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The Potency of Endophytic Fungi of Turmeric (*Curcuma longa* L.) in Biotransformation of Curcumin Compounds in Various Media

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Abstract: With the aim of investigating the potency of endophytic fungi in biotransforming curcumin, a total of 45 endophytic fungi have been isolated from rhizomes of turmeric plant (*Curcuma longa* L.) obtained from several turmeric cultivation centres in West Java and Central Java areas, i.e., Bogor, Cibinong, Parung, Serang, Yogyakarta, Cilacap and Banyumas. The isolates was selected for their potency in biotransforming curcumin into its derivative compounds in 4 different media, namely Potato Dextrose Broth (PDB), Czapek medium, Shyntetic Low Nutrient (SLN) and Saboraud media. Fermentation process was carried out at room temperature with shaking for 14 days after addition of 0.01% w/v curcumin. Extraction method of fermentation products was done applying partition method using ethyl-acetate solvent (1:1). The extracts obtained were then analyzed using TLC (Thin Layer Chromatography) chloroform: metanol eluent (10:1). The Biotransformation Products compound (BTP) was confirmed through High Performance Liquid Chromatography (HPLC). The results indicated that 4 out of 45 isolates were able to biotransform curcumin into other compound. The 4 isolates were no. Cl.Pa.4F (fom Parung), Cl.Bel.5F (from Serang), Cl.Tg.3F (from Yogyakarta) all in PDB medium, whilst isolate no.Cl.Bk.5F (from Serang) showed the ability in Saboraud medium. However, further test indicated that only Cl.Bel.5F isolate showed stability in biotransforming curcumin. It was confirmed that BTP could be detected beginning on day 5 and reached optimum at day 10. The BTP has lower color intensity and more polar compare to curcumin.

Key words: Growth media, endophytes, turmeric, fungal transformation

INTRODUCTION

Turmeric (*Curcuma longa* L.) has been known for a long time as dye and spice. It rhizomes contain curcumin which has been reported to have medicinal properties to cure various diseases such as hormonal disorders, indigestion, blood circulation, lipid metabolism etc. It is also believed to have certain other properties like anti-inflammation, anticoagulation, antibacterial and anticancer (Chattopadhyay *et al*., 2004). However, curcumin has some disadvantages such as low solubility in water and strong color intensity so that limit its uses in pharmacology (Anand *et al*., 2008). Efforts have therefore been made to explore metabolites and curcumin derivatives that are more effective as well as have better characteristics.

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Biotransformation or frequently called green chemistry has been widely used in various industries. Its wide application, the large diversity of useful micro-organism and the wide scope of chemical reaction that can be applied for, have made biotransformation processes quite promising for the future especially with regards to development of product technology of chemicals and medicines. Few constraints such as availability of enzymes, substrates and stability in operation may be overcome through development of genomic science as well as exploration of biodiversity (Borges *et al*., 2009).

Endophytic microbes are microorganisms that live within the plant tissues which are able to conduct metabolism and biotransformation processes of certain chemical compounds into their derivatives. Researches carried out by Shibuya *et al*. (2005) and Agusta *et al*. (2005) proved that endophytic microbe isolated from tea plant was able to transform (+)-catehin and (-)-epicatechin compounds into derivative compounds 3,4 dihidroxyiflavan. The compounds resulted from the biotransformation process have higher effectivity against cancer than the original compound, catechin.

The literature study carried out so far indicates that there has been no research done on endophytic microbes of turmeric plant (*C. longa*). Considering potency and future prospects of the microbial group as well as probable uses of curcuminoid derivates as drug materials such as anti oxidant and anticancer it is important to develop potency of the endophytic microbes living in turmeric plant.

In this experiment selection of potential fungi in biotransformation of curcumin has been done in various media. The Biotransformation Products (BTP) confirmed through HPLC analysis to find out the biotransformation period and changes of BTP and curcumin during the transformation process.

MATERIALS AND METHODS

Isolation of Endophytic Fungi

Curcuma longa rhizomes used in this experiment as source of the endophytic fungi were obtained from the turmeric cultivation centers in West Java and Central Java areas, i.e., Bogor, Cibinong, Parung, Serang, Yogyakarta, Cilacap and Banyumas. Samples were taken at the age of the plant about nine months, during the years 2007-2008. Isolation of endophytic fungi followed the method described by Tanaka *et al*. (1999). The rhizomes were washed in running water and then dried with tissue paper prior to soaking in 70% alcohol for 1 min, before transferring them to 5.3% sodium hypochlorate for 5 min. The rhizome was again soaked in 70% alcohol for 30 sec. After being air dried on sterile tissue paper the rhizomes were then cut into pieces vertically and horizontally. The sliced rhizomes were carefully placed on CMM media (CMA 17 g, malt extract 20 g, yeast extract 2 g, chloramphenicol 50 mg and aquadest 1 L) in petri-dishes with the position of inner side of the slice facing downward in direct contact with the media. The petri-dishes containing the sliced rhizomes were then incubated at 28-30°C until there was a sign of fungus growth appeared on the inner surface. The fungus colonies were then transferred to other petri-dishes containing PDA medium (PDA 39 g, Agar 10 r, Aquadest 100 mL) and incubated at 28-30°C for 7 days with regular checking to monitor its purity.

Screening of Endophytic Microbes for Biotransformation Ability

The screening for the purpose was done using 4 different media, namely Potato Dextrose Broth (PDB) 24 g, Synthetic Low Nutrient (SLN) consist of glucose 0.2 g, sucrose 0.2 g, KH_2PO_4 1 g, $MgSO.7H_2O$ 0.5 g, KNO_3 1 g, Kcl 0.5 g, Saboraud consist of peptone 1 g,

glucose 4 g, $MgSO.7H_2O$ 1 g, $NaNO_3$ 1 g, K_2HPO_4 1.5 g and Czapek consist of sucrose 30 g KH_2PO_4 1 g, $MgSO.7H_2O$ 0.5 g, $NaNO_3$ 3 g, KCl 0.5 g, $FeSO_4.7H_2O$ 10 mg. All media were made in 1 L aquadest.

Fermentaion was conducted by inoculating 1 mL of 3 day preculture in PDB media into 35 mL fermentation media. There were then reincubated while being shaken at room temperature. On day 3 curcumin was added to reach 0.01% final concentration. Sampling was done on day 3, 7, 10 and 14 after adding curcumin.

Extraction and Thin Layer Chromatographic (TLC) Tests

Extraction was carried out applying partition method using ethyl acetate solvent (1:1). The extracts were made more concentrated by air drying. The concentrated samples were then analyzed by using TLC (Thin Layer Chromatography) technique with chloroform methanol eluent (10:1) and silica gel GF_{254} plates as stationary phase. Identification of spots was done under UV light and then sprayed with cerium sulfate reagent to develop the spots. As a comparison the same isolates were used but without adding curcumin. Standard curcumin (S 4786854 718 Merck®), pure media extract and media extract with curcumin but without inoculation were used as control.

Purification using Column Chromatography (CC)

Fractionation was done through column chromatography using silica gel 60 F_{254} for stationary phase and n-hexane, n-hexane-ethyl acetate (20:1), n-hexane- ethyl acetate (5:1), n-hexane- ethyl acetate (2:1), Chloroform-methanol (5:1) and chloroform-methanol (1:1) for mobile phase. The samples dropping from each column were collected into vials for further TLC test. Samples having same spot patterns and Rf values were gathered and dried.

Isolation of BTP using Preparative TLC

The samples obtained after CC were dissolved in ethyl acetate to reach 150 mg mL^{-1}. They were then scratched on TLC plates (silica gel 60 $F_{254)}$ using n-hexane-ethyl acetate solvent (1:1). After identification under UV light (254 nm), the bands that indicated BTP were scraped and dissolved in ethyl acetate and separated from silica gel by decantation. The filtrates obtained were once again identified using TLC to ensure purity of the BTP which was indicated by clear single spot.

Identification through UV-Vis Spectrophotometry

Pure BTP was weighed and then dissolved in ethyl acetate. They were then identified using UV-Vis spectrophotometry at 200-800 nm wave length. As a comparison pure curcumin was used.

Identification using HPLC

Each concentrated extracts of the fermentation products of day (D) 2, 5, 7, 10, 14 and 17 was evaporated to dry and dissolved in acetonitrile p.a for identification using HPLC. Identification using Capcell Pack C_{18} as stationary phase, size 6.0 mm ø×250 mm and acetonitrile p.a-aquabidestilata (1:1) as mobile phase. UV detector was used with 290 nm wave length. Injection volume was 20 μL and flow-rate 1 mL min^{-1}. Retention time obtained was compared with that of curcumin and pure BTP as a standard.

Growth Curve

Growth curve was measured based on change of fungi biomass from day 0 to day 19 with two replications. The fungi were grown in tubes each containing 7 mL PDB, shaken at

room temperature. The biomass was harvested based on time of measurement by straining and then dried in an oven at 60°C for 24 h. The biomass dry-weight obtained after measurement were plotted as growth curve.

All of the microbiological works were conducted at the laboratory of microbiology, while analyses and purification of BTP was conducted at the Laboratory of Natural Product Chemistry, Research Center for Biotechnology, Indonesian Institute of Sciences.

RESULTS AND DISCUSSION

As microbes living in plant tissues, endophytes have to have the ability to overcome toxic compounds produced by their hosts as defense against other organisms (Zikmundova *et al*., 2002). Curcumin, as it has been widely known is an antifungal having wide spectrum against phytopatogens (Cho *et al*., 2006). The existence of biodegradation and biotransformation processes of the toxic substances by the help of certain specific enzymes have enable the endophytes to survive (Verza *et al*., 2009). Based on the phenomena it is assumed that endophytic fungi isolated from *C. longa* are able to biotransform curcumin into its derivatives.

In this experiment a total of 45 fungus isolated from rhizomes of *Curcuma longa* were successfully incubated in 4 different media (PDB, SLN, Czapek and Saboraud) containing 0.01% curcumin. The concentration is relatively low compare to curcumin concentration in fresh rhizomes which is around 3-4% (Joe *et al*., 2004). It is not surprising therefore that all isolates were able to survive and even showed best growth in PDB. However, through screening only 4 of them had the ability to transform curcumin into it derivatives. Three isolates were Cl.Bel.5F, Cl.Pa.4F, Cl.Tg.3F which showed biotransformation ability in PDB media and one isolate i.e., Cl.Bk.5F in Saboraud media. The existence of BTP in the culture media could be detected on the chromatographic patterns resulted from TLC as shown in Fig. 1a-d.

Here, BTP is shown by the spot which is absent both in the standard substance and in control. To confirm ability of the 4 isolates in biotransformation activity the experiment was repeated several times using the same procedure and media. The results showed that only isolate Cl.Bel.5F which have stable performance indicated by appearance of spot having constant position as shown in Fig. 2.

The instable performance of the isolates other than Cl.Bel.5F may be due to the changes of microenvironment as well as inconsistent expression of the fungus characters because of

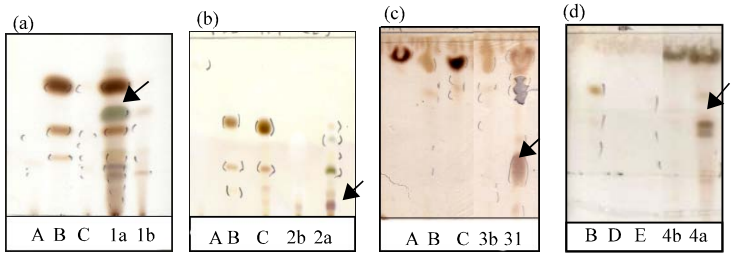

Fig. 1: (a-d) Profile of chromatograms obtained after TLC of the BTPs produced by the 4 isolates. A: PDB media, D: Saboraud media, B: Curcumin, C: A+B, E: D+B, 1=Cl.Bel.5F; II= Cl.Tg.3F; III= Cl.Pa.4F; IV= Cl.Bk.5F a: plus curcumin, b: without curcumin; arrows indicated SHB

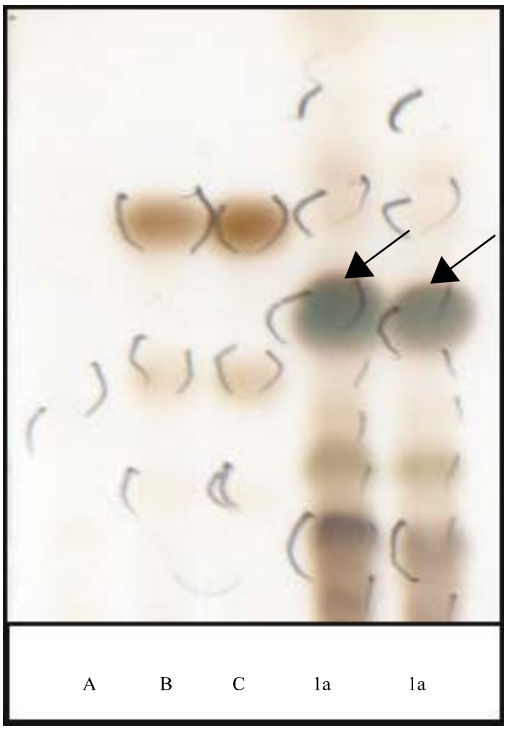

Fig. 2: Chromatogram profile of the stable BTPs produced by the CL.Bel.5F in PDB medium. A: PDB media, B: Curcumin, C: A + B, 1a: Cl.Bk.5F plus curcumin

environmental changes such as ambient temperature and air pressure in the culture room which influence oxidative pressure and physiological character of the fungus (Belo *et al.*, 2005).

Biotransformation process strongly linked with enzymes formed during fermentation and enzymatic activity of the microbes depends on nutrient content of certain substances in the growing media. As mentioned by Kaminaga *et al*. (2003), who studied about curcumin glucoxilation by *Catharanthus roseus* cells in liquid culture, glucoxilation efficiency depends on the level of culture growth and sucrose concentration in the media. The effect of nutrient composition in media against enzyme production and activity was also mentioned by Srinubabu *et al*. (2007), who studied protease enzyme from *Aspergillus oryzae*. Based on his study production and enzyme activity was very much dependent on the composition of nitrogen and carbon sources, culture time and interaction between these variables. In this study, isolate Cl.Bel.5F was able to transform curcumin in PDB media containing potato starch and dextrose. This indicates that the nutrient composition in PDB fulfilled all the requirement of the isolate to grow and to produce particular enzymes required for the curcumin biotransformation to occur. The success of biotransformation process in the PDB medium also reported by earlier researchers who biotransformed (L)-citronellal to (L)-citronellol using *Rhodotulura minuta*. Velankar and Heble (2003) cited that PDB was found to be suitable media composition for obtaining maximum (L)-citronellol concentration. Besides PDB, several other media were also reported to be suitable for biotransformation. Shibuya *et al*. (2005) on his study on biotransformation of (+)-catechin and (-)-epicatechin

Table 1: Results of pH measurement during fermentation of isolate Cl.Bel.5F in PDB media

	Culture pH on day						
Media	0	2	5	7	10	14	17
(+Cur)	5.34	5.04	5.02	5.05	5.43	5.44	5.29
(- Cur)	5.38	5.34	5.16	5.66	6.57	6.70	7.90

Cur: Curcumin

by endophytic fungus *Diaporthe* sp., used YPG medium containing yeast extract, pepton and glucose. Meanwhile, Srivastava *et al.* (2009) using Sabouraud Dextrose Broth (SDB) medium to biotransform artemisinin by fungal strains. Czapek medium was found suitable for biotransformation of tetrahydrofuran lignin by endophytic fungus *Phomopsis* sp. (Verza *et al.*, 2009). Interestingly none of the 45 isolates tested in this study successfully transformed curcumin in both Sabouraud and Czapek medium. This finding lead to the suggestion that success or failure of biotransformation process is not only determined by nutrient composition but also depend on the species of microorganisms involved and the kind of substrate to be transformed.

Beside composition of media, culture condition also important on biotransformation such as pH of the liquid medium, relative humidity, fermentation period, volume of inoculums, weight of the substrate, temperature and agitation (Panda *et al.*, 2009). In this study, the initial medium pH for biotransformation was pH 5.3. Not yet known whether the initial pH had a significant influence on the occurrence of biotransformation to produce BTP. But there was sharp indication that by increasing pH of the liquid medium will help to increase the gallic acid yield from tannin by *Rhizopus oryzae* (Panda *et al.*, 2009). While fermentation of *L. theobromae*, the initial pH influence on biomass production (Dhandhukia and Thakkar, 2007).

Biotransformation causes pH of fermentation media tends to be stable, i.e., 5.0-5.4 (Table 1). This values still in the range of optimum pH for Fungi that are known between 5.0 and 7.0 although they could grow at a wider range of pH, i.e., 3.0-8.5 (Zikmundova *et al.*, 2002). Measurement of pH during biotranformation becomes important in order to reduce initially to a significant extend the pH value of medium, which prevent the development of spoilage organisms. Change of medium pH could happen during biotranformation i.e., biotranformation of swetend winter savory (local name Rtanj tea) into kombucha showed that lowering of pH value was evident with prolonging the duration of process (Cvetkovic and Markov, 2006).

There was no research activity to measure optimum pH for produce BTP in this study. But several earlier researchers cited that biotransformation process had optimum pH 5 (Shukla *et al.*, 2007), pH 6 (Panda *et al.*, 2009), pH 5.5 (Velankar and Heble, 2003).

While the pH of culture media without curcumin tends to increase on D10. Increasing of pH was due to production of secondary metabolites by the fungus which are basic in nature.

The existence of BTP was also supported by the results of HPLC analysis. The pattern of changes in the composition of the Curcumin and BTP compounds in the culture medium seemed on Fig. 3a-c. After an incubation time of 5 days (D5) on the HPLC chromatogram began to appear one primary metabolite which showed high intensity at Retention Time (Rt) 6.7 min (indicated as 1). This primary metabolite becomes the focus of this experiment. Whilst the HPLC chromatogram of curcumin (indicated as C) is shown at Rt 17.7 min (Fig. 3). Based on the Rt value it can be assumed that BTP is more polar than curcumin so can be expected that it is more soluble in water. It is proven by a simple solubility test (Fig. 4). Reducing color intensity of BTP compare to curcumin also observed and suitable with the result analysis

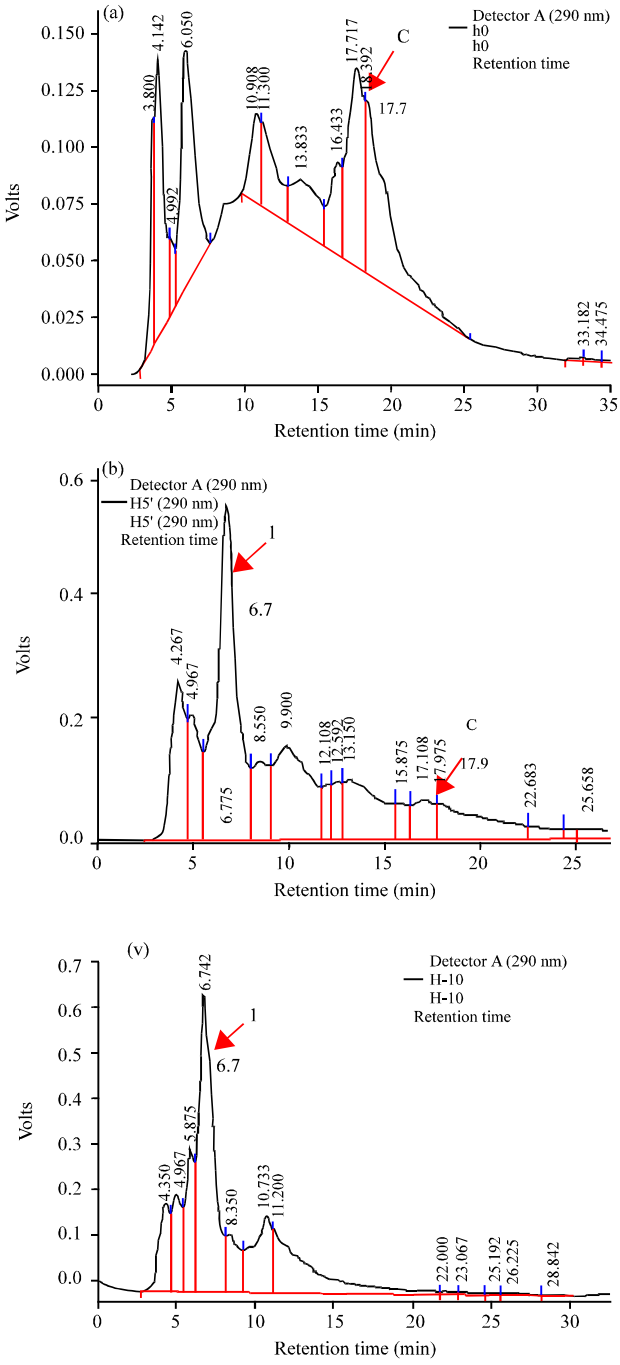

Fig. 3: HPLC chromatogram of BTP and Curcumin at (a) D0, (b) D5 and (c) D10

using UV-Vis Spectrophotometry. The observation on the λmax values indicated that λmax BTP was 285 nm belongs to the group of compounds having extremely low color intensity compare to curcumin which has λmax of 421 nm. It can be suggested that transformation of

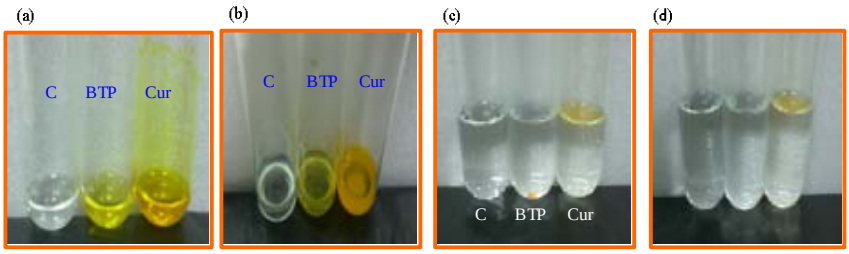

Fig. 4: Solubility test and color intensity comparison between curcumin and BTP (a) the BTP and curcumin solution in methanol, (b) BTP and curcumin solution after the addition of distilled water, (c) BTP and curcumin solution in 3 mL of distilled water and (d) solution of BTP and curcumin in 5 mL of distilled water

Table 2: Concentration of curcumin compound and BTP in ethyl acetate extracts obtained after fermentation

Day	Curcumin concentration (mol L^{-1})	BTP concentration (mol L^{-1})
0	7.8066×10^{-6}	0
2	3.1875×10^{-6}	0
5	5.1494×10^{-6}	8.3463×10^{-4}
7	0	9.6058×10^{-4}
10	0	1.0099×10^{-3}
14	0	7.5567×10^{-4}
17	0	7.5928×10^{-4}

curcumin by the endophytic fungus Cl.Bel.5F could improve physical characteristic of curcumin, i.e., reduce the colour intensity as well as increased the water solubility of compound, this will make it more applicable as material for pharmaceutical purposes.

The time course of relative abundance change of C on the biotransformation by Cl.Bel.5F was monitored by HPLC (data not shown) and measured quantitatively by using Lambert-Beer formula, the result is shown in Table 2. The data on Table 2 show that beginning from D2 after incubation there has been reduction of curcumin concentration in the media, however BTP was not yet detected on the HPLC chromatogram. BTP began to appear clearly on D5 and reached its peak on D10 and at the same time there was a reduction of curcumin concentration. Based on data on Table 2 the curcumin was totally consumed on D7.

The relation between biotransformation activity and stage of culture growth can be understood by looking at BTP content and growth curve of the fungus (Fig. 5).

In this study biotransformation process by Cl.Bel.5F fungus began at D5 which is the time when the culture was at its exponential phase and reached optimum level on D10 when the culture reached stationary phase. This research finding is in agreement with Eley and Greenwood (2005) statement who said that enzyme production in a fermentation process drastically increased at the time when growth curve reached exponential phase and reaching optimum level at the early stationary phase. Since the character of each microorganism is usually so specific, consequently the time required to achieved maximum production of a certain biotransformation process may also vary. Several earlier researchers cited that biotransformation process reached optimum results after 9 days (Verza *et al*., 2009), 5 days (Shibuya *et al*., 2005), 48 h (Srivastava *et al*., 2009), or even 44 h (Velankar and Heble, 2003) depending on the species of microorganism used.

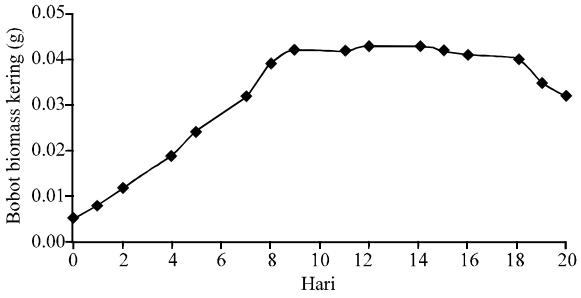

Fig. 5: Growth curve of endophytic fungus isolate Cl.Bel.5F in PDB media

The BTP began to decrease on D14 up to D17. Explanation on the decrease of BTP content is not yet clearly understood. It is presumed that there has been further conversion going on to produce other metabolites.

As a conclusion it can be suggested this experiment has laid down base line information on curcumin biotransformation and the potency of endophytes living in the rhizomes of *C. longa.* Fungus Cl.Bel.5F has a potency as a microbial model for generating curcumin analogues which has better physical characteristics. Further and more intensive study on bioactivities and elucidation of chemical structure of BTP is in progress.

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