African Journal of Microbiology Research Vol. 5(13), pp. 1619-1628, 4 July, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR11.197
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

Screening and production of antibacterial compound from *Trichoderma* spp. against human-pathogenic bacteria

Rashad M. Saleh[1], Saleh A. Kabli[1], Saleh M. Al-Garni[1] and Saleh A. Mohamed[2,3]*

[1]Department of Biology, Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudia Arabia.
[2]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Kingdom of Saudia Arabia.
[3]Department of Molecular Biology, National Research Centre, Dokki, Cairo, Egypt.

Accepted 10 May, 2011

This study focus on the production of antibacterial compound from *Trichoderma* spp. Screening of antibacterial activities in some *Trichoderma* spp. was investigated using CYS80 medium. *Trichoderma reesei* and *Trichoderma viride* were highly effective toward human-pathogenic bacteria tested. *T. viride* and *T. reesei* were separately applied on Sephacryl S–200 column. Column fractions No. 56 to 64 for *T. viride* and fractions No. 57 to 66 for *T. reesei* had inhibitory effect against the most pathogenic bacteria examined. *T. reesei* and *T. viride* Sephacryl S-200 fractions with antibacterial activity were analyzed by Gas chromatography–mass spectrometry (GC- MS). The product with highest peak (95%), using different libraries, was kojic acid. The yield of kojic acid crystals from *T. reesei* and *T. viride* Sephacryl S-200 fractions were 3 and 5 g/L, respectively. Physical analysis of kojic acid with respect to UV, IR, ^{1}HNMR analysis and melting point was examined. The minimum inhibitory concentration (MIC) of kojic acid and augmentin, as control, against human-pathogenic bacteria were evaluated. Kojic acid and augmentin showed a similar time-killing kinetics with human-pathogenic bacteria. The level of kojic acid increased with decreased level of reducing sugar during the growth of *T. reesei* and *T. viride* suggesting that the enzyme system for the synthesis of kojic acid found in the cell of these fungi.

Key words: *Trichoderma* spp., human-pathogenic bacteria, kojic acid, minimum inhibitory concentration.

INTRODUCTION

Traditional methods used to protect humans from diseases have been largely based on the use of chemical compound called antibiotics. Antibiosis is defined as the inhibition or destruction of a microorganism by substances such as specific or nonspecific metabolites, lytic agents, or enzymes that are produced by another microorganism, which operate at low concentrations: less than 10 ppm (Irtwange, 2006). Antibiotic production by *Trichoderma* spp. was first described by Weindling (1934). *Trichoderma* have been known since at least the1920s for their ability to act as biocontrol agents against phytopathogenic fungi and some strains are able to produce metabolites that enhance plant growth (Howell, 1998; Harman, 2006; Carvajal et al., 2009; Nallathambi et al., 2009). The classical mechanisms of control have included antibiosis, mycoparasitism, and competition for nutrients. *Trichoderma* strains inhibit or kill plant-pathogenic fungi through production of antifungal antibiotics and/or hydrolytic enzymes. The ability to promote growth and induce resistance in plants is activities which have also been described for members of this genus (Monte, 2001; Harman et al., 2004). In contrast to other fungi, *Trichoderma* spp. has been reported to have limited applications in biocontrol of pathogenic bacteria (Verma et al., 2007).

Kojic acid ($C_6H_6O_4$-molecular mass: 142.0266) is a major secondary metabolite produced by a limited range of microorganisms, including *Aspergillus oryzae*, *Aspergillus flavus*, and *Aspergillus tamarii*, as well as

*Corresponding author. E-mail: saleh38@hotmail.com

Penicillium species and certain bacteria in the stationary phase of growth (Wilson, 1971; Blumenthal, 2004; Bentley, 2006; Terabayashi et al., 2010). All species in *Aspergillus* section *Flavi,* except the phylogenetically outlying *Aspergillus avenaceus*, produced kojic acid (Frisvad and Samson, 2000). Most important biological functions of kojic acid are antibacterial, fungicidal, insecticidal activities, and it is approved as food and cosmetic additive (Burdock et al., 2001). The action of the enzyme peroxidase on kojic acid produces a bright greenish-yellow fluorescence (BGYF) that has been used to detect *A. flavus* (Maragos, 2004). The pyridone structure, formed by kojic acid, is found in a number of useful pharmaceuticals (Hider and Zhou, 2005). Also Kojic acid can act as a tyrosinase inhibitor (inhibition of melanin formation). Applications of Kojic acid include the prevention of discolouration of crustacean, meat and fresh vegetables, the preparation of derivative esters that is (kojic oleate, kojic stearate), the adhesives, and the chelate-forming resins and as a plant growth- regulating agent (Cabanes et al., 1994; Chemos Group, 2000; Jarchem, 2000). Kojic acid has been used in flavourings at 0.2% to add luster, to prevent discolouration on vegetables at 1.0%, in flour production at 0.1%, in meat production at 0.2%, in syrup at 0.05% and as a whitening agent in cosmetics at 0.5 to 1.0% (Chemos Group, 2000).

Kojic acid had been inhibited several genera of bacteria including *Aerobacter*, *Bacillus*, *Chromobacterium*, *Clostridium*, *Corynebacterium*, *Diplococcus*, *Escherichia*, *Gaffkya*, *Klebsiella*, *Micrococcus*, *Neisseria*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Staphylococcus* and *Vibrio* (Morton et al., 1945). Moubasher et al. (1979) extracted Kojic acid from moldy cheese and the extract was found to produce a zone of inhibition in a culture of *Bacillus megaterium*. Kojic acid was also found to be active against various human *Tubercle bacilli* grown under a variety of cultural conditions (Lee et al., 1950). Kojic acid inhibited swarming of *Azospirillum brasilense* and *Proteus mirabilis* at 1.0 and >10 mg/ml, but stimulated swarming of *Proteus mirabilis* at low concentrations. Growth inhibition of *Bacillus thuringiensis* was seen at 0.25 mg/ml (Lenz et al., 1989). Although *Trichoderma* spp. had the ability to produce antifungal compounds, very little information has been reported on the production of antibacterial compounds from *Trichoderma* spp. Therefore, the main goal of this study is focused on the production of antibacterial compound from *Trichoderm*a spp. against human-pathogenic bacteria.

MATERIALS AND METHODS

Trichoderma spp.

Trichoderma harzianum, Trichoderma viride, *Trichoderma reesei*, *Trichoderma psudokoningii*, *Trichoderma virens* and *Trichoderma V6* were obtained from Plant Pathology Unit, National Research Centre, Cairo, Egypt.

Pathogenic bacteria

Escherichia coli (EC1) ATCC 25922, *E. coli* (EC2) ATCC 35218, *Streptococcus agalactiae* (SA) ATCC12386, *Staphylococcus aureus* (SAU) ATCC 29213, *Streptococcus pneumonia* (SP) ATCC 49619, *Streptococcus pyogenes* (Spy) ATCC19615, *Entreococcus faecalis* (EF) ATCC29212, Methicillin-Resistant *S. aureus* (MRSA) ATCC 4333, *Klebsiella pneumoniae* (KP) ATCC 700603*, Shigella sonnei* (Sh.s) ATCC 25931, *P. mirabilis* (Prot.M) ATCC 14153 and *Pseudomonas aeruginosae* (PA) ATCC27853 obtained from hospital of King Abdulaziz University, Jeddah, Kingdom of Saudia Arabia.

Cultivation and culture conditions

T. harzianum, T. viride, T. reesei, T. psudokoningii, T. virens and *T. V6* were cultivated and maintained on slants of potato dextrose agar for 5 days at 28°C. Conidia were scrapped from mycelia which were grown on slants and cultivated on autoclaved CYS80 medium (sucrose 80 g /L, yellow corn meal 50 g /L, yeast extract 1 g /L) as solid state fermentation (Suay et al., 2000). Three hundred milliliters flasks were incubated for 14 days at 28°C on a laboratory incubator. Crude extract was obtained by filtration and subjected to centrifugation at 5000 rpm for 15 min. The supernatant was pooled and designated as crude extract.

Bioassay of *Trichoderma* spp. crude extract

Microbial suspension in sterile water containing 10^8 CFU/ml of bacteria was adjusted to McFarland No. 0.5 standard turbidity (Cheesbrough, 2006). The bacterial inoculum is uniformly spread using sterile cotton swab on a sterile Petri dish Muller Hinton agar. Crude antibacterial compounds were added to each of the 5 wells (7 mm diameter holes cut in the agar gel, 20 mm apart from one another). The systems were incubated for 24 h at 36 ±1°C, under aerobic conditions, except for *Streptococcus pneumonia* which incubated under anaerobic condition using carbon dioxide (8%) incubator. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in millimeter. Tests were performed in duplicate (Smania et al., 1999).

Sephacryl S-200 chromatography

Crude extracts of *T. viride* and *T. reesei* with highest antibacterial activity were concentrated by lyophilization. The concentrated crude extracts were separately loaded on a Sephacryl S-200 column (90 × 1.6 cm i.d.) equilibrated with 20 mM Tris-HCl buffer, pH 7.2. The antibacterial compounds were eluted with the same buffer. Fractions in 3 ml volume were collected at a flow rate of 60 ml/h. The eluted fractions were monitored at 280 nm and assayed for antibacterial activity.

GC–MS analysis

The Sephacryl S-200 fractions with antibacterial activities were analyzed by GC–MS (QP-2010, Shimadzu Co., Kyoto, Japan) equipped with 30 m x 0.25 mm RTX-5MS column (RESTKCrop). The carrier gas was helium. The temperature program was set as follows: 70°C hold for 5 min, raised to 280°C, and hold for 5 min. The injector and detector temperatures were set at 260 and 280°C, respectively. The ion source and interface temperatures were set

at 200 and 230°C, respectively. The mass range was scanned from 50 to 900 amu. The control of the GC-MS system and the data peak processing were controlled by means of Shimadzu's GC-MS solution software, version 2.21. Compound identification was verified based on the relative retention time and mass fragmentation pattern spectra with those of standards and the NIST147. LIB. Database of the GC-MS system (Shimadzu). The samples will be prepared in methanol before analyzed by GC/MS (Ao et al., 2008).

Crystallization of kojic acid

Kojic acid crystals were obtained according to Morton et al. (1945). Sephacryl S-200 fractions with antibacterial activity were concentrated till least volume using rotary evaporator and subjected to 0°C, where the crystals of kojic acid were formed. The kojic acid crystals were loaded on filter paper Whatman No. 1 and washed several times with benzene. The Kojic acid crystals were dissolved in ethylacetate several times for get rid the impurities and the ethylacetate solution was dried at room temperature, where the pure crystals were appeared.

Physical analysis of kojic acid

The IR spectra recorded for KBr discs on a Perkin Elemer Spectrum RXI FT-IR systems No. 55529. ^{1}HNMR was determined for solution in deutrated DMSO with a Bruker NMR Advance DPX 400 MH using TMS as an internal standard solvent. Electronic absorption spectra were recorded in DMF on Shimadzu UV and visible 3101 PC spectrophotomer. Melting point of Kojic acid was determined in an electrothermal Bibby Stuart Scientific melting point SMP (US).

Colorimetric determination of kojic acid

Kojic acid was determined by two methods: the first was used for rapidly detection of kojic acid using drop of ferric chloride with appearance of a deep red colour (Morton et al., 1945), the second method was used for quantitatively determination of kojic acid using spectrophotometric method. Tanigaki et al. (1980) developed a spectrophotometric method with 2,6-dichlorophenolindophenol (DCIP) for the determination of Kojic acid in the fermentation medium even in the presence of various organic substances. The reaction mixture included 0.5 ml of 0.2 M sodium acetate buffer, pH 6, 0.2 ml of 0.2 mM DCIP (DCIP dissolved in n-butanol and 0.2 M sodium chloride). The reduction of color was detect at 525 nm.

Determination of reducing sugar

The reducing sugar was determined by the method of Miller (1959).

Minimum inhibition concentration (MIC) test

Microdilution method

Prepare antibacterial agent: 50 mg of Kojic acid and 10 mg of augmentin (as control) were separately dissolved in 1 ml of Muller Hinton broth. Prepare a standardized inoculum using growth method as mentioned above. MIC was determined by microdilution broth following the procedure recommended by the National Committee for Clinical Laboratory Standard (2009) (formerly the NCCLS), according to protocols M7-A7. Augmentin was used as the reference compound for bacteria. Two Gram-positive bacteria (MRSA and *S. aureus*) and three Gram-negative bacteria (*E. coli*, *P. aeruginosae* and *S. sonnei*) were used as quality control strains. The volume of Muller Hinton broth in the well was 0.1 ml and the inoculum volume was 0.005 ml, then the 0.5 McFarland suspensions (1 x 10^8 CFU/ml) should be diluted 1:10 to yield 10^7 CFU/ml. When 0.005 ml of this suspension was inoculated into the broth in wells, the final test concentration of bacteria will be approximately 5 x 10^5 CFU/ml (or 5 x 10^4 CFU/well in the microdilution method). To prevent drying, seal each tray in a plastic bag, with plastic tape, or with a tight-fitting plastic cover before incubating.

Incubation

Incubate the inoculated microdilution trays at 35 ± 2°C for 16 to 20 h in an ambient air incubator. To maintain the same incubation temperature for all cultures, do not stack microdilution trays more than four high.

Determining MIC end points

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in microdilution wells as detected by the unaided eye. Viewing devices intended to facilitate reading microdilution tests and recording of results may be used as long as there is no compromise in the ability to discern growth in the wells. Compare the amount of growth in the wells or tubes containing the augmentin or Kojic acid with the amount of growth in the growth-control wells (no antimicrobial agent) used in each set of tests when determining the growth end points. For a test to be considered valid, acceptable growth (≥2 mm button or definite turbidity) must occur in the growth-control well.

Susceptibility testing (time-kill kinetics)

Time-kill kinetics has been obtained using peak concentration of MIC for Kojic acid and augmentin in Muller Hinton Broth for each bacterial strain. Prior to the time-killing kinetics experiments, several colonies of each bacterial strain were incubated aerobically overnight in Muller Hinton Broth. Cultures were incubated at 37°C in an aerobic trays, Kojic acid or augmentin solutions were added to each well to produce antibiotic concentrations as described above. No antibiotic was added to the control well. Cultures were then incubated at 37°C under aerobic conditions. At predetermined time points of 0, 2, 4, 6, 8, 10, 12 and 24 h following the incubation of Kojic acid and augmentin separately into the wells, 0.001 µl using sterilized loop samples of the cultures were withdrawn aseptically and streak on blood agar plate evenly over the surface of the medium in three directions, rotating the plate approximately 60° to ensure even distribution. With the petri dish lid in place, allow 3 to 5 min (no longer than 15 min), for the surface of the agar to dry and incubated at 36±1°C for 24 h. Bacteria colonies were counted using plate counter.

Statistical analysis

Each value of enzyme activity represents the mean of three runs ±S.E.

RESULTS AND DISCUSSION

Screening of antibacterial activity in *Trichoderma* spp.

Screening of antibacterial activity in crude extracts from

Table 1. Antibacterial activity of *Trichoderma* spp. extracts on growth of *Escherichia coli* (EC1) ATCC 25922, *Escherichia coli* (EC2) ATCC 35218, *Streptococcus agalactiae* (SA) ATCC12386, *Staphylococcus aureus* (SAU) ATCC 29213, *Streptococcus pneumonia* (SP) ATCC 49619, *Streptococcus pyogenes* (Spy) ATCC19615, *Entreococcus faecalis* (EF) ATCC29212, Methicillin - Resistant *S. aureus* (MRSA) ATCC 4333, *Klebsiella pneumoniae* (K.P) ATCC 700603, *Shigella sonnei* (Sh.s) ATCC 25931, *Proteus mirabilis* (Prot.M) ATCC 14153 , *Pseudomonas aeruginosae* (PA) ATCC27853 in aerobic condition.

	Inhibition zone (mm)											
Bacteria / Fungi	EC1	EC2	SA	SAU	SP*	Spy	EF	MRSA	K.P	Sh.s	Prot.M	PA
T. V6	-	-	-	18±1.2	12±0.6	10±0.6	-	30±1.5	20±1.3	25±1.2	20±1.3	-
T. pseudokoningii	-	-	-	20±1.3	20±0.9	-	-	-	-	-	-	-
T. reesei	15±0.9	10±0.4	10±0.5	13±0.8	10±0.2	13±0.8	-	10±0.8	20±1.1	25±1.9	10±0.7	20±1.2
T. viride	20±1.0	14±0.6	15±1.0	28±1.2	15±0.4	12±0.8	10±0.2	20±01.6	20±1.4	25±0.9	23±1.5	22±0.7
T. herzanium	-	14±0.7	-	18±0.8	-	-	-	10±0.5	20±0.8	21±1.5	17±0.7	12±0.2
T. virens	-	12±0.9	-	10±0.3	10±0.3	10±0.5	-	12±0.8	-	-	-	-

Medium: CYS80 medium, *Anaerobic condition, each value represents the mean of three runs ±S.E.

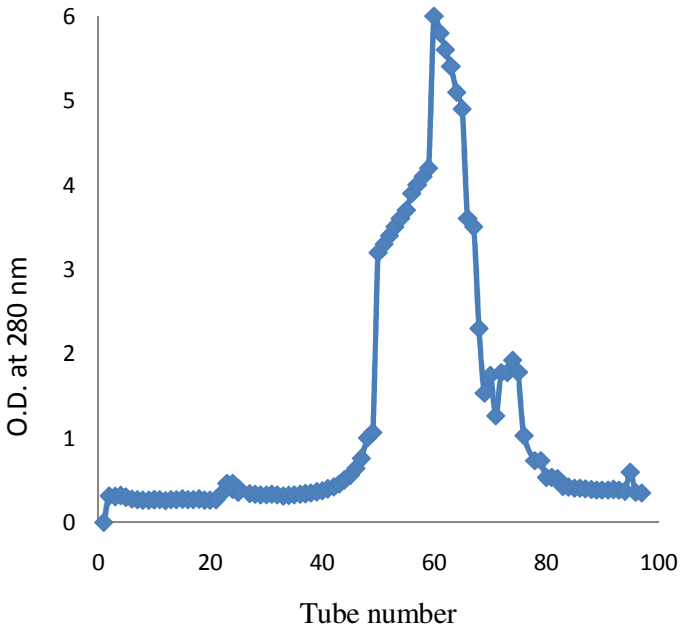

Figure 1. Chromatography of *T. viride* crude extract on Sephacryl S-200 colum (90 x 1.6 cm i.d) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions.

some *Trichoderma* spp. was investigated using CYS80 medium. Most *Trichoderma* spp. had inhibitory effects against twelve of tested human-pathogenic bacteria (Table 1). *T. reesei* and *T. viride* were highly effective toward pathogenic bacteria tested *E. coli* (EC1), *E. coli* (EC2), *S. agalactiae* (SA), *S. aureus* (SAU), *S. pneumonia* (SP), *S. pyogenes* (Spy), *E. faecalis* (EF), (MRSA), *K. pneumoniae* (KP), *S. sonnei* (Sh.s), *P. mirabilis* (Prot. M) and *P. aeruginosae* (PA). The inhibition zones caused by extracts of these fungi ranged from 10 to 28 mm inhibition zones. Different inhibition zones ranged from 1 to 42.3 mm were reported for *sobolifera, T. koningii, Chaetomium cupreum* and *Penicillium sclerotiorum* against EC1 and EC2 (Imtiaj and lee, 2007; Takahashi et al., 2008). SP, Spy and PA were inhibited by *Verticillium albo-atrum* (3 mm inhibition zone), *Verticillium lecanii* (2 mm inhibition zone) and *Verticillium albo-atrum* (2.3 mm inhibition zone) (Mekawey, 2010). On the contrary, SP, Spy, EF and PA not inhibited by *Agarius* cf. *nigrecentulus, Agrocybe perfecta,* Basidiomycetes, *Climadocon pulcherrimus, Phellinus* sp. and *Tyromyces duracinus* (Rosa et al., 2003). Very little information has been reported on the inhibition of SA, MRSA, KP, Sh.s and Prot.M by fungi.

Chromatography on sephacryl S-200

The crude extracts of *T. viride* and *T. reesei* with highest antibacterial activity were separately applied on Sephacryl S – 200 column (90 × 1.6 cm i.d.) and eluted by Tris-HCl buffer, pH 7.2 and monitored by measuring O.D at 280 nm (Figures 1 and 2). Fractions No. 56 to 64 for *T. viride* and fractions No. 57 to 66 for *T. reesei* had inhibitory effect against the most human-pathogenic bacteria examined.

Analysis of the Sephacryl S-200 fractions by GC-MS

T. viride and *T. reesei* Sephacryl S-200 fractions with antibacterial activity were analyzed by GC-MS to determine their chemical composition that may contribute to the antibacterial activity. GC-MS analysis showed that these fractions contained a variety of compounds. The product with highest peak (95%) was Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone). This is the first information reported on the production of Kojic acid by *Trichoderma* spp. Generally, Kojic acid was produced from *Aspergillus* spp. such as *Aspergillus candidus* (Wei et al., 1991), *Aspergillus flavus* (El-Sharkawy, 1995),

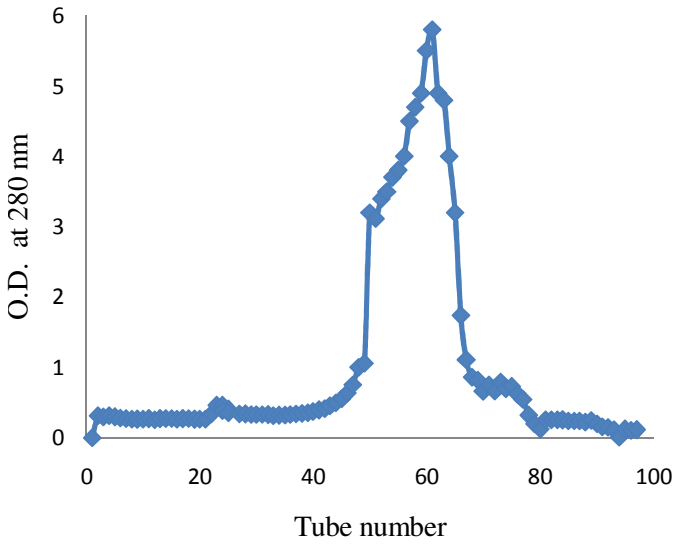

Figure 2. Chromatography of *T. reesi* crude extract on Sephacryl S-200 colum (90 x 1.6 cm i.d) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.2 at a flow rate of 60 ml/h and 3 ml fractions.

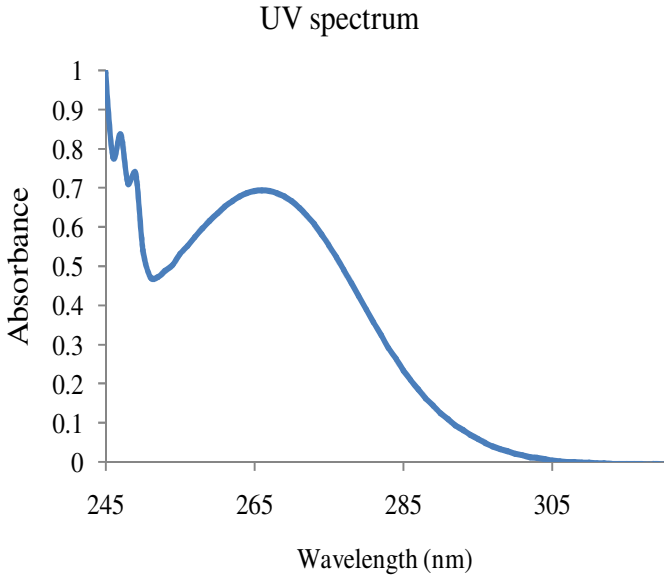

Figure 3. UV spectrum of kojic acid.

Aspergillus parasiticus (Ansari and Shrivastava, 1991), *A. flavus* and *A. fumigatus* (Kharchenko et al., 1993; Moubasher et al., 1977).

Crystallization of kojic acid

After crystallization procedure, the yield of kojic acid from *T. reesei* and *T. viride* Sephacryl S-200 fractions (CYS80 medium contained 80 g sucrose/L) were 3 and 5 g/L, respectively. Similarly, Morton et al. (1945) produced 50 g of pure Kojic acid from *A. luteo-virescens* using 400 g glucose/L medium. The ability of 98 strains of *A. flavus* to form Kojic acid were studied. Of the 98 strains, 14 with a high activity of synthesis were further studied. The maximum Kojic acid formation was found to take place during the exponential growth phase. Carbohydrates such as glucose, sucrose, maltose, and galactose provided the best feedstock. A comparatively high yield of kojic acid (8.5 to 9.5 g/kg) was obtained by the method of solid-phase fermentation on the grain and grain forage with large amounts of proteins and carbohydrates (Kharchenko, 1999).

Physical analysis of kojic acid

Spectroscopic investigation

UV absorption spectrum of Kojic acid exhibited λ_{max}(t) at 265 (t= 1.5) nm which indicate the presence of n-π^* and π-π^* electronic transitions as hetroconjugated system (α,β-unsaturated ketone) (Figure 3). IR absorption spectrum showed characterized bands at 3200-3148 (b, OH), 2922, 2851 (aliphatic CH), 1703 (cyclic ketone), 1607 (C=C), 1465 (deformation of CH_2), 1067 (cyclic C-O-C), 938, 859 and 755 cm^{-1} (1,4-α-disubstituted of ring) (Figure 4). ^{1}HNMR (DMSO- d6) spectrum reported the resonated signals at 9.1 (s; 1H, OH of ring), 8.35, 6.349 (s.s.1H, H, α, β- CH=CH), 5.116 (t, 1H, OH of $–CH_2$-OH), 4.304- 4.294 (b, 2H, CH_2) ppm (Figure 5).

Melting point of kojic acid

The melting point of Kojic acid was determined at 150°C and this value is closed to the melting-point of Kojic acid detected at 153.5°C (Lide and Milne, 1996). The physical analysis data indicated that the separated solid crystals are Kojic acid.

Minimum inhibitory concentration

MIC is the most basic parameter in pharamcokinetics and pharamacodynmics. The MIC of Kojic acid from *T. viride* and *T. reesei* comparing with augmentin, as control, were carried out for five types of human-pathogenic bacteria. The maximum growth of bacteria was achieved at 24 h for the broth microdilution test (Table 2). Kojic acid was found to be potent against MRSA, PA, SAU, EC1 and Sh.s (MIC: 0.78 to 6.25 mg/ml). Augmentin was more potent against the most of bacteria examined comparing with Kojic acid, except of MIC of PA. The MIC of kojic acid was examined for *P. aeruginosae* (MIC 128 µg/ml), *S. aureus* (MIC 256 µg/ml) and *E. coli* (128 µg/ml) (Aytrmir et al., 2003).

Time-killing kinetics

Kojic acid and augmentin showed similar time-killing

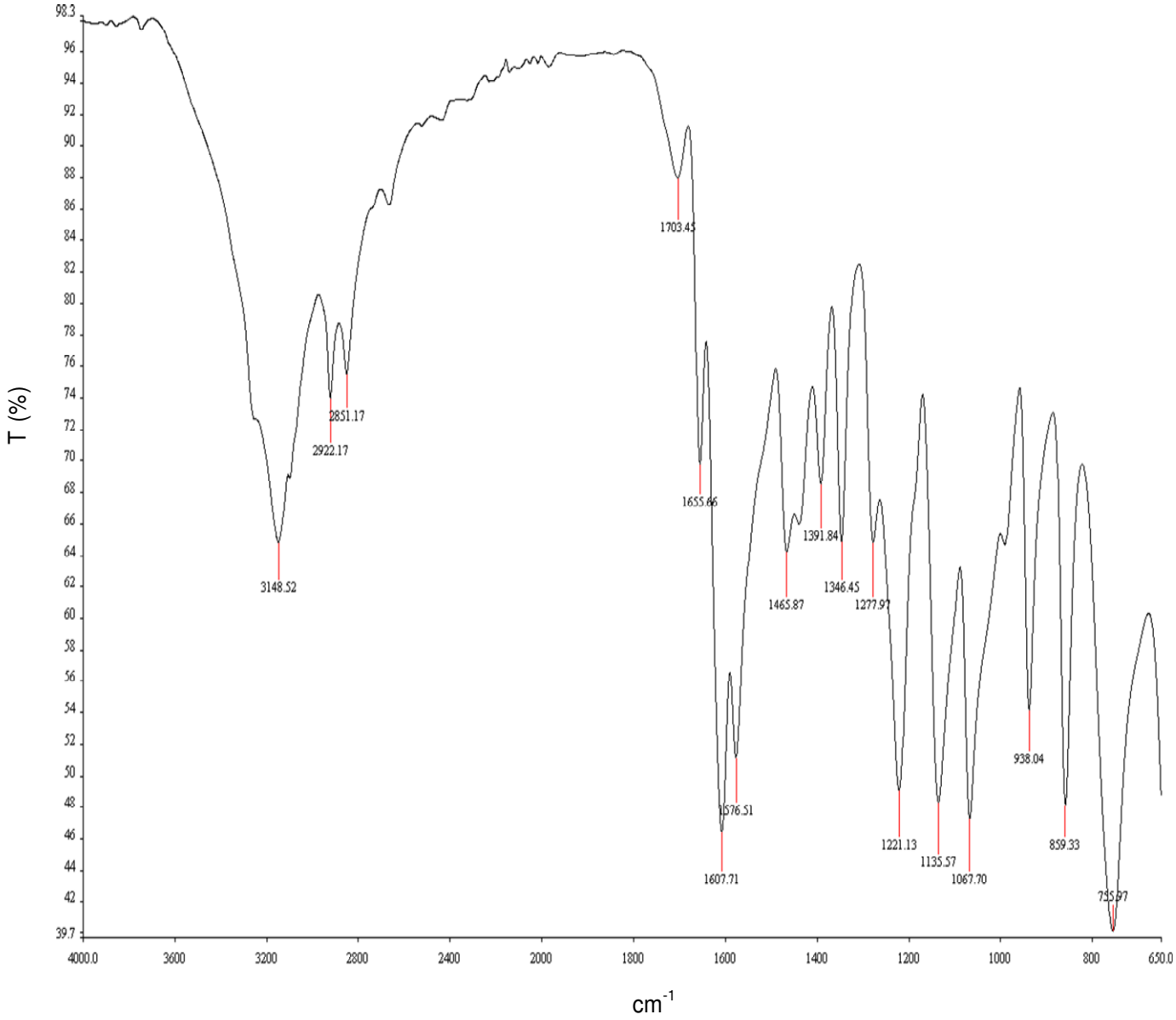

Figure 4. IR spectrum of kojic acid.

kinetics with pathogenic bacteria. In the present study bacterial density for all strains rapidly increased to a plateau of 5 x 10^5 bacteria/ml in absence of antibiotics. For the susceptible strains, Kojic acid at MIC for MRSA, PA, SUA, EC1, and Sh.s mentioned above initially reduced bacterial numbers. After 2 h of incubation 87.6, 89.4, 90, 87.6 and 90% of MRSA, PA, SUA, EC1, and Sh.s, respectively, were killed, whereas between 8 and 24 h of incubation bacterial outgrowth was zero. Similar results were with augmentin at MIC for the same bacteria.

After 2 h of incubation 82, 50, 98.6, 81and 88.8% of MRSA, PA, SUA, EC1, and Sh.s, respectively, were killed, whereas between 6 and 24 h of incubation the bacterial density was zero. The MIC and the rate of killing of bacteria were illustrated in Figure 6. The most time-killing kinetics were reported for antibiotics, whereas little studies on time-killing kinetics were reported for acids such as nalidixic acid against *E. coli* (Guerillot, et al., 1993) and fusidic acid against a group of staphylococci (Farber et al., 1986).

Screening of kojic acid and reducing sugar during growth of *T. viride* and *T. reesei*

From the above results, it should be screened that the production of kojic acid comparing with reducing sugar in

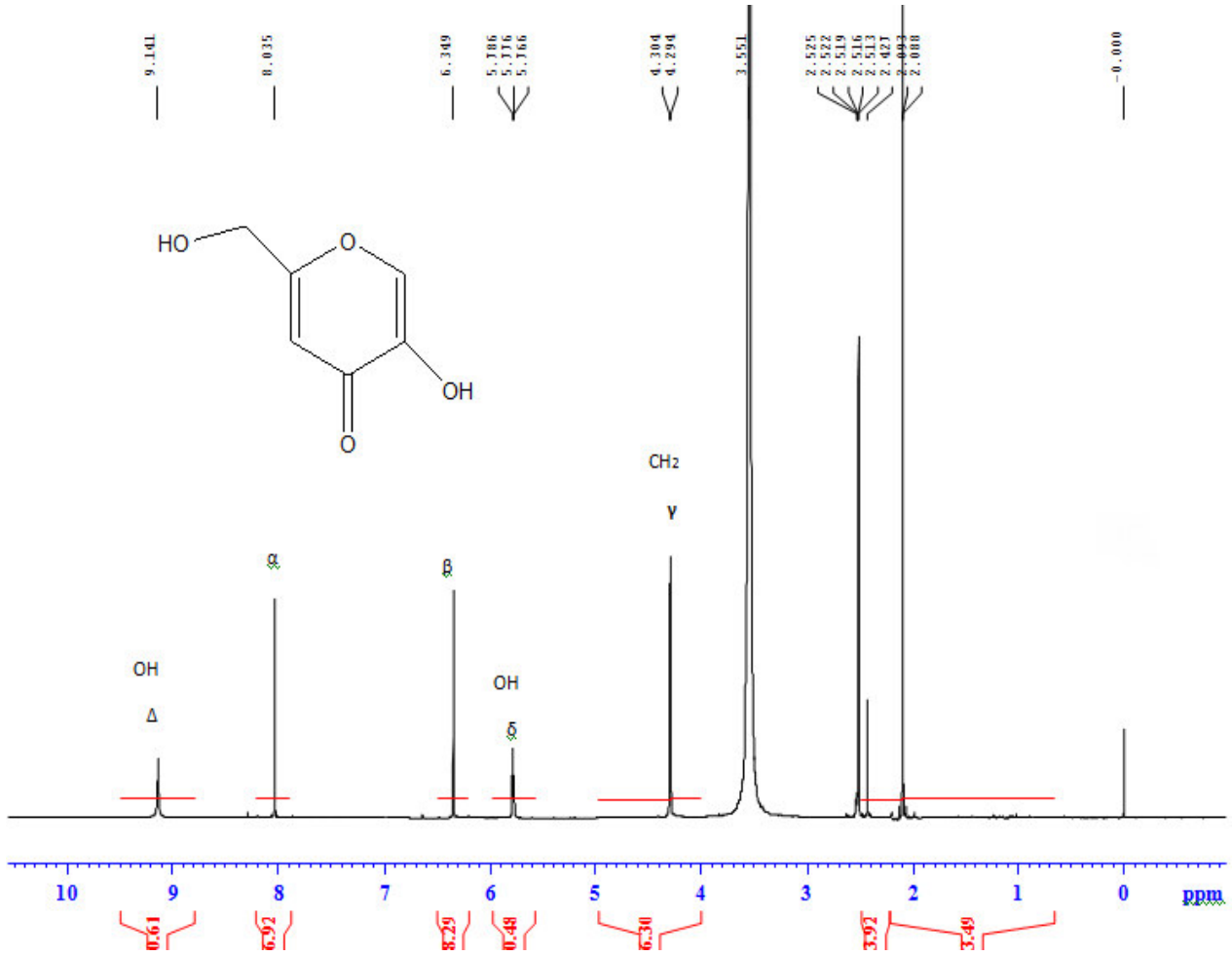

Figure 5. [1]HMNR spectrum of kojic acid.

Table 2. Determination of minimum inhibition concentration.

Bacteria	MIC mg/ml				
	MRSA	PA	SAU	EC	Sh.s
Kojic acid	6.25	0.78	3.12	1.56	1.56
Augmentin	1.25	2.5	0.625	0.039	0.313

T. viride and *T. reesei* crude extracts during growth in CYS80 medium. This is the first study reported on the production of Kojic acid from *Trichoderma* spp. The level of Kojic acid increased with decreasing reducing sugar during the incubation time, the highest level for reducing sugars for *T. viride* (22 mg reducing sugar/ml) and *T. reesei* (26 mg reducing sugar/ml) were detected on day 2 during incubation, while the highest level of kojic acid for *T. viride* (12 mg kojic acid/ml) and *T. reesei* (16 mg kojic acid / ml) were detected on days 9 and 13, respectively (Figures 7 and 8). The results indicated that approximately half of reducing sugars converted to kojic acid and these fungi produced invertase which hydrolyzed sucrose to glucose and fructose. Also, these fungi had enzymatic system responsible for the production of Kojic acid from glucose. Similar results were reported for production of Kojic acid from *A. flavus* Link 44-1 (Bajpai et al., 1982).

ACKNOWLEDGEMENT

This work was supported by King Abdulaziz City for Science and Technology, Saudi Arabia.

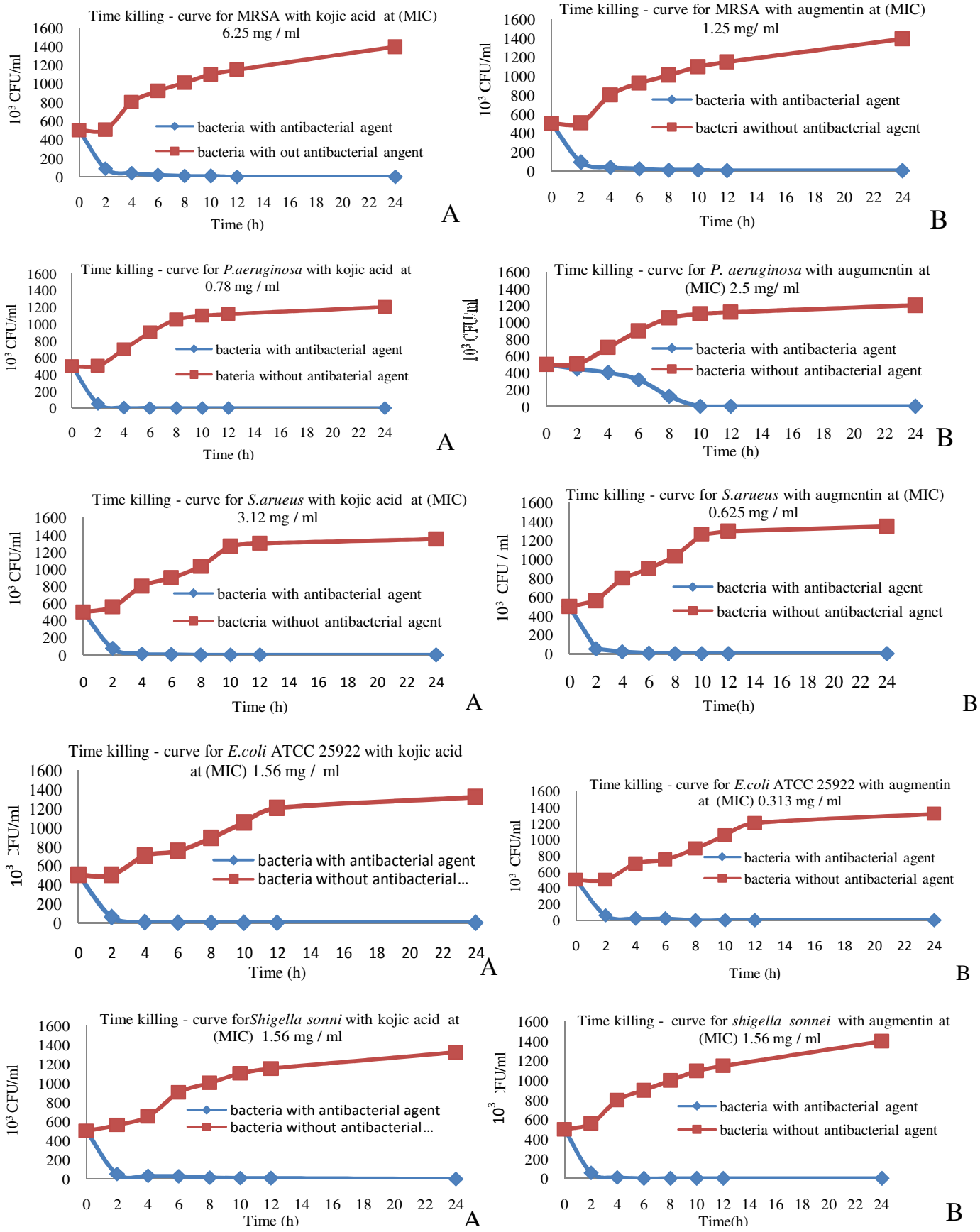

Figure 6. Time-killing curves for (A) MIC of kojic acid and (B) augmentin against some bacteria.

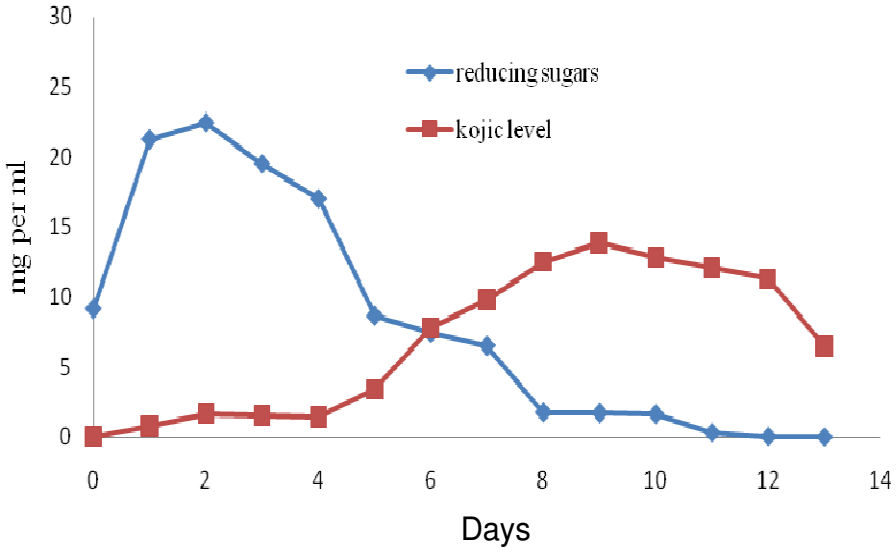

Figure 7. Determination of kojic acid and reducing sugars in crude extract during growth of *T. viride*

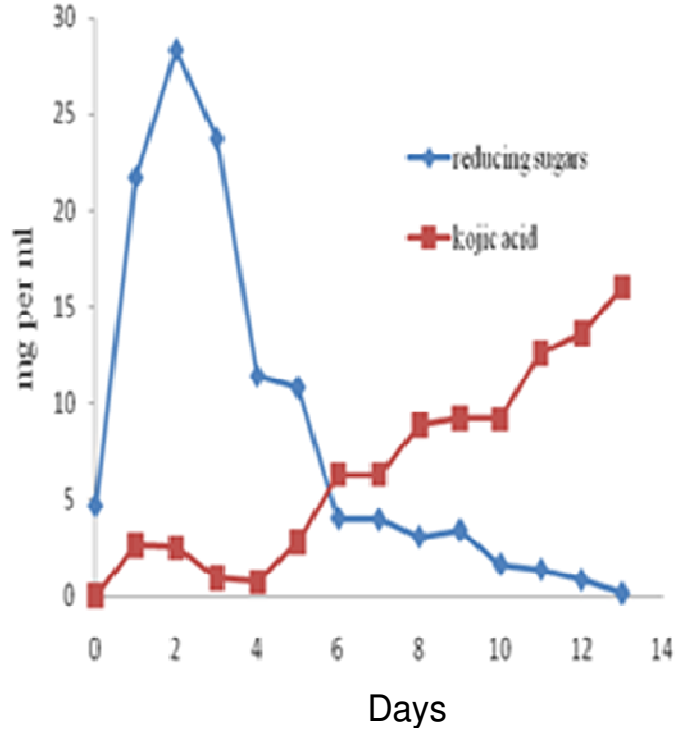

Figure 8. Determination of kojic acid and reducing sugars in crude extract during growth of *T. reesei*.

REFERENCES

Ansari AA, Shivastava AK (1991). High performance liquid hromatographic analysis of norsolorinic acid produced by *Aspergillus parasiticus* mutant. Proceedings of the Indian National Science Academy. Part B. Biol. Sci., 57: 403–405.

Ao C, Li A, Elzaawely AA, Xuan TD, Tawata S (2008). Evaluation of antioxidant and antibacterial activities of *Ficus microcarpa* L. fil. Extract. Food Control, 19: 940–948.

Aytemir MD, EROL DD, Hider RC, Ozalp M (2003). Synthesis and evaluation of antimicrobial activity of new 3-Hydroxy-6-methyl-4-oxo-4*H*-pyran-2- carboxamide derivatives. Turk. J. Chem., 27: 757- 764.

Bentley R (2006). From miso, sake and shoyu to cosmetics: a century of science for kojic acid. Nat. Prod. Rep., 23: 1046–1062.

Blumenthal CZ (2004). Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae,* and *Trichoderma reesei*: justification of Mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regul. Toxicol. Pharm., 39: 214-228.

Bajpai P, Agrawala PK, Vishwanathan L (1982). Production of kojic acid by resuspended mycelial of *Aspergillus flavus*. Can. J. Microbiol., 28: 1340–1346.

Burdock GA, Soni MG, Carabin IG (2001). Evaluation of health aspects of kojic acid in food. Regul. Toxicol. Pharm., 33: 80–101.

Cabanes J, Chazarra S, Garcia-Carmona F (1994). Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. J. Pharm. Pharmacol., 46: 982–985.

Carvajal LH, Orduz S, Bissett J (2009). Growth stimulation in bean (*Phaseolus vulgaris* L.) by *Trichoderma*. Biol. Control, 51: 409–416.

Cheesbrough M (2006). District Laboratory Practice in Tropical Countries. Part 2. 7.16 p. 137.

Chemos Group (2000). Data Sheet*:* Kojic Acid, Prague [http://www.chemos-group.com].

El-Sharkawy SH (1995). Kojic acid production from cocoa juice by *Aspergillus flavus* entraped in calcium alginate. Boll. Chim. Farm., 134: 316–319.

Farber BF, Yee YC, Karchme AW (1986). Interaction between rifampin and fusidic acid against methicillin-resistant coagulase-positive and -negative staphylococci. Antimicrob. Agents Chemother., 31: 174-175.

Frisvad JC, Samson RA (2000). Neopetromyces gen. Nov. and an overview of teleomorphs of *Aspergillus subgenus* Circumdati. Stud. Mycol. (Baarn), 45: 201–207.

Guerillot F, Carret G, Flandrois JP (1993). Mathematical model for comparison of time-killing curves. Antimicrob. Agents Chemother., 37: 1685-1689.

Harman GE (2006). Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology, 96: 190-194.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004). *Trichoderma* spp. Opportunistic avirulent plant symbionts. Nat.

Microbiol. Rev., 2: 43–56.
Hider RC, Zhou T (2005).The design of orally active iron chelators. Ann. N. Y. Acad. Sci., 1054: 141–154.
Howell CR (1998). The role of antibiosis in biocontrol. In *Trichoderma* and *Gliocladium*. Vol. 2. Enzymes, Biological Control and Commercial applications (C. P. Kubicek, G. E. Harman, eds), Taylor & Francis, London, pp. 173–184.
Imtiaj A, Tae-Soo L (2007). Screening of antibacterial and antifungal activities from Korean wild mushrooms. World J. Agric. Sci., 3: 316-321.
Irtwange SV (2006). Application of biological control agents in pre- and postharvest operations. Agricultural Engineering International: the CIGR Ejournal. Invited Overview, 3(8): 3.
Jarchem Industries (2000). Technical Information Sheet: Kojic Acid, Newark, NJ.
Kharchenko SN, Iatsyshin AI, Tea EM, Potoskii NK, Pavlenko OI (1993). The species composition of the micromycetes in feed and their role in animal kojic acid toxicosis. Mikrobiol. Zh. (Ukraine) 55: 78–84.
Kharchenko SN (1999). The biosynthesis of kojic acid by *Aspergillus flavus* link starins isolated from feed. Mikrobiol. Zh. (Ukrainian), 61: 15–21.
Lee HF, Boltjes B, Eisenman W (1950). Kojic acid as an inhibitor of *Tubercle bacilli*. Am. Rev. Tuberc., 61: 738-741.
Lenz P, Suessmuth R, Seibel E (1989). A test battery of bacterial toxicity assays and comparison with LD50 values. Toxicol. Assess., 4: 43–52.
Lide DR, Milne GWA (1996). Properties of Organic Compounds, Version 5.0, Boca Raton, FL, CRC Press [CD-ROM].
Maragos CM (2004). Emerging technologies for mycotoxin detection. J. Toxicol. Toxin Rev., 23: 317-344.
Mekawey AAI (2010). The antibiotic properties of several strains of fungi. Aust. J. Basic Appl. Sci., 4: 3441-3454.
Miller GL (1959). Use of dinitrosalicylic acid reagent for the determination of reducing sugar. Anal. Chem., 31: 426-429.
Monte E (2001). Understanding Trichoderma: between biotechnology and microbial ecology. Int. Microbiol., 4: 1–4.
Morton HE, Kocholaty W, Junowiczkocholaty R, Kelner A (1945). Toxicity and antibiotic activity of kojic acid produced by *Aspergillus* luteo-virescens. J. Bacteriol., 50: 579–584.
Moubasher AH, Abdel-Daker MIA, El-Dady IA (1979). Toxigenic fungi isolated from Roquefort cheese. Mycopathol., 66: 187 -190.
Moubasher AH, El-Kady IA, Shoriet A (1977). Toxigenic *Aspergilli* isolated from different sources in Egypt. Ann. Nutr. Aliment., 31: 607–615.
Nallathambi P, Umamaheswari C, Thakore BBL, More TA (2009). Post-harvest management of ber (*Ziziphus mauritiana* Lamk) fruit rot (*Alternaria alternata* Fr. Keissler) using *Trichoderma* species, fungicides and their combinations. Crop Protec., 28: 525–532.
National Committee for Clinical Laboratory Standards (2009). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved Standard M7-A7, Volume 26.
Rosa LH, Machado KMG, Jacob CC, Capelari M, Rosa CA, Zani CL (2003). Screening of Brazilian Basidiomycetes for Antimicrobial Activity. Mem. Inst. Oswaldo. Cruz., Rio de Janeiro, 98: 967-974.
Smania A, Monache FD, Smânia EFA, Cune RS (1999). Antibacterial activity of steroidal compounds isolated from *Ganoderma applanatum* (Pers.) Pat. (Aphyllophoro-mycetideae) Fruit body. Int. J. Med. Mushrooms, 1: 325-330.
Suay I, Arenal F, Asensio FJ, Basilio A, Cabello MA, Diez MT, Garcia JB, del Val AG, Gorrochategui J, Hernandez P, Pelaez F, Vicente MF (2000). Screening of basidiomycetes for antimicrobial activities. Antonie Van Leeuwenhoek, 78: 129–139.
Takahashi JA, Monteiro de Castro MC, Souza GG, Lucas EMF, Bracarense AAP, Abreu LM, Marriel IE, Oliveira MS, Floreano MB, Oliveira TS (2008). Isolation and screening of fungal species isolated from Brazilian cerrado soil for antibacterial activity against Escherichia coli, *Staphylococcus aureus*, *Salmonella typhimurium*, *Streptococcus pyogenes* and *Listeria monocytogenes*. J. Mycol. Méd., 18: 198-204.
Tanigaki H, Obata H, Tokuyama T (1980). The determination of kojic acid using the stopped-flow method. Bull. Chem. Soc. Jpn., 35: 3195–3197.
Terabayashi Y, Sano M, Yamane N, Marui J, Tamano K, Sagara J, Dohmoto M, Oda K, Ohshima E, Tachibana K, Higa Y, Ohashi S, Koike H, Machida M (2010). Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*. Fungal Gen. Biol., 47: 953–961.
Verma M, Brar SK, Tyagi RD, Surampalli RY, Valero JR (2007). Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. Biochem. Eng. J., 30: 1-20.
Wei CI, Huang TS, Chen JS, Marshall MR, Chung KT (1991). Production of kojic acid by *Aspergillus candidus* in three culture media. J. Food Prot., 54: 546–548.
Weindling R (1934). Studies on a lethal principle effective in the parasitic action of *Trichoderma lignorum* on *Rhizoctonia solani* and other soil fungi. Phytopathology, 24: 1153–1179.
Wilson BJ (1971). Fungal toxin. In: Ciegler, A., Kadis, S., Ajl, S.J. (Eds.), Microbial Toxins, Academic Press, New York, 6: 235–250.