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ORIGINAL ARTICLE

# *In vitro* study of biological activity of four strains of *Burkholderia gladioli* pv. *agaricicola* and identification of their bioactive metabolites using GC–MS

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**Abstract** This research was carried out to study *in vitro* antibacterial activity of 4 strains of *Burkholderia gladioli* pv. *agaricicola* (*Bga*) against G + ve *Bacillus megaterium* and G− ve *Escherichia coli*, haemolytic activity against the cell membrane of erythrocytes, the production of extracellular hydrolytic enzymes and finally, the pathogenicity against *Agaricus bisporus* flesh blocks. Chemical structure of bioactive substances of the most bioactive strain (ICMP 11096) was established using gas chromatography–mass spectrometry (GC–MS). All the studied *Bga* strains inhibited the growth of the two tested bacteria although some growing substrates negatively influenced the antimicrobial substance production. The same *Bga* strains showed highly haemolytic activity and were able to produce 3 hydrolytic enzymes, i.e. chitinase, glucanase and protease. In pathogenicity assays, the considered *Bga* strains resulted virulent for *A. bisporus*. The GC–MS for compounds from *Bga* ICMP 11096 were compatible with the structure of two bioactive fatty acids identified as methyl stearate and ethanol 2-butoxy phosphate with mass spectrum m/e 298 and 398, respectively.


## 1. Introduction

The public concern to search novel microbial natural biocides has recently been increasing in order to avoid the negative impact of synthetic pesticides either on the environment and/or animal and human health. Many *Burkholderia* spp. produce *in vitro* secondary metabolites with relevant biological activities and potential practical applications. The genus *Burkholderia* contains several species which have a wide host range, including many clinically important microorganisms as well as

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phytopathogens (Sawana et al., 2014), and inhabit a wide range of ecological niches, ranging from soil to human respiratory tract (Yabuuchi et al., 1992; Jiao et al., 2003; Coenye and Vandamme, 2003).

*Burkholderia gladioli* Yabuuchi is a species that causes disease in human, plants and fungi (Coenye and Vandamme, 2003). In particular, *B. gladioli* pv. *agaricicola* (*Bga*) is considered a dangerous pathogen in the mushroom industry (Gill, 1995). In fact, it causes soft rot disease on a number of commercially important mushrooms such as *Lentinula edodes* (Berkeley) Pegler, *Pleurotus ostreatus* (Jacq.) P. Kumm, *Flammulina velutipes* (Curtis) Singer, *Pholiota nameko* (T. Itô) S. Ito & S. Imai, *Hypsizygus marmoreus* (Peck) H.E. Bigelow and *Grifola frondosa* (Dicks.) Gray in Japan and different cultivated *Agaricus* species in New Zealand and Europe (Chowdhury and Heinemann, 2006).

The biocontrol effect exhibited by diverse microorganisms depends on their antagonistic action against phytopathogenic microbes which operates through synthesis of antibiotics with fungicidal effect and cell-wall degradation enzymes as well as production of siderophores (Glick, 1995; Ciccillo et al., 2002; Lucy et al., 2004; Elshafie et al., 2013).

Most of *Burkholderia* species can potentially be used as biocontrol agents against phytopathogenic fungi, bacteria, protozoa and nematodes in several different crops such as corn, sweet corn, cotton, grapevine, pea, tomato, pepper and some citrus and apple fruit trees (Cain et al., 2000; Perin et al., 2006; Scuderi et al., 2009) due to the production of antimicrobial substances (El-Banna and Winkelmann, 1998; Elshafie et al., 2010, 2012; Lamorte et al., 2010).

*B. gladioli* has been indicated for *in vitro* and *in vivo* diseases biocontrol because it can completely inhibit conidial germination of *Penicillium digitatum* Sacc., *Rhizoctonia solani* (Cooke) Wint., and *Botrytis cinerea* Pers. (Walker et al., 2001; Elshafie et al., 2013). Metabolites produced by *B. gladioli* also induced a significant inhibition of *P. expansum* Link growth (Altindag et al., 2006). The mode of action of *B. gladioli* as biocontrol agent is apparently the consequence of the synergic combination between its competition for nutrients and/or space and production of antimicrobial metabolites (Altindag et al., 2006). Previous studies on the biological and chemical characterization of toxic metabolites produced by *Bga* ICMP 11096 suggested the lipodepsipeptide nature of the above bioactive molecules (Andolfi et al., 2008).

In the current research, the biological characterization of four *Bga* strains (11096, 11097, 12220 and 12322) from the International Collection of Microorganisms from Plants (ICMP) has been carried out. The antibacterial activity of the above studied strains was evaluated against *Escherichia coli* (Migula) Castellani & Chalmers (*E. coli*) and *Bacillus megaterium* de Bary (*B. megaterium*), together with their haemolytic, hydrolytic activities and eventually their pathogenicity against *A. bisporus* flesh blocks. The current research studied also the main bioactive substances produced by *Bga* ICMP 11096 by Gas Chromatographic and Mass Spectrometry (GC–MS) analysis.

## 2. Materials and methods

### 2.1. Bacterial strains used in this study

The following *Bga* strains, obtained from ICMP, were used in this study: 11096, 11097, 12220 and 12322. The target microorganisms *B. megaterium* ITM100 and *E. coli* ITM103 (ITM: Institute of Tropical Medicine in Antwerp) have been obtained from stock cultures of the same prokaryotes kept freeze-dried in collection at the Laboratory of Mycology of School of Agricultural, Food-Forestry and Environmental Sciences of University of Basilicata (Potenza, Italy), recultured on King B (KB) media (King et al., 1954) and stored at 4 °C.

### 2.2. In vitro antibacterial assay

The studied *Bga* strains were evaluated for their ability to inhibit the growth of target organisms in dual agar plate assay following the method of Lavermicocca et al. (1997) with some minor modifications. More specifically, single small masses from fresh *Bga* cultures were transferred in the center of 9 cm diameter Petri dish series, each containing 14 ml of three different nutrient media, i.e. KB, potato dextrose agar (PDA) and minimal mineral agar (MMA). The different plate series were then sprayed with a target bacterial suspension containing $10^8$ - CFU $ml^{-1}$ of *B. megaterium* or *E. coli* and incubated for 48 h at 24 ± 2 °C. The antagonistic activity was registered measuring the diameter of bacterial inhibition zone after a 72 h incubation period at room temperature and expressed using the following equation: $BIP\ (\%) = 100 - [(GC - GT)/GC \times 100]$, where BIP represents the bacterial inhibition percentage, GC the average diameter of bacterial grown in control plate in cm and GT the average diameter of inhibition zone in cm. The test was repeated twice with three replicates.

### 2.3. Biological characterization of Bga strains

#### 2.3.1. Haemolytic assay

The haemolytic activity of studied *Bga* strains was evaluated against cell membrane of erythrocytes (RBCs) using blood agar base (BAB) media supplemented with fresh bovine blood following the method reported by Munsch and Alatossava (2002) and Lo Cantore et al. (2006) with some minor modifications. Blood sample was treated with heparin 25 µl 1000 U/5 ml blood, washed three times in buffer (0.72 g Tris–HCl, 1.16 g NaCl, 0.07 g EDTA at pH 7) and then centrifuged at 20.000 g for 3 min at room temperature. RBCs were successively added at 0.25% to BAB. Ten ml of the suspension was later poured in each Petri dish. A loopful of bacterial mass of studied *Bga* strains was added in the Petri dish and incubated at 24 ± 2 °C. Diameter of haemolysis zone was scored after 48 h. The test was repeated twice with three replicates.

#### 2.3.2. Extracellular hydrolytic enzymes assay

Chitinase and protease activities of studied *Bga* strains were determined according to Tahtamouni et al. (2006) on plates of KB containing chitin 1% and skim milk 1%, respectively. Cellulase activity was detected according to the method of Essghaier et al. (2009) using carboxymethyl cellulose 0.4%. Glucanase activity was detected according to the method of Teather and Wood (1982) using lichenan 0.2%. Amylase, pectinase and polygalacturonase activities were detected using soluble starch 1%, pectin 0.5% and polygalacturonic acid 1%, respectively (Sung et al., 1993; Bhardwaj and Garg, 2010). After a five day incubation at 28 ± 2 °C, plates were flooded with specific staining solutions: congo red 0.03% for chitinase, cellulase and glucanase; lugol solution for amylase; CTAB 2%

for pectinase and ruthenium red 0.1% for polygalacturonase. The enzymatic activity was taken as evidence by the appearance of clear zones around the colonies and their diameters were measured in mm.

### 2.4. Pathogenicity assay

The pathogenicity tests against *A. bisporus* flesh blocks were performed by inoculation of 40 µl broth at 3 different concentrations ($10^8$, $10^6$, $10^4$ CFU $ml^{-1}$) of studied *Bga* strains. Blocks of the same mushroom treated only with sterile distilled water were used as negative control (NC). Results were registered after 3 days of incubation at 24 ± 2 °C through intensity differentiation of the flesh block color. The bioassay method was lightly modified from the basic technique of Chowdhury and Heinemann (2006).

### 2.5. Gas chromatographic analysis of bioactive metabolites

#### 2.5.1. Preparations of cell-free culture filtrate of Bga ICMP 11096

The selected strain (ICMP 11096) was grown at 25 °C under shaking conditions at 200 rpm in a 500 ml Erlenmeyer flask filled with 150 ml of liquid MM nutrient medium inoculated with 1.5 ml of bacterial suspension at optical density O. D. ≈ 0.2 at 590 nm ($10^8$ UFC·$ml^{-1}$). Ten ml of liquid filtrates was then loaded on a cartridge syringe (SPE-Strata C18-T) prewashed with 2 ml methanol followed by 2 ml distilled water. The cartridge was washed after that with 1 ml distilled water and recovered by 1 ml of methanol that was collected in sterilized eppendorf. The purified filtrate was injected later in GC–MS for identification the bioactive compounds.

#### 2.5.2. Experimental procedures of GC–MS

A qualitative analysis of the purified filtrate fraction has been carried out using Solid Phase Micro Extraction method (SPME) as following: an SPME fibre coated with 100 µm of non-grafted poly (dimethylsiloxane) phase (Supelco 57300-U, mounted on a Supelco 57330 support) was conditioned for 1 h. at 250 °C in a stream of helium. A single fibre was used for the complete study. A blank run was performed after each analysis in order to confirm that no residual compounds were polluting the fibre or the column. The fibre was later introduced into the injection port of a HP6890 plus gas chromatograph equipped with a Phenomenex Zebron ZB-5 MS capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness). A HP 5973 mass selective detector (mass range: 15–800 mAU; scan rate: 1.9 scan/s; EM voltage: 1435) was used as detector, helium at 0.8 ml/min was used as carrier gas. The injection port, equipped with a glass insert (internal diameter 0.75 mm) was split at 250 °C. The desorption time of 1.0 min was used. Detector was maintained at 230 °C. Oven was maintained at 40 °C for 2 min, then the temperature was increased until 250 °C (8 °C/min) for 10 min. All analyses were performed in triplicate. The chromatograms obtained from the total ion current were integrated without any correction for coelutions and the results were expressed as percent of the total area of peaks. All peaks were identified from their mass spectra by comparison with spectra in Wiley 6 N and NIST98 libraries.

### 2.6. Statistical analysis

The experimental data were statistically analyzed using statistical Package for the Social Sciences SPSS (version 13.0, Prentice Hall: Chicago, IL, USA, 2004). Experimental values were expressed as mean ± SD and comparisons were employed by a one way ANOVA followed by Tukey *post hoc* test for showing any significance differences at $P < 0.0\ 5$.

## 3. Results and discussion

### 3.1. *In vitro* antibacterial assay

Results of *in vitro* antibacterial test showed that all studied *Bga* strains inhibited the growth of *B. megaterium* and *E. coli*. In particular, strain *Bga* ICMP 11096 showed the highest antimicrobial activity towards both target microorganisms in MMA (Fig. 1). The used nutrient media order according to the bioactivity significance of produced metabolites is the following: MMA > PDA > KB. The obtained results demonstrated that the nutrient components in growing medium have influenced the production of antimicrobial substances. This effect could be due to the chemical components of each media such as phosphate and different minerals which may influence on the growth of studied bacteria.

### 3.2. Biological characterization of Bga strains

#### 3.2.1. Haemolytic effect of Bga strains on erythrocytes

Results of haemolytic tests showed that all *Bga* strains are able to haemolyse the cell membrane of erythrocytes. In particular, there is no significant difference ($P < 0.05$) of haemolytic activity between *Bga* strains ICMP 11096 and 12220. In fact, the two strains showed haemolysis zones 75 and 70 mm in diameter, respectively.

The possible haemolytic activity mechanism could be clarified by the production of some specific enzymes called *hemolysin* such as streptolysin, an exotoxin. This enzyme, produced by some bacteria, is able to cause lysis the red blood cells by completely interacting with the cholesterol of the eukaryotic cell membrane. *Bga* strains undergo a process called Beta haemolysis (β-haemolysis), which leads to complete haemolysis around the colonies observed as a lightened and transparent zone. These results agree with the hypothesis of Munsch and Alatossava (2002) who reported that several *Pseudomonas* and other related bacteria such as *Burkholderia* sp., which are associated with cultivated mushrooms *A. bisporus*, are haemolytic.

#### 3.2.2. Production of extracellular hydrolytic enzymes by Bga strains

All tested *Bga* strains produced, without significant differences ($P < 0.05$), 3 hydrolytic enzymes: protease, glucanase and chitinase forming clear zones on skim milk 1%, lichenan media and chitin plates, where hydrolysis zone diameter ranged between 25 to 36 mm, 20 to 26 mm and 13 to 25 mm, respectively (Fig. 2). The production of chitinase and glucanase might have a contribution in degradation of the fungal cell wall and could explain why *Bga* can apparently infect the cuticle and flesh of *A. bisporus* according to Ordentlich et al.

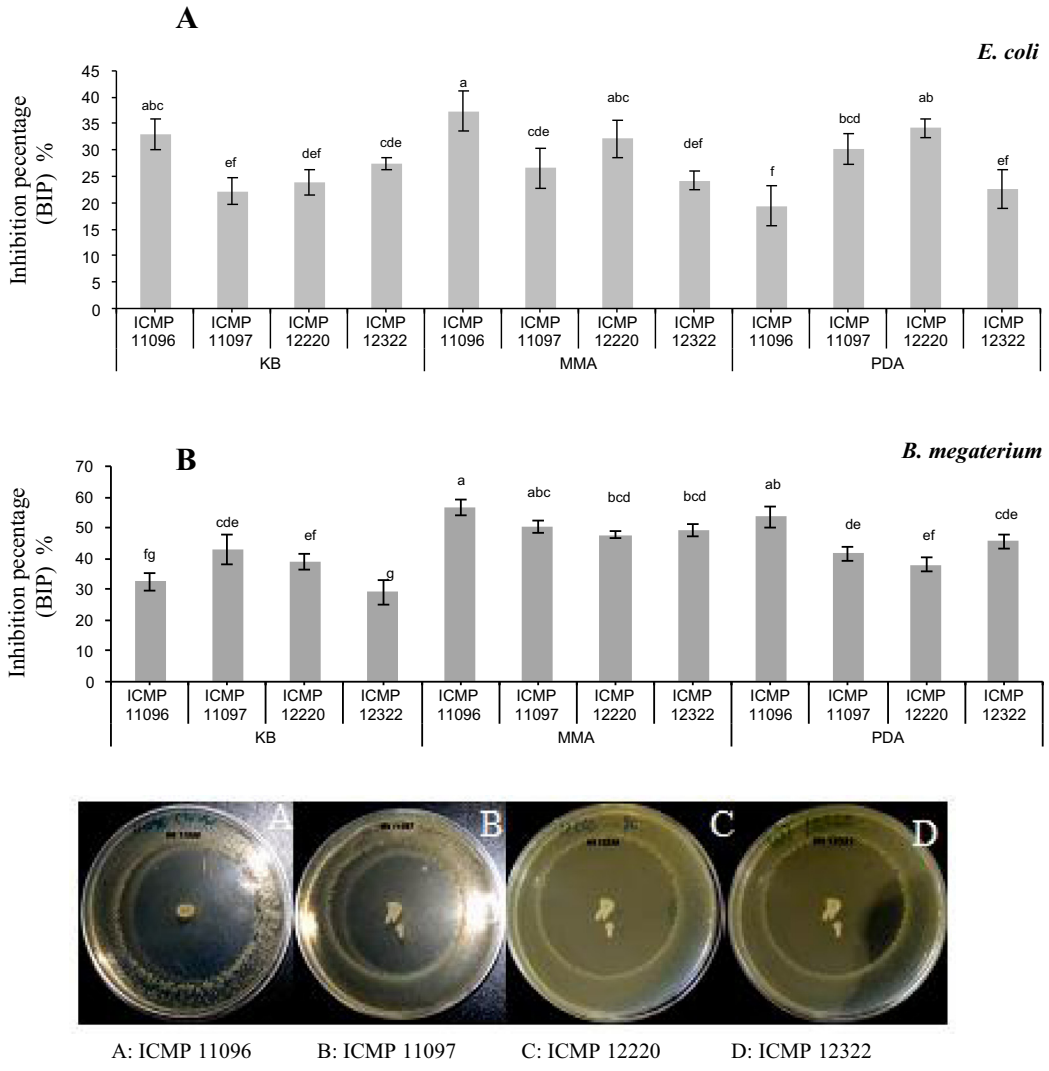


**Figure 1** *In vitro* antibacterial assay of studied *Bga* strains in (KB, MMA and PDA); A: *E. coli* and B: *B. megaterium*, where: KB: king B media, MMA: minimal mineral agar, PDA: potato dextrose agar. Bars with different letters indicate mean values significantly different at $P < 0.005$ according to Tukey test. Data are expressed as mean of three replicates ± SD.

(1988). Protease enzymes such as cell wall lytic enzyme, may play a significant role in degrading the fungal cell wall which is mainly constituted of skeletal components (Saligkarias et al., 2002). The production of hydrolytic enzymes is considered one of the most important mechanisms explaining the biocontrol of phytopathogenic fungi and bacteria. Their mode of action could be explained through the degradation of cell walls and cell membrane components and hence increasing the permeability of cytoplasm particles and organelles (Cherif et al., 1992). Nevertheless, no hydrolysis activity due to cellulase, amylase, pectinase and polygalacturonase was evidenced.

### 3.3. Pathogenicity assay

The color change induced by the four tested *Bga* strains on *A. bisporus* flesh blocks ranged between dark brown and faint brown which confirmed their mycopathogenicity. In the negative control, *A. bisporus* flesh blocks did not show any color variation. The highly visible chromatic alterations. i.e. dark brown color of the whole pileus tissue was determined by the highest concentrated bacterial suspensions containing $10^8$ CFU $ml^{-1}$ (Fig. 3). The appearance of soft rot symptoms is probably due, as hypothesized by Chowdhury and Heinemann (2006), to secretion of the hydrolytic enzymes responsible for the hydrolytic activities.

### 3.4. Identification of secondary metabolites of Bga ICMP 11096 using GC–MS

Two fatty acids were identified by GC–MS analysis: (i) methyl stearate (Fig. 4) with probability of 95% and (ii) ethanol, 2-butoxy, phosphate (3:1) (Fig. 5) with probability of 91%. The mass spectrum of the two fatty acids obtained showed characteristic ions at m/e 298 and 398, respectively

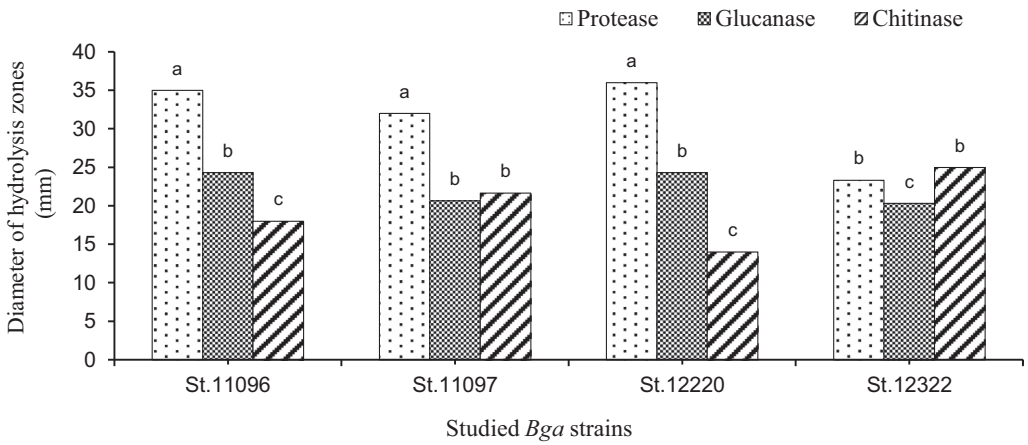


**Figure 2** Hydrolytic enzyme activity of studied *Bga* strains. Bars with different letters indicate means values significantly different at $P < 0.05$ according to Tukey test. Data are expressed as mean of three replicates.

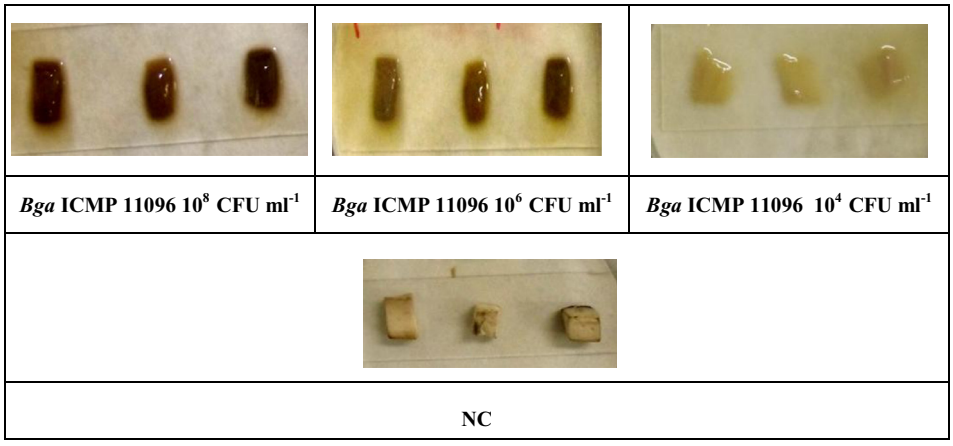


**Figure 3** Pathogenicity assay of studied *Bga* strains ($10^8$, $10^6$, $10^4$ CFU $ml^{-1}$) compared to control, where: NC is negative control.

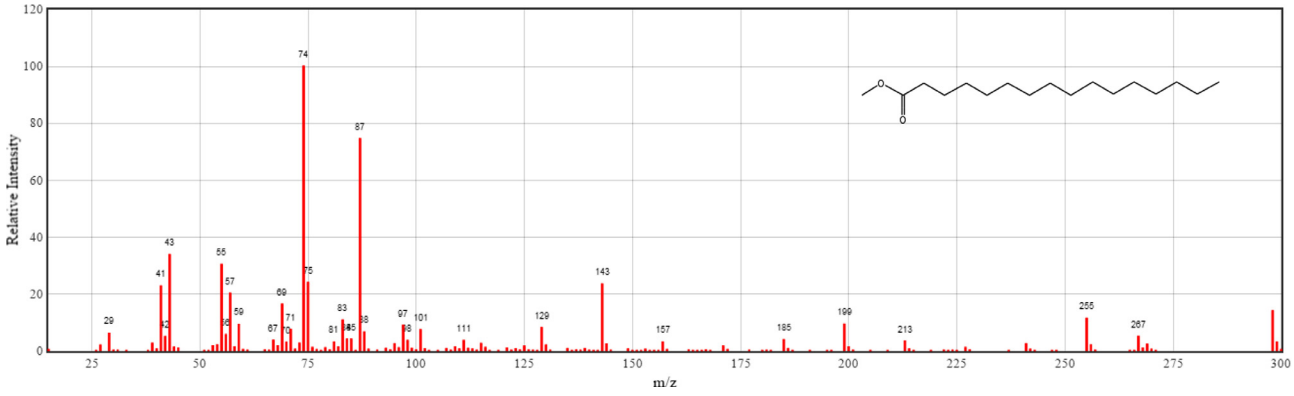


**Figure 4** MS spectrum of methyl stearate.

(Figs. 4 and 5). This results are in agreement with Makula and Finnerty (1975) who reported that 3-hydroxy hexadecanoate is linked to the amino group of ornithine containing lipid, and several fatty acids are linked through the hydroxyl group of 3-hydroxy hexadecanoate. This amino lipid compound demonstrated diverse biological effects in microorganisms and mammals (Makula and Finnerty, 1975; Kawai et al., 1996; Aygun-Sunar et al., 2006).

Generally, fatty acids are known to have antibacterial and antifungal properties. The two identified fatty acids demonstrated diverse biological effects in microorganisms and mammals as reported by Makula and Finnerty (1975), Kawai et al. (1996) and Aygun-Sunar et al. (2006). In particular, methyl esters are able to inhibit different clinical strains of the pathogenic fungus and were also active against some pathogenic bacteria as reported by Lima et al. (2011).

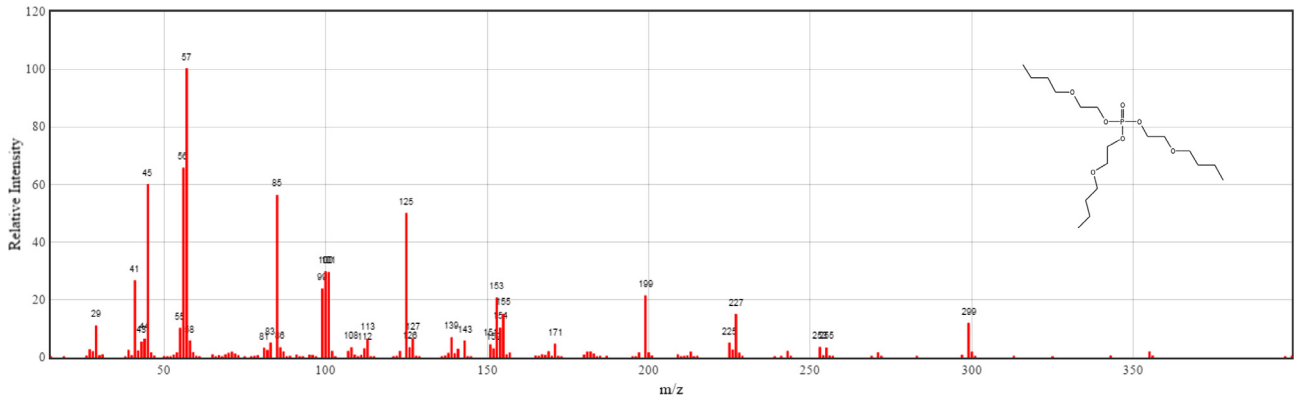


**Figure 5** MS spectrum of ethanol 2-butoxy, phosphate.

Kettout et al. (2013) reported the isolation and identification of 2-butoxy-phosphate (1:3) produced by two protagonists date palm (*Phoenix dactylifera* l.) – *Fusarium oxysporum* f. sp. *albedinis* and explained that this fatty acid has a higher antimicrobial activity. Abd El-Fattah et al. (2014) studied the antimicrobial activity of chamomile extract against some skin infections induced by *Staphylococcus aureus* and *Candida albicans* and showed that butoxy-phosphate substance is able to inhibit the growth of the tested pathogens than the traditional drugs.

## 4. Conclusion

In the present study the biological properties of investigated *Bga* strains were evaluated, which can be exploited for the biological control of some phyto and human pathogens. Strains of *Bga* showed the ability to antagonize *B. megaterium* and *E. coli*, haemolyse the cell membrane of erythrocytes and produce 3 hydrolytic enzymes: chitinase, protease and glucanase. The studied strains confirmed their mycopathogenicity against *A. bisporus* tissues. The GC–MS analysis of fermentation broth of *Bga* strain ICMP 11096 led to identification of two bioactive fatty acids as methyl stearate and ethanol, 2-butoxy, phosphate, which could be responsible for the antagonistic activity of *Bga* strains together with extracellular hydrolytic enzymes.

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