

Growth Characteristic of Fungus strain MGS-2 in a Defined Medium Containing Organic Acids Derived from Peat Soil

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Some organic acids derived from lignin degradation in peat soil have significant role in lowering pH of peat soil and have phytotoxic effect for plant. A series of experiments has been conducted to decrease the amount of organic acids in peat soil by microbial treatment. The aim of the study was to characterize the growth of selected fungus using organic acids extracted from peat soil as a sole carbon source. The fungus strain MGS-2 was grown in a liquid medium containing various concentrations of organic acids extracted from peat soil, nitrogen and salt at initial pH of 3.8. The cell growth, pH of medium and the content of organic acids were analyzed. The identification of fungus MGS-2 was based on the 28S rDNA. The ability of *Hypocrea* sp. MGS-2 to degrade toxic organic acid derived from peat soil has never been reported. The organic acid and nitrogen optimum for the growth were 33.1 mN-NaOH and 2 g (NH₄)₂SO₄ per liter medium, respectively. The interaction between carbon and nitrogen source was found to be significantly influenced by the increment of pH medium, however this interaction did not effect to the cell growth and reduction of organic acids. The carbon source affected significantly to the cell growth and acid metabolism by fungus strain MGS-2. The fungus could not grow well in the medium without yeast extract, but grew well in the limitation of NH₂SO₄, suggested that yeast extract was metabolized as nitrogen source. The optimum degradation of organic acids extracted from peat soil by MGS-2 was obtained at pH 3.8. These result suggested that the cell mass production of MGS-2 can be performed in optimal defined medium utilizing organic acid derived from peat soil. Molecular identification revealed that the MGS-2 was closed to *Hypocrea* sp.

Key words: acid tolerant-fungus, peat soil, organic acid degrading-fungus

Beberapa asam organik yang berasal dari degradasi lignin dalam tanah gambut memiliki peran penting dalam menurunkan pH tanah dan toksisitas terhadap tanaman. Serangkaian riset telah dilakukan untuk mengurangi jumlah asam organik dalam tanah gambut dengan perlakuan mikroba. Tujuan dari riset ini adalah untuk mengkarakterisasi pertumbuhan mikroba terpilih menggunakan asam organik yang diekstraksi diekstraksi dari tanah gambut sebagai sumber karbon tunggal. Strain jamur MGS-2 ditumbuhkan dalam medium cair yang mengandung berbagai konsentrasi asam organik, nitrogen dan garam pada pH awal 3,8. Pertumbuhan sel, pH dan kandungan asam organik dianalisis. Identifikasi jamur MGS-2 didasarkan pada 28S rDNA. Kemampuan *Hypocrea* sp. strain MGS-2 dalam menurunkan asam organik beracun yang berasal dalam tanah gambut belum pernah dilaporkan. Asam organik dan nitrogen yang optimal untuk pertumbuhan adalah 33,1 mN-NaOH dan 2 g (NH₄)₂SO₄ per liter media, masing-masing. Interaksi antara sumber karbon dan nitrogen berpengaruh terhadap peningkatan pH, namun interaksi ini tidak berpengaruh terhadap pertumbuhan sel dan pengurangan asam organik. Sumber karbon berpengaruh secara nyata terhadap pertumbuhan sel dan metabolisme asam oleh strain MGS-2. Strain *Hypocrea* sp. MGS-2 tidak bisa tumbuh dengan baik di media tanpa ekstrak ragi, tetapi tumbuh baik dengan pemberian NH₂SO₄ secara terbatas. Degradasi optimum asam organik oleh strain *Hypocrea* sp. MGS-2 berlangsung pada pH awal pH 3,8. Hasil ini menunjukkan bahwa produksi sel MGS-2 dapat dilakukan dengan medium minimal menggunakan asam organik dari tanah gambut. Identifikasi molekuler mengungkapkan bahwa MGS-2 adalah *Hypocrea* sp.

Kata kunci: asam organik, *Hypocrea* sp. strain MGS-2, jamur toleran asam, tanah gambut

Tropical peat consists of lignin, hemicellulose, cellulose, and protein. Lignin biodegradation in peat soil produces several organic acids. The organic acids resulted in lignin biodegradation process are from the groups of aliphatic and aromatic compounds. Aliphatic compounds found in peat soil are originated from carboxylic acid derivatives such as acetic, formic,

propionic, and butyric acids, while the aromatic compounds are from phenolic acid derivatives such as vanillic, *p*-hydroxybenzoic, *p*-coumaric, ferulic, and syringic acids (Alexander 1977; Stevenson 1994; Hartley *et al.* 1984).

Phenolic acid generally has negative effects to plant nutrient absorption and growth (Tadano *et al.* 1992). Phenolic acid, with a range of concentration between 4-30 mM, showed toxic effects in several

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species of plant (Guenzi *et al.* 1966). Phenolic acids such as ferulic, *p*-coumaric, and *p*-hydroxybenzoic acids are considered more harmful compared to carboxylic acids, because of its greater toxicity. Other than harming plants, organic acid found in peat soil also causes low pH.

Several microbes in soil have the ability to quickly decompose organic acids. Studies have been conducted to develop the utilization of organic acids as carbon source for microbes (McCarty *et al.* 1986). Phenolic acid, which was chosen as one of the organic acid that can be utilized by the microbes in soil and rhizosphere, has been studied by adding one type of phenolic acid with a concentration of $\geq 0,25 \mu\text{mol g}^{-1}$ of soil. The addition of *p*-hydroxybenzoic acid in soil showed that microbes were able to utilize the acid as their single source of carbon (Mohamed *et al.* 2009).

Several genera of bacteria are able to degrade phenolic acid and use it as a carbon source, one of them is *Azotobacter* (Juarez *et al.* 2005), while from the group of actinomycetes, *Streptomyces sannanensis* can grow optimally by using ferulic acid with a concentration of 5 mM as its carbon source (Ghosh *et al.* 2007). Beside bacteria and actinomycetes, there are also several fungi that are able to utilize phenolic acid, such as the white rot fungus *Schizophyllum commune*, *Pycnoporus cinnabarinus*, and *Trametes* sp., using coumaric and ferulic acids as their single carbon source (Sachan *et al.* 2010). According to Ghosh *et al.* (2006), the fungi *Paecilomyces variotii* grows optimally by using ferulic acid as its carbon source with a concentration of 10 mM.

We have been conducting a series study to decrease the amount of phytotoxic organic acid in peat soil. The aim of the current study was to characterize the growth of selected fungus using organic acid extracted from peat soil as a sole carbon source.

MATERIALS AND METHODS

Extraction of Organic Acid and Liquid Medium. Organic acids were extracted from peat soil using a modified method from Maciak and Harms (1986). Ten grams of soil was added by 100 mL of 2 M NaOH and then agitated on a shaker for 3 h at room temperature. The solution was separated from the solid fraction by centrifugation for 20 min at 8 000 rpm (8770 x g). H₂SO₄ solution was added gradually to the liquid fraction while being mixed, until precipitation was fully formed. Liquid fraction was separated by centrifugation for 20 min at 8 770 x g and then used as

carbon source in this study.

Fungi and liquid medium. A medium containing organic acid at low pH (3.8) was used to isolate a potent fungal-degrading organic acid of peat soil. The growth characterization of MGS-2 was done using modified Czapek's media (Labeda, 1990), with the following composition (g L⁻¹): 0.5 g of MgSO₄·7H₂O; 3 g of (NH₄)₂SO₄; 1 g of KH₂PO₄; 0.5 g of yeast extract; and 33.1 mN-NaOH organic acid, with the pH medium 3.8.

Analysis of Total Acid and Phenolic Acid. Total acid was quantified by titration method (AOAC, 19190). Two mL of sample was added to a 250 mL Erlenmeyer. The liquid was diluted with aquades until it reached 20 mL of volume (Dilution factor = 10) and was added several drops of phenolphthalein indicator. The solution was titrated with 0.1 N NaOH until a pink color was formed. The concentration of phenolic acid derivatives was detected by HPLC. Ten mL of sample or medium was acidified to pH 2.5 with 1 M HCl. The solution was then diluted by adding 10 mL of methanol (CH₃O) : water (H₂O) : acetic acid (C₂H₄O₂) solution. The solution was then used in HPLC to determine the phenolic acid concentration. The types of phenolic acid which constitute the sample was determined by partition separation system using a reversed-phase column (Bondapak C18 column, 3.9 x 300 mm) and a UV detector with D2 light at 240 nm wavelength.

Fungal Growth Characterization. Growth characterization was determined by optimizing carbon and nitrogen source. The concentrations of organic acid used as carbon source (C1, C2, C3, and C4) are 33.1; 66.2; 99.3; 132.4 mN-NaOH L⁻¹ of medium, while the concentrations of (NH₄)₂SO₄ as nitrogen source (N1, N2, N3, N4) are 1, 2, 3, 4 g L⁻¹ of medium. 10 mL of MGS-2 culture was inoculated into 100 mL medium (with composition as stated in Table 1) and incubated at room temperature for 48 hours with agitation at 120 rpm. Observation was conducted by measuring total acid, pH, cell growth (dry cell mass). The study was done with two repetitions for each treatment.

28s rDNA-based Identification of MGS-2. The 28S rDNA of the MGS-2 were amplified by PCR using a pair of specific primers, NL1(5'-GCATATCAATAAG CGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTT CAAGACGG-3') (Park *et al.* 2002). The PCR product was analyzed using 1% agarose gel electrophoresis. After the DNA band was confirmed, to obtain nucleotide sequences of 28s rDNA gene, the PCR product was purified and directly sequenced using both primers by an automated DNA sequencer

Table 1 Carbon source and nitrogen source variation in growth medium

Carbon Source = organic acid (mN-NaOH L ⁻¹ medium)	Nitrogen Source = (NH ₄) ₂ SO ₄ (g L ⁻¹ medium)			
	N1=1	N2=2	N3=3	N4=4
C1=33.1	C1N1	C1N2	C1N3	C1N4
C2=66.2	C2N1	C2 N2	C2N3	C2N4
C3=99.3	C3N1	C3N2	C3N3	C3N4
C4=132.4	C4N1	C4N2	C4N3	C4N4

*) Other compositions of the growth media include MgSO₄.7H₂O; KH₂PO₄ and yeast extract.

(Applied Biosystem 3130). The sequences were then analyzed for identity using NCBI BLAST (<http://www.ncbi.nlm.nih.gov>) with 28s rDNA sequences available at the NCBI database. Multiple sequence alignment of MGS-2 sequence and its BLAST result sequences was carried out using Clustal X software (<http://www.clustal.org/clustal2/>). Phylogenetic tree was then constructed by distance method in PHYLIP software (<http://evolution.genetics.washington.edu/phylip.html>).

Statistical Analysis. Data resulted from this study was analyzed as a factorial experiment with completely randomized design (2 factors). Every treatment was repeated two times. Significant difference between treatments was carried out by Duncan analysis.

RESULTS

Growth Characterization of MGS-2. MGS-2 reached its optimum growth when cultured in media with a concentration of organic acid of 33.1 mN-NaOH, and a concentration of nitrogen source of 2 g L⁻¹. The pH of the medium increased to 5.43 at optimum growth (Table 2, Fig 1), followed by a decrease in organic acid as much as 4.8 mN-NaOH during 48 hours of fermentation (Fig 2).

The results of variety analysis towards pH showed that the interaction of C and N significantly influenced the pH at 5% level ($p < 0.05 \rightarrow 0.00 < 0.05$). This result suggested that the C and N source was strongly needed by strain MGS-2 in degrading organic acid. The Duncan test also indicated that the combination factor of C1N2 (33.1 mN-NaOH of carbon source and 2 g L⁻¹ of nitrogen source) significantly produced a different pH compared to other combination factors of treatment.

The results of variety analysis towards cell growth

showed that interaction of C and N did not significantly affects the cell growth at 5% level ($p < 0.05 \rightarrow 0.00 < 0.05$). A single factor analysis showed that only C factor significantly influenced the cell growth at 5% level ($p < 0.05 \rightarrow 0.00 < 0.05$). The Duncan test towards the C factor also showed that there were two treatment groups produced a significant influence to the cell growth. The Duncan test also indicated that carbon source was strongly required for the growth of strain MGS-2.

In total acid analysis, the interaction of C and N did not affects the total acid reduction at 5% level ($p < 0.05 \rightarrow 0.00 < 0.05$). However, in a single factor analysis, C factor significantly influenced the total acid reduction at 5% level ($p < 0.05 \rightarrow 0.00 < 0.05$), and the N factor also significantly influenced the total acid reduction at 5% level ($p < 0.05 \rightarrow 0.00 < 0.05$). The Duncan test towards the C factor showed that two groups of treatment exhibited different effects to the cell growth. The Duncan test towards the N factor also showed a similar result to the cell growth. The analysis of variance and Duncan test indicated that each carbon and nitrogen source is strongly required by strain MGS-2 during degradation of organic acid.

Optimization of Nitrogen. Strain MGS-2 was unable to grow in medium without yeast extract. In this medium, the pH increase to 4.39 at 36 h incubation. In medium without ammonium sulphate, the strain MGS-2 was still capable to grow, and the pH medium was found increasing to 5.79 at 36 h incubation (Fig 4).

Optimization of pH. Effect of initial pH value on strain MGS-2 growth was investigated by treatment of initial pH variation in medium with optimum C and N concentration range from 3.8-6.5. The result showed higher initial pH gave slower rate of organic acid degradation (Fig 5). It can be concluded that strain MGS-2 is tolerant towards low pH and showed highest

Table 2 Final culture pH of strain MGS-2 with C & N source variations after 48 hours of incubation

Nitrogen Source (g L ⁻¹)	Carbon Source (mN-NaOH)			
	33,1	66,2	99,3	132,4
1	5,34 ^{ef}	5,2 ^{bc}	5,22 ^{bcd}	5,32 ^e
2	5,43 ^g	5,25 ^{cd}	5,18 ^b	5,22 ^{bcd}
3	5,39 ^{fg}	5,22 ^{bcd}	5,19 ^b	5,08 ^a
4	5,26 ^d	5,34 ^{ef}	5,2 ^{bc}	5,12 ^a

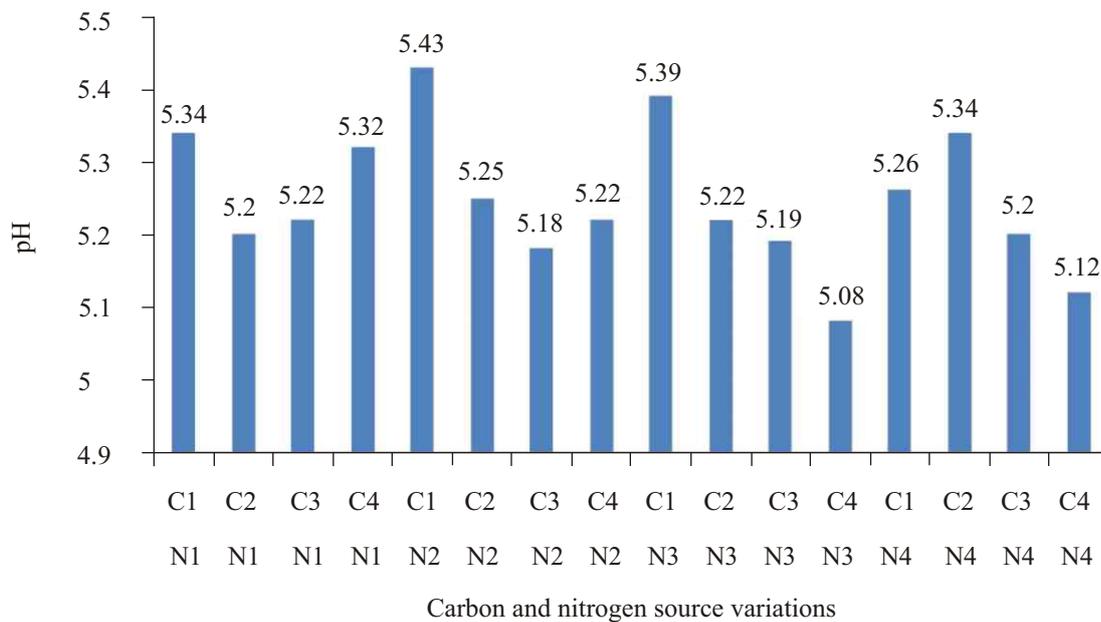


Fig 1 pH value of strain MGS-2 growth media with carbon and nitrogen source variations.

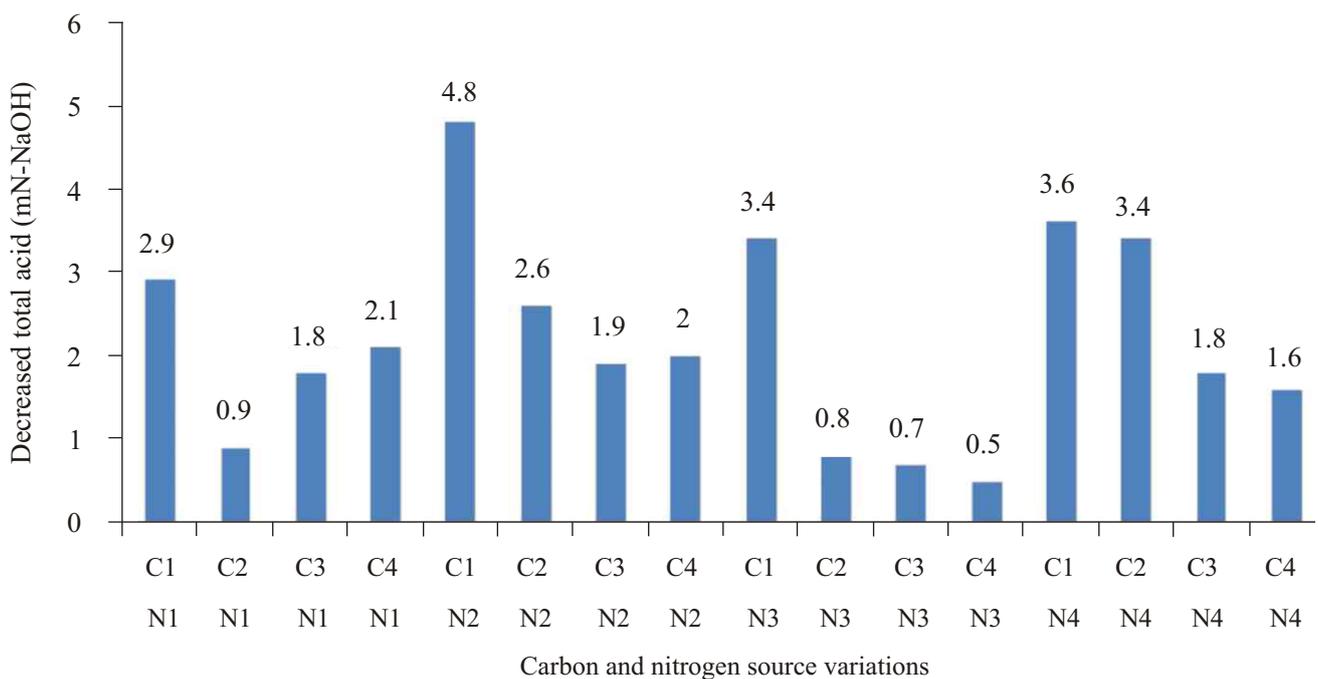


Fig 2 Decreased total acid consumed by strain MGS-2 in growth media with carbon and nitrogen source variations

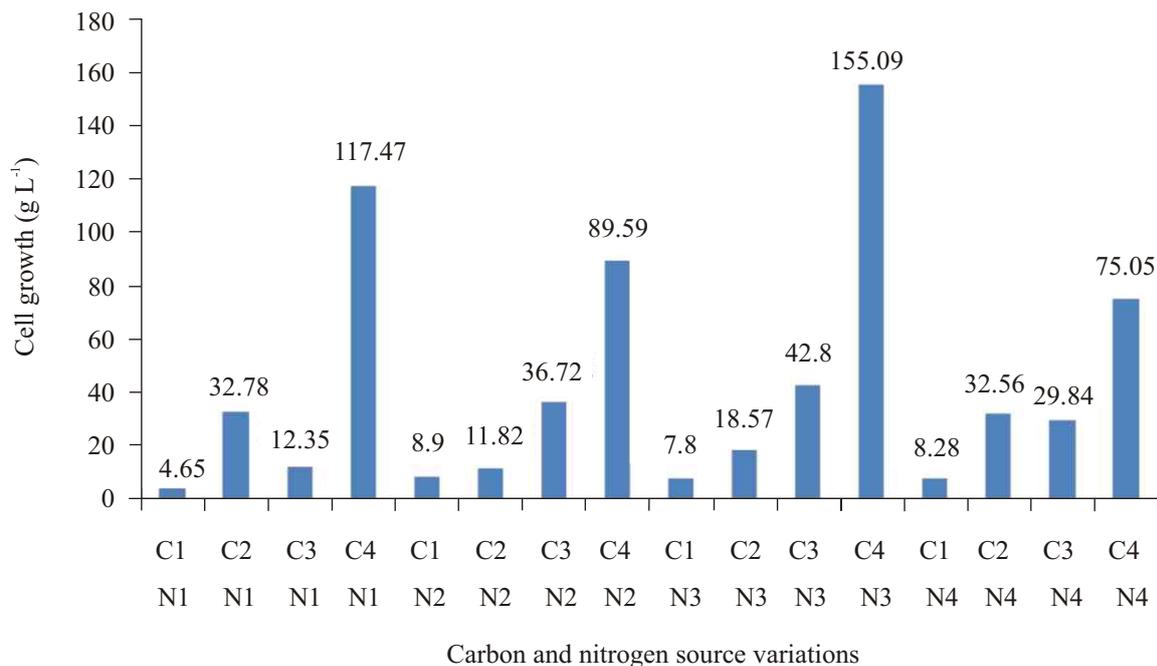


Fig 3 Cell growth of strain MGS-2 in growth media with carbon and nitrogen source variations.

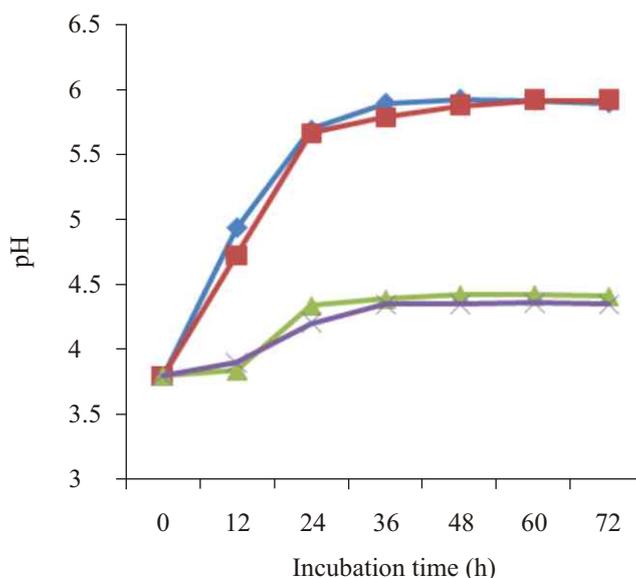


Fig 4 Yeast extract effects on the growth of strain MGS-2. —◆— : carbon and nitrogen optimal, —■— : non (NH₄)₂SO₄, —▲— : non yeast, and —×— : non non (NH₄)₂SO₄ and yeast.

degradation activity of organic acid as a sole carbonat pH 3.8.

Growth of MGS-2 in the Optimum Medium.

The rise of pH in the medium until 12 h incubation showed that strain MGS-2 was not only capable in consuming phenolic acid (*p*-hydroxybenzoic acid), but also capable to utilize non-phenolic organic acids for their growth (Fig 6 and Fig 8). Non-phenolic organic acids consumption possibly started from the initial growth phase until the maximum growth phase (6-12 h incubation). The increment of culture pH was also followed by reduction in total acid, started from 6 to 12

h incubation (Fig 6). During the period of incubation, the cell growth was inclined, and then declined after the 12 h (Fig 7). Depleting of organic acid in the medium possibly caused lysis in the fungal cells. The *p*-hydroxybenzoic acid was even already consumed during the early growth phase. In addition, the phenolic acid (*p*-hydroxybenzoic acid) was also possibly already degraded during the early growth phase (6-12 h incubation), because it was not detected in the medium during that incubation period (Fig 8).

Molecular Identification. The BLAST result of the 28S rDNA sequence of strain MGS2 showed 100%

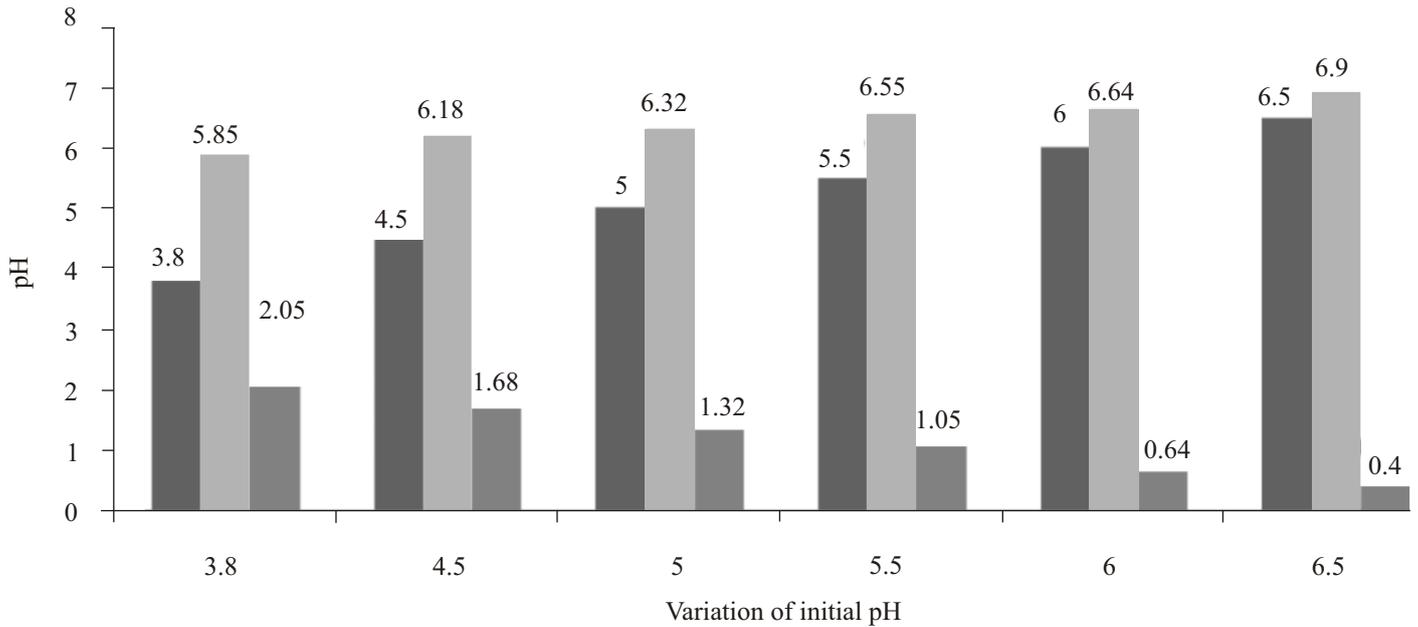


Fig 5 Influences of initial pH on the growth of strain MGS-2. ■ : Initiated pH, □ : final pH, and ▒ :Δ pH.

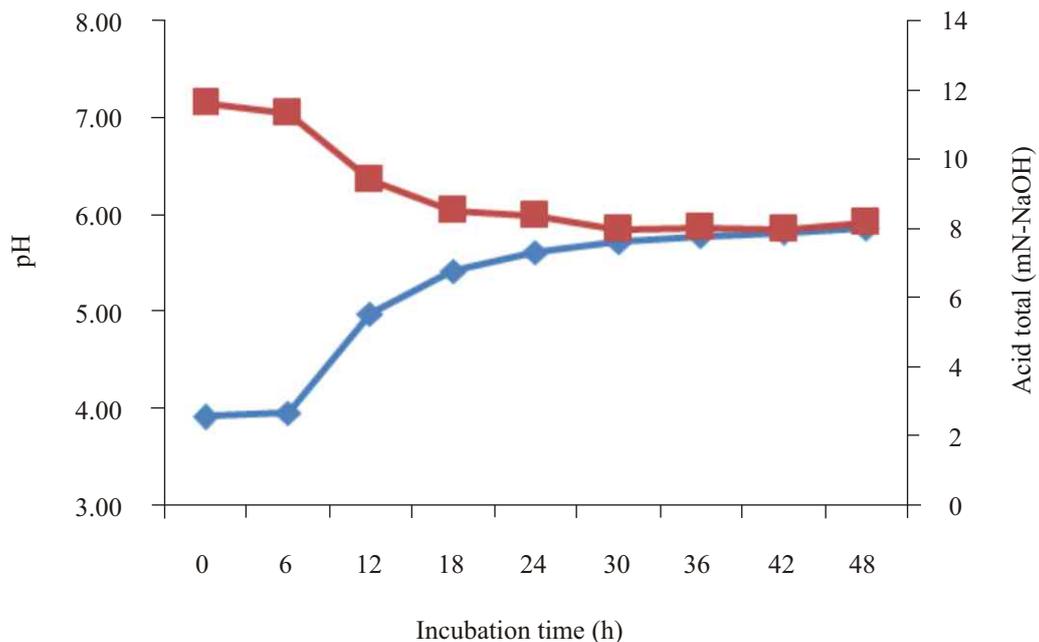


Fig 6 pH and total acid value during the growth of strain MGS-2 in optimum medium for 48 h. ◆ :pH and ■ :acid total.

similarity and query coverage to several species of *Trichoderma/Hypocrea*. The highest similarity is *Hypocrea* sp. (GU048594.1). The phylogenetic tree (Fig 9) indicated that strain MGS-2 showed a close phylogenetic relationship to *Hypocrea* sp. (Gu048594.1) Genus *Hypocrea* is a teleomorph state (sexual) of *Trichoderma*.

DISCUSSION

A fungus utilizing organic acid extracted from peat

soil as a sole carbon source has been isolated and characterized. Carbon and nitrogen are essential nutrition required by microbes for their growth and metabolism. Gao *et al.* (2007) stated that carbon and nitrogen demand varies in different strains of fungi depending on each strains growth characteristic. Strain MGS-2 can grow optimally in medium containing 2 g L⁻¹ of ammonium sulphate. The same concentration of ammonium sulphate was also reported capable to increase the growth of *Phytophthora capsici* (Perveen, 2010). *Hypocrea* sp. strain MGS-2 was also optimally

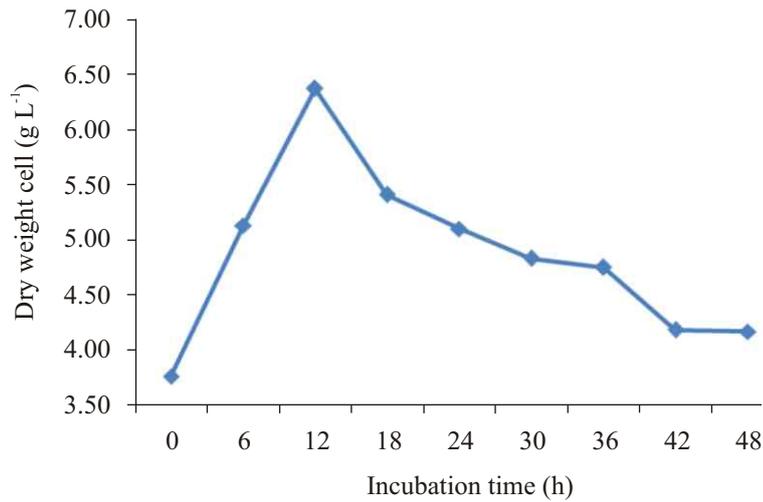


Fig 7 Cell growth of strain MGS-2 in optimum medium during 48 h incubation.

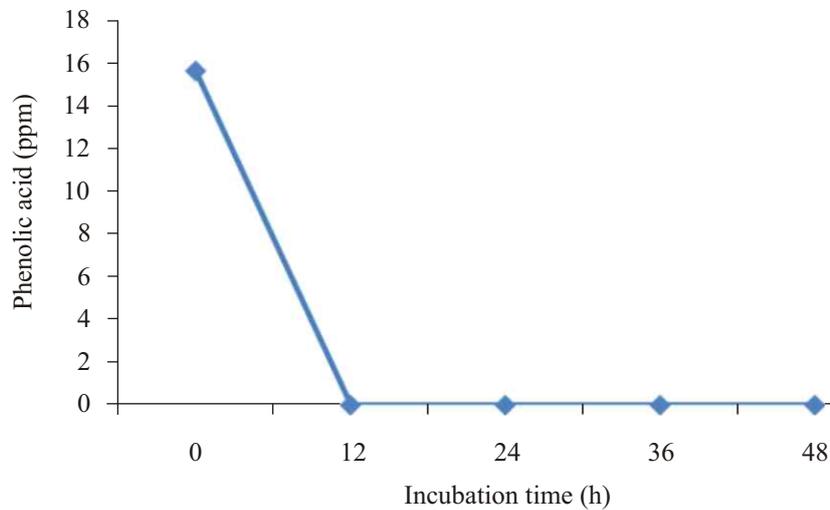


Fig 8 Phenolic acid consumption by strain MGS-2 in optimum medium.

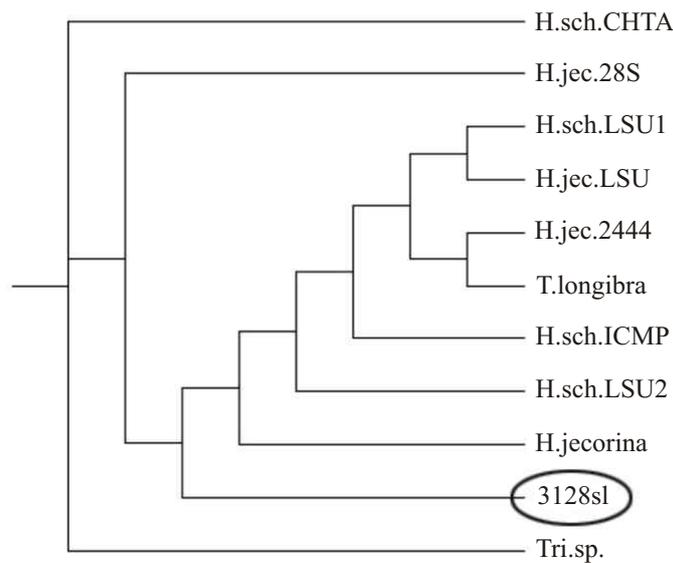


Fig 9 Phylogenetic analysis of strain MGS-2 based on 28s rDNA analysis. The MGS-2 strain is identified as *Hyprocrea* sp (circled). The other strain are H.sch.CHTA: *Hypocrea schweinitzii* (GU048594.1), H.jec.28S : *Hypocrea jecorina* (AF127154.1), H.sch.LSU1: *Hypocrea schweinitzii* LSU,(AY281095.1), H.jec.LSU: *Hypocrea schweinitzii* LSU(AY281097.1), H.jec.2444: *Hypocrea jecorina* 24449 LSU (HM466681.1, T.longibra: *Trichoderma longibrachiatum* ATCC 18648 LSU (HM466682.1), H.sch.ICMP: *Hypocrea schweinitzii* ICMP1694 LSU (AY283549.1), H.sch.LSU2: *Hypocrea schweinitzii* LSU (AF279395.1), H.jecorina : *Hypocrea jecorina*, Tri.sp.: *Trichoderma* sp. (KJ372238.1).

grown at 33.1 mM-NaOH. Several species of microbes have the ability to degrade phenolic acid and utilize it as their carbon source, these include *Azotobacter*, *Streptomyces sannanensis*, *Schizophyllum commune*, *Pycnoporus cinnabarinus*, *Trametes* sp., *Paecilomyces variotii* (Juarez *et al.* 2005; Ghosh *et al.* 2006, 2007; Sachan *et al.* 2010). The studies on microbial degradation of hydroxybenzoic acids provide a wealth of knowledge on the metabolism of this compound (Chatterjee *et al.* 1987; Suemori *et al.* 1995).

During the growth of *Hypocrea* sp. strain MGS-2, the availability of yeast extract was proven as essential factor. Yeast extract possibly contains vitamins or certain amino acids which are important for the growth of *Hypocrea* sp. strain MGS-2. Other studies, using *Clostridium thermoaceticum* grown in mineral medium, showed that only nicotinic acid derived from yeast extract essentially needed in the formation of NAD⁺ during its growth (Koesnandar *et al.* 1990). The requirement of yeast as a growth factor by *Hypocrea* sp. is an interesting phenomenon, because fungi usually require a simple medium. It is well known that bacteria require a more complex medium requirements. The ability *Hypocrea* sp. strain MGS-2 to utilized organic acids as sole carbon sources derived from peat soil, suggested that the increment of culture pH was due to the deletion of organics acid in the culture.

The ability of *Hypocrea* sp. to degrade toxic organic acid has never been reported. This phenomenon is advantages because some organic acids derived from lignocellulose in peat soil is toxic to plant to grow (Guenzi and McCalla, 1966). Mendonca *et al.* (2004) reported that *Fusarium flocciferum* was able to degrade phenolic acid, while according to Sachan *et al.* (2010), strain *Schizophyllum commune* was able to convert coumaric acid into *p*-hydroxybenzoic acid. *Streptomyces sannanensis* was also reported for its ability to degrade ferulic acid (Ghosh *et al.* 2007). Among the group of bacteria, *Azotobacter chroococcum* was able to degrade *p*-hydroxybenzoic acid in 24 hours of incubation (Juarez *et al.* 2005). *Bacillus* sp. and *Pseudomonas* sp. produce *p*-hydroxybenzoic acid hydroxylase which is used in degrading *p*-hydroxybenzoic acid into protocatechuic acid (Karegoudar *et al.* 2000). Since hydroxybenzoic acid are the most important intermediate compound in the microbial degradative pathways of various aromatic compound (Karegoudar *et al.* 2000), this is might be the reason that hydroxybenzoic acid was metabolized by *Hypocrea* sp. strain MGS-2 very fast

(Fig 8), followed by utilization of other organic acids derived from peat soil (Fig 8). Thus capability *Hypocrea* sp. strain MGS-2 to metabolize organic acid is important to release toxic compound and decreased soil acidity. Previously we reported that a mixture of undefined microbes was able to improve pH and other chemicals properties of peat soil (Nurani, *et al.* 2007), suggested that the effectivity of *Hypocrea* sp. strain MGS-2 to improve peat soil characteristics is required.

The present result showed the ability of *Hypocrea* sp. MGS-2 to metabolize organic acid derived from peat soil in a defined medium. The application the *Hypocrea* sp. MGS-2 to eliminate phytotoxic and improve peat soil characteristics as well as increase plant production is under investigation.

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