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Full Length Research Paper

Application of lipase treatment on carotenoids extraction from Chili (*Capsicum annuum* L.)

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Five strains of lipase-producing bacteria were selected and determined for enzyme activity. As well as, the potential of enzymatic extraction on carotenoids from dried chili was also investigated. Among five strains, *Bacillus subtilis* yielded the highest lipase activity (25.5±0.2 U/ml) within 60 days of cultivation followed by *Candida tropicalis* (19.1±0.3 U/ml), *Candida albicans* (10.6±0.2 U/ml), *Bacillus stearothermophilis* (14.1±0.0 U/ml) and *Bacillus sphaericus* (0.6±0.2 U/ml). Lipase from *B. subtilis* and *C. tropicalis* were applied for carotenoids extraction from chili samples (*Capsicum annuum* L.). During the experiments, the chili was cut, dried with freeze dryer and characterized. The chili sample dried with freeze-drying method had a final moisture content and fat content at 4.05±0.19% and 6.62±0.11%, respectively. The chili powder was applied for pigment extraction using enzyme and solvent extraction. The highest carotenoids content (6.26 g of total carotenoids/ kg of powder) was obtained from dried chili extracted with lipase from *B. subtilis* at lipase concentration of 25 U/ml. In particular, extraction with lipase from *B. subtilis*, yielded an increase of 1.56 folds for carotenoids content as compared to control.

Key words: *Bacillus subtilis*, *Candida tropicalis*, *Capsicum annuum*, carotenoids, enzymatic extraction, lipase.

INTRODUCTION

In recent years, lipase has a wide range of potential applications such as hydrolysis of fats, tranesterification, stereo specific hydrolysis of racemic esters, organic synthesis as well as, their use in the detergent, textile, dairy industry, oil processing, production of surfactants, synthesis of chiral pharmaceuticals and paper industry was also developed (Inoita et al., 2001; Ertugrul et al., 2007). Many microorganisms such as bacteria, yeast and fungi are known to secret lipases during growth on insoluble organic substrate. The literature reports that several *Bacillus* and *Candida* species isolated from several diverse environments produce lipase enzyme.

Capsicum annuum is the most cultivated chili in the world due to its unique flavor and pungency (Salgado-Roman et al., 2008). *C. annuum* L. are widely used to obtain carotenoids for applications in the food industry as natural colorants. *Candida pubescen* and *Candida chinense* are used as a source of capsaicinoids for the pharmaceutical industry as therapeutic agents (Kozukue et al., 2005). However, some of chili such as *C. frutescens*, *C. annuum cayenne* and *C. annuum bydagi* are source of both carotenoids and capsaicinoids (Dull et al., 2000). Currently, many researchers attempted to develop chili extraction process by using several of enzymes such as Viscoenzyme L, Extrazyme, pectinase and multienzyme complex (Santamaría et al., 2000; Desikacharya et al., 2004). However, only little information is available about carotenoids extraction from dried chili using lipase.

In this study, three strains of *Bacillus* (*Bacillus sphaericus, B. stearothermophilis*, *B. subtilis*) and two strains of *Candida* (*C. albicans* and *C. tropicalis*) were selected to investigate their production of lipase as well

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as, their lipase activity. Therefore, lipase from selected bacteria was applied to improve an enzymatic treatment that relies on enzymatic extracts from chili powder as compared to solvent extraction.

MATERIALS AND METHODS

Bacteria, culture media and cultivation conditions

Three strains of *Bacillus* species including *B. sphaericus* TISTR 678, *Bacillus stearothermophilis* TISTR 329 and *Bacillus subtilis* TISTR 1248 (Culture collection, Thailand Institute of Scientific and Technological Research, Thailand) were used in all the cultivation experiments.

Liquid medium containing (w/v): Rhodamine B 0.001, nutrient broth 0.8, NaCl 0.4 and olive oil 3, were used as a selected media for lipase production. The initial pH of media was 6.5 incubated at 55^{o}C for 48 h.

Cultivation medium containing (w/v): peptone 1, yeast extract 0.5 and NaCl 0.5. Medium was adjusted to pH 6.5 incubated at 55℃ for 18 h. Submerged medium containing (w/v): nutrient broth 0.325, $CaCl_2$ 0.325, olive oil 2.5 and gum arabic 1. The initial pH of media was 6.5 incubated at 55℃ with agitation speed at 1 50 rpm for 96 h.

Yeasts, culture media and cultivation conditions

Two strains of *Candida* species including *C. albicans* TISTR 5779 and *C. tropicalis* TISTR 5045 were utilized as lipase producer. Liquid medium containing (w/v): gum arabic 10 and olive oil or tributylin emulsion 10 have been used as a selected media for lipase production. The initial pH of media was 5.6 incubated at 28℃ for 96 h. Cultivation medium for yeast namely YED medium containing (g/L): yeast extract 10, anhydrous dextrose 10. Medium was adjusted to pH 5.6 incubated at 28℃ for 96 h.

Assay of enzyme lipase activity

Cells were separated from the incubation or cultivation medium by centrifugation at 15,994 x g for 5 min at 4℃ and the supernatant was used as the source of extracellular enzyme. The harvested cells were washed with distilled water twice in microcentrifuge tubes, kept on ice and then sonicated (20 kHz, 5 min). The cell debris was removed by centrifugation at 15,994 x g rpm for 5 min at 4℃ and the clear supernatant was used to determine the intracellular lipase activity.

Lipase activity was determined using *p*-nitrophenol pamitate (*p*NPP) as substrate. The substrate solution was prepared by adding the solution A (30 mg of *p*NPP in 10 ml of isopropanol) into solution B (0.1 g of gum arabic and 0.4 ml Triton X-100 in 90 ml of 50 mM Tris-HCl buffer, pH 8) with stirring until all was dissolved. The mixture of 9 ml of substrate solution and 1 mL of suitably diluted enzyme solution was incubated at 30±1℃ for 15 min and absorbance was measures for 15 min at 410 nm in a spectrophotometer (Shimadzu UV 2001). One unit of activity (U/ml) was expressed as µmol of *p*-nitrophenol released per minute under the assay conditions (Ertugrul et al., 2007).

Determination of cell growth

Cell growth was determined by diluting the culture supernatant to the appropriate concentration with 0.9% NaCl. Cell concentration was calculated as the difference in optical density values between the sample and blank at 560 nm.

Raw materials

C. annuum L. was obtained from Phatthalung market (Thailand). During the experiments, the chili was cut, the whole fruit of chili was used with stems and seeds, and dried with freeze dryer. After drying, the samples were ground into powder with the grinder.

Enzymatic extract

Starter culture was cultivated on cultivation medium. 10% of starter was transfer to submerge medium (pH 6.5) incubated at 55℃ with agitation speed at 150 rpm for 48 h. These conditions led to morphological development in the form of pellets and high lipase synthesis. After 48 h, the biomass was separated by centrifugation (15,994 x g, 5 min), and supernatant obtained was used as an enzymatic extract. The enzyme concentration was determined using *p*-nitrophenol pamitate (*p*NPP) as substrate (Ertugrul et al., 2007).

To evaluate the effect of the enzymatic extract on chili fruits, the enzyme solution was blended with 2 g of dry chili and kept on rotary (150 rpm) at 30℃ for 60 min. Samples were taken and determined for carotenoids contents compared with the effect of commercial enzyme extraction.

Solvent extraction

Two grams of chili samples were extracted using 20 mL of acetone or hexane. Sample and solvent mixtures were agitated on rotary (150 rpm) at 30℃ for 60 min. Samples were taken and determined for carotenoids contents.

Carotenoids analysis based on the AOAC (970.64) method

When extraction was finished, 20 ml of the solvent mixture was added. A sample (10 ml) of the liquid phase was mixed with 20 ml of the solvent mixture and 2 ml of a solution of potassium hydroxide (Sigma Chemical) in methanol (40% w/v). The resulting solution was incubated for 20 min at 56℃ for saponification. Subsequently, the solution was mixed with 30 mL of hexane and 38 ml of sodium sulfite (Sigma Chemical) solution (10% w/v). After settling, the immiscible phases were separated. The light phase (that contained the free carotenoids) was analyzed by a UV-vis spectrophotometer at 460 nm, and the concentration of carotenoids was obtained on the basis of the following expression: total carotenoids = [($A_{460\ nm}$ × 11.57)/wt of sample] (Salgado-Roman et al., 2008).

Partial characterization of chili (*Capsicum annuum* L.) powder

Moisture

Moisture content of the paprika powders were determined by drying at 60℃ in a convection cabinet dryer until insignificant consecutive weight changes (Topuz et al., 2011).

Fat content

Five grams of sample was transferred to a thimble and sealed the end. Extraction thimble with the sample was placed in the soxhlet apparatus and fixed a previously dried and weighed round bottom flask. 200 ml of extracting solvent (petroleum ether) was added to the flask containing pumice chips. Then the flask and the condenser were connected to the soxhlet extractor. Sample was

Table 1. The extracellular and intracellular lipase activities of five bacterial tests in this study.

Microorganism	Lipase activity (U/ml)	
	Intracellular	Extracellular
Bacillus sphaericus TISTR 678	6.0±0.2	3.4±0.2
B. stearothermophilis TISTR 329	14.1±0.4	9.3±0.7
B. subtilis TISTR 1248	25.5±0.2	10.2±0.3
Candida albicans TISTR 5779	10.6±0.2	6.5±0.4
C. tropicalis TISTR 5045	19.1±0.3	10.3±0.5

allowed to reflux for about five hours. After the extraction flask was removed from the apparatus and kept in the water bath and then in the oven. Then the flask was cooled and weight was taken (Topuz et al., 2011). % of crude fat was calculated as follow:

$$\% \text{ of crude fat} = \frac{(X - F) \times 100}{W}$$

Where X = weight of flask with fat and chips, F = weight of flask and chips, W = weight of sample

RESULTS AND DISCUSSION

Production of lipase from *Bacillus* and *Candida* species

Three strains of *Bacillus* were firstly cultivated on agar plates containing rhodamine B to evaluate the feasibility of selected bacterial for lipase production. After 18 h of incubation all *Bacillus* strains began to show an orange fluorescence; with continuing incubation time orange fluorescent halos were formed around the colonies of lipase producing strains. However, *Candida* sp. showed the zone of clearing on agar plates containing tributyrin after 80 h of incubation. The result indentified all selected bacterial in this study as lipase producers, in accordance with the results of a titrimetric assay of culture supernatants.

In the experiments intracellular and extracellular lipase activity were determined, 1 ml of culture which was formerly activated in the medium for lipase activity, was inoculates in 100 ml of cultivation medium. Table 1 show the intracellular and extracellular lipolytic activities of bacteria tested in this study. As aforementioned, *Bacillus* sp. and two of *Candida* strains showed the ability to produced lipase during its growth. *B. subtilis* yielded the highest lipase activity mainly extracellularly (25.5±0.2 U/mL) within 60 h of cultivation followed by *C. tropicalis* (19.1±0.3 U/ml), *C. albicans* (10.6±0.2 U/ml), *B. stearothermophilis* (14.1±0.0 U/ml) and *B. sphaericus* (0.6±0.2 U/ml). The enzymatic extract obtained as earlier mentioned increased the cell-wall permeability of several substrates including chili, and it facilitates the diffusive processes that are inherent to an extraction process. Therefore, lipase from *B. subtilis* and *C. tropicalis* were selected and utilized as enzyme for pigment extraction from chili throughout this study.

Analysis of dried chili (*Capsicum annuum* L.) sample

The chili samples (*Capsicum annuum* L.), an abundant chili found in Phatthalung (Thailand), is one of the most widely used food colourants for culinary and industrial purposes such as soups, stews, sausage, cheese, snacks, salad dressing, sauces, pizza, and confectionary products because of its high colouring capacity (Topuz et al., 2011). Therefore, *C. annuum* L. were selected for carotenoids extraction. During the experiments, the chili was cut and dried with freeze dryer. After drying, the samples were ground into powder with the grinder. The chili sample dried with freeze-drying method had a final moisture content and fat content at 4.05±0.19% and 6.62±0.11%, respectively.

Traditional drying methods are known to be associated with microbial proliferation and quality deterioration in the dehydrated and milled fruit of certain varieties of red peppers (*Capsicum annuum* L.). Traditionally, dried chili has been processed by sun drying (Condori et al., 2001). It is a lengthy process, taking up to 10 days to bring moisture content to 9.9% (Oberoi et al., 2005). The moisture content of dried chili from various drying method were shown in Table 2.

Effect of enzymatic treatment and solvent extraction on carotenoids content in chili powder

Dried samples were treated with the *B. subtilis* and *C. tropicalis* enzymatic extract at lipase concentration of 25 U/ml. The highest carotenoids content was obtained from dried chili extracted with lipase from *B. subtilis* (6.26 g of total carotenoids/ kg of powder (dry weight). An increase in the yield of carotenoids content compare with the control (non-treated chili powder), was clearly correlated with enzymatic activity. In particularly, extraction with lipase from *B. subtilis*, yield increases of 1.56 folds for carotenoids content compared to control (4.02 g of total carotenoids/ kg of powder). However, lower carotenoids content (3.47 g of total carotenoids/ kg

Table 2. Moisture content value of dried chili (*Capsicum annuum* L.) powders.

Samples	Moisture content (%)	Reference
Fresh chili	80 - 85	This study
Freeze drying	4.05±0.19	
Freeze drying	4.65±0.40	Topuz et al. (2008)
Oven drying	4.86±0.19	
Refractive window drying	4.76 ± 0.32	
Natural convective drying	5.36 ± 0.26	

Table 3. Concentration profiles after lipase treatment (enzymatic concentration at 25 U/ml) and solvent extraction on carotenoids in the chili (*Capsicum annuum* L.) samples compared to the other studies.

Chili	Extraction method	Samples	Absorbance (OD_{460nm})	Carotenoids concentration [g / kg of powder]	Reference
Capsicum annuum L.	Blank		0	0	This study
	Control		0.695	4.02	
	Lipase	*B. subtilis*	1.082	6.26	
		C. tropicalis	0.599	3.47	
		Commercial lipase (*Candida* sp.)	0.447	2.59	
	Solvent	Acetone	0.347	2.01	
		Hexane	0.387	2.24	
		Acetone : Hexane (1:1)	0.354	2.05	
Bydagi chili	Lipase	*Rhizopus nigricans* (concentration 114.039 µg/ml)	NG[a]	7.1	Salgado-Roman et al. (2008)
Cayenne chili				2.6	
Paprika (Cv., Jalapeno)					
Freeze drying	Solvent	Acetone	NG	1.61	Topuz et al. (2008)
Natural convective drying				2.53	

[a]NG = Not given.

of powder) was obtained from *C. tropicalis* suspension (Table 3). Extraction of carotenoids from various solvent gave lower content compared to enzymatic extraction. Carotenoids content from solvent extraction was 2.01 to 2.24 g of total carotenoids/ kg of powder. Hexane is an appropriate solvent for pigment extraction, which gave highest carotenoids content (2.24 g of total carotenoids/ kg of powder) among the other. The combination of acetone and hexane yield moderate carotenoids content (2.05 g of total carotenoids/ kg of powder) and the lowest carotenoids content achieved when acetone was utilized as a sole solvent (2.01 g of total carotenoids/ kg of powder).

However, the highest carotenoids content from chili (*C. annuum* L.) powders treated with the *B. subtilis* (6.26 g/kg powder) obtained from this study was 1.13 folds lower than Salgado-Roman et al (2008). The highest carotenoids was extracted from two varieties of chili samples including bydagi and cayenne chilli fruits. The samples were treated with the *Rhizopus nigricans* enzymatic extract at a protein concentration of 114.039 µg/ml showed an increase in the yield of carotenoids at 7.1 and 2.6 g of total carotenoids/ kg of powder (dry weight), respectively. Therefore, the strain of chili may play an important role in carotenoids content. Not only is the strain of chili but also drying methods affected on pigment extraction. Topuz et al. (2008) reported that the carotenoids compositions of the chili samples were significantly ($P < 0.05$) altered after drying. Their results showed that the carotenoids concentrations of paprika puree were decreased by the freeze drying (1.61±0.01 g/kg), oven drying (1.47±0.08 g/kg) and refractive window drying methods (1.53±0.04 g/kg). No significant differences were found among these drying methods with regards to their effect on the carotenoids contents.

However, it is interesting to point out that the natural

convective dried samples generally had the highest carotenoids concentrations (2.53±0.08 g/kg) in comparison to the paprika powders produced by the other methods, as well as those in the puree (Topuz et al., 2011).

Conclusion

All strain of microorganisms tested in this study showed the ability to produced lipase which *B.subtilis* yield the highest lipase activity (25.5±0.2 U/ml) within 60 days of cultivation followed by *C. tropicalis* (19.1±0.3 U/ml).

The chili samples (*C. annuum* L.) were selected for carotenoids extraction. The chili sample dried with freeze-drying method had a final moisture content and fat content at 4.05±0.19% and 6.62±0.11%, respectively. The chili powder was applied for pigment extraction using enzyme and solvent extraction. The highest carotenoids content [6.26 g of total carotenoids/ kg of powder (dry weight)] was obtained from dry chili extracted with lipase from *B*. *subtilis* at lipase concentration of 25 U/ml. Extraction of carotenoids from various solvent gave lower content compared to enzymatic extraction. Carotenoids content from solvent extraction was 2.01 to 2.24 g of total carotenoids/ kg of powder. This study carried out is significant because carotenoids is currently needs for applications in the food industry as natural colorants. The process of extraction is environmental friendly and cause minimum environmental or atmospheric pollution.

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