomato by Pcc21 possessed substantial anticandidal effect against various clinical and pathogenic isolates of *Candida* species. *Candida* infections are getting serious worldwide due to the pathogenic disorders in human beings, although control measures are available but limitedly effective (6). Hence, microbial conversion of vegetable products may be considered as an effective alternative biological approach to develop new and novel types of anticandidal agents for the preventive treatment of serious fungal infections in human beings.

Recently we reported the antifungal potential of bioconverted vegetable products (1, 2). We also confirmed the industrial potential of several bioconverted products, obtained from the microbial conversion of unsaturated fatty acids (3, 4). Besides, the microbially bioconverted products of leptomycin B by a bacterial strain *Streptomyces* sp. ATS 1287 had significant anti-proliferative activity (11). The production of antifungal materials through bioconversion using microorganisms has also been confirmed by others (18). Hence, microbial conversions of vegetables may yield diverse range of potential and value-added products similar to other types of microbial products. However, there has been a continuous requirement to further improve the productivity and efficiency of microbial systems in order to develop more efficient bioconversion processes which can be obtained by employing better microbial strains. In addition, microbial conversion of vegetables as a feasible technology could reduce the wastes of the natural resources and the chemical impact on environmental quality. If this newly developed, cheap and easily accessible technique is going to be practical, there could be a high medicinal potential of microbially bioconverted vegetable products controlling *Candida* species.

Conclusions

In the present study, bioconverted products of tomato by Pcc21 evoked higher controlling efficacy for *Candida* species than the standard antibiotic treatment. Anticandidal effects of microbially bioconverted products against the pathogenic and clinical isolates of

Candida species may offer new applications of clinical trials. Hence, the availability of microbially bioconverted products may contribute to providing sustainable anticandidal tools. These findings conclude that bioconverted products obtained from the microbial conversion of tomato by Pcc21 could be served as affective bio-medicinal products for their possible applications and clinical efficacy in medicine industry. Future research is warranted to identify bioactive molecules from the bioconverted products of tomato that inhibited the growth of *Candida* species.

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