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Antifungal Activity of Silver Loaded Zeolite A from Bagasse Ash against Candida Albicans

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Abstract: Normally, *Candida albicans* is found in the gastrointestinal tract, the upper respiratory tract and the genital mucosa of mammals. *Candida albicans* can form biofilm because of the increasing population and the resistance of the existing antifungal. Silver loaded zeolite A is an anti-fungal material that is effective in inhibiting the pathogen microorganism. This study aims to demonstrate the preparation of silver loaded zeolite A from bagasse ash and its antifungal activity against *Candida albicans*. Silver loaded zeolite A was obtained by ion exchange mechanism on zeolite A. It was characterized using XRD and XRF. The inhibition of the growth of *Candida albicans* by silver loaded zeolite A was observed from cell viability of *Candida albicans*. The optimum concentration of silver loaded zeolite A to inhibit the growth of *Candida albicans* was 4.5 g/L at 60 hours. This inhibition mechanism was resulted from the releasing of silver from zeolite A.

Key Words: *Candida albicans*, silver loaded zeolite A, bagasse ash, viability *cell, antifungal*

INTRODUCTION

Candida albicans is a normal flora found in respiratory, gastrointestinal tract, mucous membranes, vagina, urethra, and skin. If the immune system becomes compromised, *Candida albicans* can infiltrate the bloodstream and diffuse to organs such as kidney, heart and brain [1]. Several diseases caused by *Candida albicans* are vulvaginistis candiduria, gastrointestinal candidiasis that can cause gastric ulcers and cancer [2]. *Candida albicans* forms biofilms due to an overdose of antifungal consumption [3]. Biofilms as a *Candida albicans* protection cause the body to exhibit toward immune system and antifungal agents. Biofilms can absorb the nutrients of the host cell therefore promote the growth colonies. The growth of biofilms is along with the increase of clinical infection in the host cell.

Fungal infections can be treated with the proper use of antifungal agents. The use of antifungal agents should be accompanied by caution for the dangers of biofilm resistance. A new of antifungal substance is needed to prevent this effect. Therefore, It is necessary to conduct research to develop a new antifungal which is more effective against clinical disease mainly caused by bacteria, fungi or virus. One effort to develop new antifungal agents is the using of zeolite as an unique, cheap, and easy to synthesize material. Zeolite as a molecular sieves has been used for membranes, catalysts, and ion exchange [4-5]. Zeolite also has been used in the biomedical field as detoxifier, decontaminant, antibacterial, drug screen, biosensors and anti-tumour [6].

Zeolite clipnotilolite and zeolite A are widely used in the biomedical field [7-8]. Unfortunately, zeolite clipnotilolite as a zeolite has impurities such as montmorillonite, apatite, quartz, oxide of Ca, Al, Si, Fe, and other elements [9]. The presence of impurities can affect to the activity of zeolite and also impact the negative effects on health. On the contrary, zeolite A is an antifungal material that is safe, nonteratogenic agent, and it does not induce toxicity and carcinogenicity. The antifungal ability of zeolite A has been reported in previous studies [10-11]. The studies demonstrated that the activity of zeolite A against *Acinetobacter junii* with EC_{50} is 0.138 to 0.328 g/L, *Saccaromyces cereviceae* with EC_{50} is 2.88 to 5.47 g/L, *Ceriodaphnia dubia* with EC_{50} is 0.425 g/L[10]. Antifungal ability of zeolite A results generation hydroxyl ions during hydrolysis. The hydroxyl ions will increase the release of silicon and aluminium from zeolite framework and formed positively charged complex $[Al_mH_nNa_pO_qSi_{3\text{-}5}]^{2+}$ or $[(NaOH)_x(AlO(OH))_y\ (Si(OH)_4)_{3\text{-}5}]^{2+}$. The positively charged complex will interact with the

electronegative cell walls of microbes (phosphoryl, carboxyl, and hydroxyl) through electrostatic interaction [10].

Meanwhile, silver is also an antifungal agent that is effective to inhibit the pathogenic microorganisms such as virus, bacteria and eukaryotic microorganisms [12]. The antifungal activity of silver is effective against about 650 types of bacteria. Silver ions can provide antifungal effects at low concentrations [13]. Submillimolar concentration of $AgNO_3$ is lethal to gram-negative and gram-positive bacteria. In this paper, we present a preparation of zeolite A from bagasse ash, modification of preparation zeolite using silver, and investigation of the antifungal activity of silver loaded zeolite A against *Candida albicans*. This research also utilized the waste of sugar industry through the using of bagasse ash as a source of silica in the synthesis of zeolite A.

EXPERIMENTAL SECTION

Materials and Instruments

In this study, bagasse were obtained from Sugar Company Candi Baru Sidoarjo, Indonesia. All of chemicals were analytical grade and used as received without further purification, sodium aluminate (Sigma Aldrich), hydrochloric acid (Merck), sodium hydroxide (Merck), silver nitrate (Merck), tetrazolium XTT (Biomedicals), menadione (Sigma Aldrich), and Difco Yeast Extract (Merck). The instruments used were an autoclave (OSK 6508 Steam Pressure Apparatus Ogawa Seiki Co.,Ltd.), centrifuge (Universal 320R Zentrifugen), laminair air flow cabinet (Kotterman 8580), X-ray diffraction (JEOL JDX-3530, Philips), X-ray fluorescence (JEOL JSX 3400R, Philips), and UV-Vis spectrophotometer (Pharmaspec UV-1700, Shimadzu).

Methods

Synthesis of amorphous silica from bagasse

The procedure of synthesis of amorphous silica from bagasse ash was adopted from the previous method [14]. Amorphous silica was synthesized using dried bagasse. Bagasse was dried in an oven at 190°C for 1 hour. The dried bagasse was calcined at 300°C for 30 minutes and continued at 600°C for 60 minutes for ashing process. The resulting ash was then characterized using XRD to evaluate the formation of amorphous silica.

Bagasse ash was placed into a beaker and poured with hot water. Then, concentrated HCl was poured on it and evaporated. This treatment was repeated three times. Furthermore, the mixture was poured into water and concentrated HCl (20:1 v/v) in water bath for 5 minutes. The mixture was then filtered and washed 4-5 times with hot water. The resulted powder was calcined at 300 °C for 30 minutes, followed at 600 °C for 1 hour to produce the amorphous silica. Sample was characterized by XRF to determine the concentration of silica.

Synthesis of zeolite A from bagasse ash

Zeolite A was synthesized based on the previous research with the molar composition was 3.9 Na_2O:1Al_2O_3:1,8 SiO_2:270 H_2O [15]. The gel was prepared using aluminate and silicate precursor solution. The precursor solution of aluminate was prepared by dissolving $NaAlO_2$ in NaOH solution. The precursor solution of silicate was prepared by dissolving SiO_2 in NaOH solution. Both of precursor solution was combined under vigorous stirring. The mixture was heated at 100 °C for 12 hours. After/that, the mixture was filtered, washed, dried, and calcined at 450 °C for 4 hours. The solid zeolite A was then characterized using XRD.

Synthesis of silver loaded zeolite A

The silver loaded zeolite A was synthesized based in the previous method [16]. 1.5 g of zeolite was combined with 10 mL of 0.05M, $AgNO_3$. The mixture was stirred and heated at 80 °C for 2 hours and dried at 100°C for 24 hours. The dried mixture was calcined at a temperature of 450 °C for 4 hours. Silver loaded zeolite A were characterized using XRF to determine the amount of silver ions.

Study of antifungal activity of silver loaded zeolite A against Candida albicans

Before being used for testing antimicrobial activity against *Candida albicans,* silver loaded zeolite A was sterilized by drying at 105 °C for 16 hours. Silver loaded zeolite A was added to 100 mL of distilled water with stirring at 30 °C for 24 hours. The suspension was used in an antifungal test against *Candida albicans* [16]. The liquid medium of *Candida albicans*, YPD was prepared by combining peptone, dextrose, and yeast extract in aquades. The mixture was sterilized at 121°C for 15 minutes and stored at cold temperature.

The test of antifungal activity was done by adding 0.25 mL of inoculum *Candida albicans* in 5 mL YPD liquid medium that contained silver loaded zeolite A with variation of concentrations (0 to 4.5 g/L), The suspension was incubated at 37 °C and shaken as a function (0 to 60 hours). The growth of *Candida albicans* was determined by cell viability. Through this method, the concentration and the optimum time of silver loaded zeolite A to inhibite the growth of *Candida albicans* can be determined.

Analysis of cell viability

Candida albicans was suspended in 3 mL of phosphate buffer saline (PBS) and which was added with 50 mL of XTT solution and 8 mL of menadione. Then, it was incubated at 37 °C for 4 hours in the dark room and centrifugated at 2000 rpm for 15 minutes. The resulting supernatant was measured for absorbance using Spectrophotometer UV-Vis at 469 nm.

RESULTS & DISCUSSION

Amorphous Silica from Bagasse Ash

Two basic process in the synthesis of amorphous silica from bagasse are drying and calcinations. Drying occurs when heating bagasse at 190 °C for 1 hour to remove water in in the materials. The presence of water in the bagasse affects the purity of sugarcane bagasse. The calcination is done at 300 °C for 30 minutes to convert bagasse into carbon. The next calcination step is heated at 600 °C for 60 minutes to remove carbon so that white ash of silica is obtained. White ash is purified by washing using hydrochloric acid to dissolves oxides of metals such as P_2O_5, K_2O, MgO, Na_2O, CaO, dan Fe_2O_3 to form chloride salts [17]. The diffractogram of amorphous silica from bagasse ash is shown in Figure 1. It can be seen that there is a hump in 2θ 15-35°. According to the literature [18], a hump is observed in the 2θ ranging from 16° to 39°, indicating disordered structure. Herein, a hump from the prepared solid which supposed to be the characteristic of amorphous silica. The same results were also reported by Malek and Yusof [19-20], which displayed the hump at 2θ 15-30° with a peak at 23° when calcined the rice husk and bagasse at high temperatures. Based on analysis result of XRF, the amount of silica in the bagasse ash obtained is about 88.7%. Moreover, this data was applied as a basic of calculation in the synthesis of zeolite A with molar composition 3.9 Na_2O: Al_2O_3: 1.8 SiO_2 · 270 H_2O.

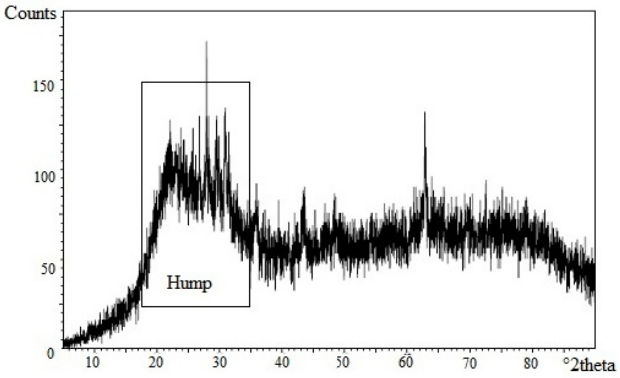

Fig 1. The diffractogram of amorphous silica from bagasse ash

Silver Loaded Zeolite A from Bagasse Ash

Synthesis of zeolites using the hydrothermal method involve a crystallization process of solution through heating and high pressure in water solvent. Water is used as a media for transformation of amorphous into a crystalline solid. The hydrothermal process requires a relatively low temperature to prevent changes in the structure of the zeolite A. According to the literature, zeolite A can be transformed into another stucture at high temperatures, so the temperature is one of important factor in the synthesis process [21]. High purity of zeolite A is obtained at hydrothermal temperatures of 100 °C for 12 hours [19]. The structure of zeolite A was analyzed using XRD. The diffractogram shows the intensive peak at 2θ = 14.26; 24.87; 32.36; 35.49; 38.48; and 43.90°. In this study, the structure of zeolite A is crystalline for stable form when applied as an antifungal material.

Silver loaded zeolite A was prepared by mixing zeolite A, and a solution of 0.05M, $AgNO_3$. The ions of silver exchange the position of sodium ions in zeolite A framework. From the results of XRD analysis, it can be seen that there is a decreasing of peak intensity at 2θ 14.26; 24.87; 32.36; 35.49; 38.48; and 43.90°. In the silver loaded-zeolite A, the diffractogram indicates the presence of silver that is proved by peak at 2θ 38.10 and 44.24°. That peaks also represented the (111) and (200) hkl as Bragg's reflection of face centered cubic crystalline silver. The pattern of diffractogram is similar with the previous report [22]. The addition amount of Ag in the zeolite A leads to a decrease in the amount of silica because the collapsed structure of zeolite. XRD pattern of zeolite A and silver loaded zeolite A from bagasse ash shown in Figure 2.

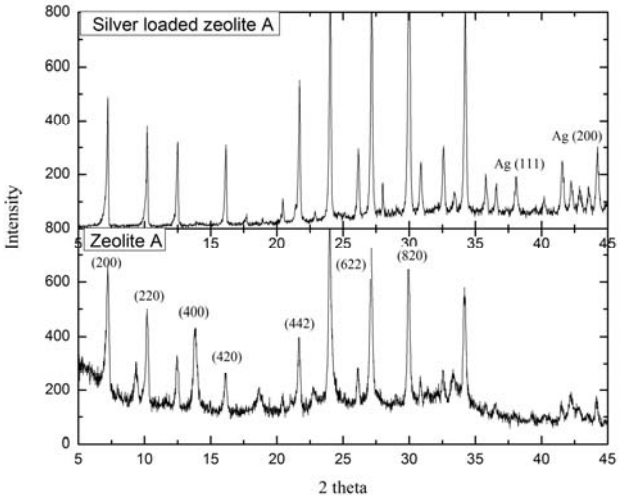

Fig 2. The diffractogram of (a) zeolite A from bagasse ash (b) silver loaded zeolite A from bagasse ash

Antifungal Activity of Silver Loaded Zeolite A against *Candida Albicans*

The influence of silver loaded zeolite A on the growth of *Candida albicans* is obtained through determination of cell viability using spectrophotometer UV-Vis at 469 nm. This analysis is based on the reducing ability of active cell metabolism towards XTT into a formazan orange with the presence of the dehydrogenase enzyme of mitochondria and menadione. The dehydrogenase enzyme of mitochondria is an oxidizing agent, thereby converting NADH into NAD^+ and H^+ by releasing electrons. Dehydrogenase enzyme consists of three kinds of complex enzymes (NADH-Q reductase, cytochrome reductase, and cytochrome oxidase). The presence of dehydrogenase enzyme of mitochondria can be an indicator of living cells. In contrast, dehydrogenase enzyme does not work in the death cell.

XTT is not soluble in lipids so that it can not diffuse through the membrane. Therefore, in cell viability tests, menadione is needed as a media for the XTT to diffuses to mitochondria through cell membranes. Menadione acts as an electron carrier that is soluble in lipid. Dehydrogenase enzymes of mitochondrial reduced menadione into menadiol and generated NADH. Furthermore, menadiol diffuse out of the cell membrane and reacts with XTT, forms colored formazon compound [23-24]. The intensity of the colored formazan is analogue with the number of surviving cells.

The profile of *Candida albicans* growth is influenced by the concentration of silver loaded zeolite A (0 to 4.5 g/L), as shown in Figure 3. The growth profile consists of four phases: lag, exponential, and a death phase. Lag phase is an adaptation phase that occur when the population of *Candida albicans* is inoculated into a new medium. Exponential phase is a phase which at *Candida albicans* reproduces by itself. Death phase (lysis) is a phase of decreasing the number of *Candida albicans* due to nutritional deficiencies or unsupported medium.

There are three phases of growth profile in the control media (0 g/L) and in the concentration of silver loaded zeolite A 1.5 and 3 g/L. They are lag exponential, and death phase. Lag phase occurs in the 0 to 12 hours, whereas exponential phase occurs at 12 to 36 hours, and death phase occurs after 36 hours. In the concentration of silver loaded zeolite A 4.5 g/L, one phases of growth profile was obtained, death phase. The formation of biofilm was occured at 48 and 60 hours in the control medium (0 g/L).

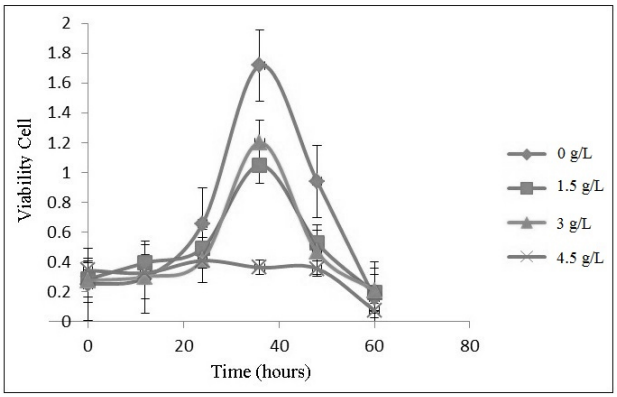

Fig 3. The growth profile of Candida albicans with the addition of silver loaded zeolite A. Determination of the growth profile is done in YPD medium for 60 hours with various concentrations of silver loaded zeolite A

The graph indicates that the silver loaded zeolite A is able to inhibit the growth of *Candida albicans*. The greater the concentration of silver loaded zeolite A, the larger inhibitory activity against *Candida albicans*. The mechanism of inhibition is due to the releasing of silver ions from zeolite A. Table 1 presents the amount of silver ion that release from zeolite framework. The silver ion inhibited cellular respiration system and binded with

sulfyhydrals enzyme, therefore destroyed the protein bonds [25-27]. Therefore, the optical density is decreased, the amount of the growth of *Candida albicans* should also be decreased.

Table 1. The concentration of releasing silver ion from zeolite A framework based on analysis of AAS

Mass of silver loaded zeolite A	Concentration of silver ion (%)
1.5 g/L	3.860
3 g/L	5.782
4.5 g/L	7.854

From the results of antifungal activity of silver loaded zeolite A with various concentration, it concluded that silver loaded zeolite A with a concentration of 4.5 g/L is an optimum concentration to inhibit the growth *of Candida albicans*. In this condition, the concentration of silver is 1.8 mg/L. Meanwhile, the occupational exposure limit for silver ion is 10 mg/L [28]. Therefore, silver loaded zeolite A potential to apply in manufacturing of medicine, medical devices, household items, and textiles where antifungal properties are required.

Despite of the mechanism of silver ions releasing, the inhibition mechanism *Candida albicans* is also due to hydroxyl ions that are produced by zeolite A due to hydrolysis, resulting higher pH. Hrenovic et al. [10] has studied this phenomenon. The possible reaction of generating of hydroxyl ions from zeolite is:

$$O\text{-}(Al,Si)_{(s)} + H_2O_{(l)} \rightleftharpoons [HO\text{-}(Al,Si)]^+_{(s)} + OH^-_{(aq)}$$

In this research, the pH of water is increased respectively 7.33 to 5.84 on zeolite concentration of 1.5 g/L; 5.89 becomes 7.84 on zeolite concentration of 3 g/L; and 6.01 to 8.47 at a concentration of zeolite 4.5 g/L. The greater the silver loaded zeolite A concentration, the higher the media basicity (Figure 4). According to the previous research [10], the hydroxyl ions will increase the release of silicon and aluminum from the zeolite framework forming positively charged complex $[Al_mH_nNa_pO_qSi_{3\text{-}5}]^{2+}$ or $[(NaOH)_x(AlO(OH))_y\ (Si(OH)_4)_{3\text{-}5}]^{2+}$. When *Candida albicans* is planktonic, the positively charged complex will interact with the electronegative cell membrane of microbes, such as phosphoryl, carboxyl, and hydroxyl through electrostatic forces.

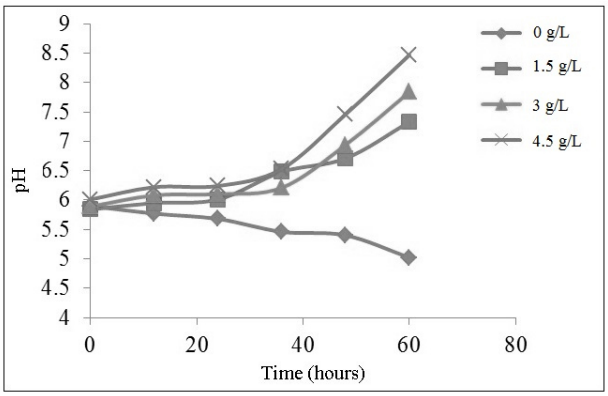

Fig 4. The pH of YPD media contains various concentrations of silver loaded zeolite A

Moreover, the alkalinity is not a major factor in inhibiting the growth of *Candida albicans*. At 36 hours, all of media generate a similar pH but different value of the cell viability. It can be concluded that the amount of released silver ions has a more substantive role than hydroxyl ions to inhibite the growth of *Candida albicans.*

CONCLUSION

This study successfully synthesized silver loaded zeolite A from bagasse through ion exchange mechanism. Silver loaded zeolite A has antifungal activity against *Candida albicans* through the release mechanism of silver ions. The optimum concentration of silver loaded zeolite A in inhibiting the growth of *Candida albicans* is 4.5 g/L at the optimum time of 60 hours. The use of silver loaded zeolite A with this concentration is appropriate for the use in manufacturing of medicine, medical devices and other industries that required the antifungal properties.

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REFERENCES

1. Nett J, Andes, *Microbiology*, 2006, 9, 1-6.
2. Rintiswati N, Praseno, Setyawan I, Suharyono, *Periodic Medical Sciences*, 2003, 35(1), 17-22.
3. Jin Y, Samaranyake LP, Samaranyake Y, Yip HK, *Archive of Oral Biology*, 2004, 49, 789-798.
4. Flanigen EM, *Pure Applied Chemistry*, 1980, 52, 2191-2211.
5. Barthomeuf D, *Catalysis Review*, 1996, 38, 521-612.
6. Auerbach SM, Carrad KA, Dutta PK, Handbook of Zeolite Science and Technology, Marcel Dekker Inc., 2003.
7. Cerri G, De'Gennaro M, Bonferoni M, Caramella C, *Applied Clay Science*, 2004, 27, 141-150.
8. Rodriguez-Fuentes G, Barrios MA, Iraizoz A, Perdomo I, Cedre B, *Zeolites*, 1997, 19, 441-448.
9. Estiaty LM, Fatima D, presented at the Exposure Research awl-LIPI Geotechnology-Indonesia, 2009, ISBN: 978-979-8636-16-5.
10. Hrenović J, Željezić D, Kopjar N, Sarpola A, Bronić J, Sekovanić L, *Journal of Hazardous Materials*, 2010, 183, 655-663.
11. Warne MStJ, Schifko AD, *Ecotoxicology and Environmental Safety,* 1999, 44, 196-206.
12. Yaohui L, Liu H, Wang Z, Hao L, Zhan J, *Polymer for Advanced Technologies*, 2008, 19, 1455-1460.
13. Lee S, Kim H, Patel R, Joon Im S, Kim J, Min B, *Polymer for Advanced Technologies*, 2007, 18, 562-568.
14. Wibowo, *Journal of Civil Engineering*, 2006, 6(2), 124-136.
15. Pera-Titus M, Bausach M, Liorens J, Cunill F, *Separation and Purification Technology*, 2008, 59(2), 141-150.
16. Rivera-Garza M, Olguin MT, Garcia-Sosa I, Alcantara D, Rodriguez F, *Microporous and Mesoporous Materials*, 2002, 39, 431- 444.
17. Yalcin N, Sevinc V, *Ceramic International*, 2001, 27, 219-224.
18. Singh D, Kumar R, Kumar A, Rai KN, *Ceràmica*, 2008, 54, 203-213.
19. Malek NA, Yusof AM, *The Malaysian Journal of Analytical Sciences*, 2007, 11(1), 76-83.
20. Sompech S, Dasri T, Taomola S, *Oriental Journal of Chemistry*, 2016, 32(4), 1923-1928.
21. Perego C, *Catalyst Today*, 1997, 34, 281-305.
22. Cheng Hsin-Han H, Chu-chin, Tsai Chia-His, *Aerosol and Air Quality Research*, 2012, 12, 409-419.
23. Stryer L, Berg JM, Tymoczko JL, Biochemistry, 7th Edition, New York: WHFreeman and Company, 2012;
24. Rabinowitz JD, Vacchino JF, Beeson C, McConnell MH, *American Chemical Society*, 1997, 120(10), 2464-2473.
25. Matsumura Y, Yoshikata K, Kunisaki S, Tsuchido T, *Applied and Environmental Microbiology,* 2003, 69(7), 4278-4281.
26. Russell AD, Hugo WB, *Progress in Medicinal Chemistry*, 31, 351-370.
27. Matsuura T, Abe Y, Sato Y, Okamoto K, Ueshige M, Akagawa Y, *Journal of Dentistry*, 1997, 25(5), 373-377.
28. Drake PL, Hazelwood KJ, *The Annals of Occupational Hygiene*, 2005, 49(7), 575-585.