Food Bioprocess Technol (2012) 5:1317–1327
DOI 10.1007/s11947-010-0402-9

ORIGINAL PAPER

Use of Protein Hydrolysate from Yellow Stripe Trevally (*Selaroides leptolepis*) as Microbial Media

Vilailak Klompong · Soottawat Benjakul · Duangporn Kantachote · Fereidoon Shahidi

Received: 26 April 2010 / Accepted: 23 June 2010 / Published online: 7 July 2010

Abstract The objective of this study was to investigate the potential use of protein hydrolysate from yellow stripe trevally as a nitrogen source for the growth of different microorganisms. Protein hydrolysates from yellow stripe trevally with different degrees of hydrolysis (5, 15 and 25%) prepared using Alcalase (HA) or Flavourzyme (HF) were determined in comparison with commercial Bacto Peptone. For bacteria, *Staphylococcus aureus* and *Escherichia coli*, HF with 25% DH (HF_{25}) yielded the highest cell density and specific growth rate (μ_{max}) and the lowest generation time (t_d) ($p<0.05$). For yeasts, *Saccharomyces cerevisiae* and *Candida albicans*, Bacto Peptone yielded the higher growth rate than did HA and HF ($p<0.05$), whereas no differences in μ_{max} and t_d were observed for fungus, *Aspergillus oryzae* ($p>0.05$). The pH of culture broth containing HF_{25} decreased markedly during the first 8 hours of cultivation of *S. aureus* and *E. coli* ($p<0.05$). This directly lowered the colony size of *S. aureus* ($p<0.05$). However, buffered culture broth containing HF_{25} rendered the similar growth and colony size of *S. aureus* ($p>0.05$), compared with that containing Bacto Peptone. Scanning electron microscopic study revealed no differences in size and shape of microorganisms cultured in HF_{25} and Bacto Peptone ($p>0.05$).

Keywords Protein hydrolysate · Yellow stripe trevally · Microbial growth · Peptone · Alcalase · Flavourzyme

V. Klompong · S. Benjakul (✉)
Department of Food Technology, Faculty of Agro-Industry,
Prince of Songkla University,
Hat Yai 90112, Thailand
e-mail: soottawat.b@psu.ac.th

D. Kantachote
Department of Microbiology, Faculty of Science,
Prince of Songkla University,
Hat Yai 90112, Thailand

F. Shahidi
Department of Biochemistry, Faculty of Science,
Memorial University of Newfoundland,
St. John's, NL A1B 3X9, Canada

Introduction

Fish processing by-products and under-utilized species are commonly recognized as low-value resources with negligible market value. In addition, their inappropriate disposal is a major cause for environmental pollution. To tackle these problems, hydrolysis processes have been developed to convert such resources into marketable and acceptable forms. Protein hydrolysates from different fish species such as tuna, cod, salmon and unspecified fish have been used as nitrogenous sources for microbial growth (Dufosse et al. 2001). Soluble fish protein hydrolysate from extensive hydrolysis comprises free amino acids and low-molecular-weight peptides, which can be used as an excellent nitrogen source for microbial growth and thus can be used as microbial peptone. The peptides with the molecular weight of 6,500 Da, di-peptides and amino acids from tuna treated with Alcalase served as a suitable nitrogenous source in microbial media (Guerard et al. 2001). Recently, protein hydrolysates from yellow stripe trevally have been produced successfully using Alcalase and Flavourzyme (Klompong et al. 2007a, b). Degree of hydrolysis (DH) can affect the functional properties and antioxidative activity of resulting protein hydrolysate (Klompong et al. 2007a). DH influencing peptide chain length may have the impact on the ease of utilization by microorganisms. In addition, different peptides produced by different proteases

can be used by microorganisms at varying degrees. Therefore, the objective of this study was to investigate the potential use of protein hydrolysate from yellow stripe trevally produced by Alcalase or Flavourzyme as a nitrogen source for the growth of different microorganisms including bacteria, yeasts and mold.

Materials and Methods

Enzymes and Cultivation Media

Alcalase and Flavourzyme were obtained from Novo Nordisk (Bagsvaerd, Denmark) and East Asiatic Company Ltd. (Bangkok, Thailand), respectively. Bacto Peptone was purchased from Difco Laboratories (Sparks, MD, USA). Nutrient Broth (NB) was procured from Merck (Darmstadt, Germany), and Potato Dextrose Broth (PDB) was obtained from Himedia Laboratories (Mumbai, Maharashtra, India).

Fish Sample Collection and Preparation

Yellow stripe trevally (*Selaroides leptolepis*) caught along the coast of the Andaman Sea with the size of 65 g per fish, off-loaded approximately 24–36 hours after capture, were obtained from the fishing port in Satul province, Thailand. Fish were washed, and the meat was separated manually. The meat was minced using a mincer with 0.4-cm-diameter holes. The mince was stored in ice until use.

Production of Protein Hydrolysates from Yellow Stripe Trevally

Mince (60 g) was suspended in 240 ml of distilled water. The mixture was homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 minute. The homogenate was adjusted to pHs 8.5 and 7.0 and preincubated at 60 or 50 °C for 20 minutes prior to enzymatic hydrolysis using Alcalase and Flavourzyme, respectively. The hydrolytic reaction was started by the addition of 0.0076, 0.05 and 0.33% Alcalase (w/w) or 0.26, 1.58, 9.77% Flavourzyme (w/w) based on the protein content of mince (Klompong et al. 2007a). The reaction was conducted as per the pH-stat method (Adler-Nissen 1986) for 20 minutes to obtain the DH of 5, 15, 25% as described by Klompong et al. (2007a).

Media Formulas

Different media including Nutrient Broth (NB) and agar (NA), yeast–malt broth (YMB) and agar (YMA) and Malt Extract Broth (MEB) and agar (MEA) were prepared for culturing bacteria, yeasts and mold, respectively. To study the use of HA or HF as the replacer for commercial peptone, Bacto Peptone was substituted by HA with DHs of 5% (HA_5), 15% (HA_{15}) and 25% (HA_{25}) or HF with DHs of 5% (HF_5), 15% (HF_{15}) and 25% (HF_{25}) at the amount yielding an equivalent level of nitrogen content to Bacto Peptone determined by Kjeldahl method (AOAC 2000).

Preparation of Microorganisms and Inocula

Staphylococcus aureus TISTR 118, *Escherichia coli* TISTR 780 and *Saccharomyces cerevisiae* TISTR 5017 were obtained from the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. *Candida albicans* PSSCMI 7010 and *Aspergillus oryzae* PSSCMI 3004 were gifts from the Department of Microbiology, Prince of Songkla University, Hat Yai, Thailand. Bacteria were kept on NA slants, whereas yeasts and mold were kept on PDA slants at 4 °C until use. To activate microorganisms before culturing, the inocula were prepared. The inocula were adjusted to obtain A_{660} of 0.02 and then subjected to centrifugation at 3,000×*g* (Hettich, Mikro 20, Zentrifugen, Germany) for 20 minutes at 25 °C. Supernatants were discarded, and the cells were washed twice with normal saline (0.85% NaCl solution). The fresh culture media were added to the cell pellet to obtain A_{660} of 0.02. The obtained cell suspension was used as late log phase inocula. For mold, *A. oryzae* was sub-cultured onto PDA slant and incubated at 25 °C for 48 hours. This process was conducted twice to produce spores. Spore suspension with the concentration of 10^6 spores/ml measured by direct count using hemacytometer was prepared.

Determination of Growth Kinetic of Microorganisms

Growth rates were determined by submerged cultivation. To 50 ml of tested broths containing HA or HF at different DHs or standard broths containing Bacto Peptone, 1.5 ml of inocula with A_{660} of 0.02 or 10^6 spores/ml was added. NB, YMB and MEB were used for culturing bacteria, yeasts and mold, respectively. Incubation was carried out at 37 °C for 24 hours for bacteria and at 25 °C for 48 hours for yeasts with continuous shaking at 120 rpm. The growth of bacteria and yeasts were monitored by turbidity (A_{660}) measurements every 4 hours. For mold, dry cell weight (DCW) measurement was used to monitor the growth every 4 hours up to 48 hours. Briefly, 2 ml of liquid culture was centrifuged at 3,000×*g* for 20 minutes at 25 °C (Hettich, Mikro 20, Zentrifugen, Germany). The cell pellet was resuspended in 2 ml normal saline and centrifuged under the same conditions. Washed cells were dried at 105 °C until the constant dry weight was obtained.

Growth curves of each microorganism cultured in different media containing HA or HF (5, 15 and 25% DH) or commercial peptone (Bacto Peptone) were obtained by plotting A_{660} for bacteria and yeasts or DCW for mold against incubation time. Specific growth rate (μ_{max}) and generation time (t_d) were then calculated (Lynch and Poole 1979) as follows:

$$\mu_{max} = \frac{\ln x_t - \ln x_o}{t_t - t_o}$$

$$t_d = 0.693/\mu$$

where x_o is initial population; x_t is population at instant t; t_o is initial time and t_t is instant time.

Protein hydrolysate with the DH rendering the highest growth rate of all microorganisms was selected for further study.

Measurement of Growth and Colony Size of Microorganisms Cultured in Medium Containing Protein Hydrolysate

To study the effect of HF_{25} on qualitative microbial growths, the colony size was determined. One loopful of each bacterium cultured in NA slants for 15 hours and yeast cultured in YMA slant for 24 hours was sub-cultured on NA and YMA containing Bacto Peptone or HF_{25} at equivalent total nitrogen (HF_{25tn}) or total solid (HF_{25ts}) contents to Bacto Peptone. The culture was incubated at 37 °C for 15 hours for bacteria and at 25 °C for 24 hours for yeasts. Thereafter, bacteria and yeasts were inoculated into NB and YMB containing HF_{25tn}, HF_{25ts} or Bacto Peptone for bacteria and yeasts, respectively, and then incubated at 37 °C for 15 hours for bacteria and at 25 °C for 24 hours for yeasts with continuous shaking at 120 rpm. Culture broths obtained were adjusted with corresponding culture media to obtain A_{660} of 0.5. For mold, *A. oryzae* was sub-cultured onto MEA slant containing HF_{25tn}, HF_{25ts} or Bacto Peptone and incubated at 25 °C for 48 hours twice to produce spores. Spore suspension was prepared as previously described and adjusted to 10^6 spores/ml using sterile normal saline. Serial dilutions of bacteria, yeasts and mold were prepared. Thereafter, aliquots of 0.1 ml dilution were pipetted onto corresponding agar plates containing HF_{25tn}, HF_{25ts} or Bacto Peptone and then spread using a sterile spreader. The plates were incubated at 37 °C for 24 hours for bacteria and incubated at 25 °C for 48 hours for yeasts and mold before counting and measuring colony diameter. The colony counts were recorded, and the diameters of 20 colonies were measured per plate.

Measurement of Growth and Colony Size of *S. aureus* Cultured in Medium Containing Protein Hydrolysate Supplemented with Buffer

To study the effect of buffer to improve the colony size of *S. aureus*, all NB and NA used for culturing *S. aureus* were supplemented with 0.2% dipotassium hydrogen phosphate (K_2HPO_4) and the pH was adjusted to 6.8±0.2 using 1 M NaOH or HCl. One loopful of *S. aureus* cultured in NA slants for 15 hours was sub-cultured on NA, which was supplemented with 0.2% K_2HPO_4 and contained HF_{25} at equivalent total nitrogen (HF_{25tn}) and total solid (HF_{25ts}) contents to Bacto Peptone or NA containing Bacto Peptone and 0.2% K_2HPO_4 (pH 6.8). The culture was incubated at 37 °C for 15 hours. Thereafter, *S. aureus* was inoculated into NB, which was supplemented with 0.2% K_2HPO_4 and contained HF_{25tn}, HF_{25ts} or Bacto Peptone and then incubated at 37 °C with continuous shaking at 120 rpm. Culture broths were taken after cultivation for 4, 8 and 12 hours, representing the log phase of *S. aureus*. Culture broths obtained were adjusted with NB to obtain A_{660} of 0.5. Serial dilutions were prepared with sterile normal saline. Thereafter, aliquots of 0.1 ml dilution were pipetted onto NA, which was supplemented with 0.2% K_2HPO_4 and contained HF_{25tn}, HF_{25ts} or Bacto Peptone and then spread using a sterile spreader. The plates were incubated at 37 °C for 24 hours before counting and measuring colony diameter. The colony counts were recorded, and the diameters of 20 colonies were measured per plate.

Determination of Microbial Morphology

Morphology of the late log phase of bacteria and yeasts and the stationary phase of mold cultured in NA and YMA and MEA containing HF_{25ts} or Bacto Peptone was analyzed by a scanning electron microscopy (JEOL JSM-5800LV, Tokyo, Japan).

Statistical Analysis

One-way analysis of variance (ANOVA) was used, and mean comparison was performed by Duncan's multiple range tests (Steel and Torrie 1980). Statistical analysis was carried out using SPSS statistic program (Version 11.0) for Windows (SPSS Inc. Chicago, IL, USA).

Results and Discussion

Effect of Protein Hydrolysate from Yellow Stripe Trevally Prepared Using Alcalase or Flavourzyme with Different DHs on Microbial Growth

Growth curves of bacteria, yeasts and mold cultured in the media containing HA or HF, in comparison with those

cultured in media with Bacto Peptone, are depicted in Figs. 1, 2 and 3, respectively. As the cultivation time increased, cell densities of all microorganisms increased, regardless of media used, and reached the plateau differently, depending on the types of microorganism ($p<0.05$). pHs of media were changed differently and varied with microorganisms. μ_{max} and t_d also varied with media as well as types of hydrolysate and DHs used (Table 1).

Bacteria

For *S. aureus*, no changes in cell density were found within the first 4 hours of cultivation period. Thereafter, the log phase started and reached stationary phase at hour 16 (Fig. 1a). The media containing HA or HF with 25% DH

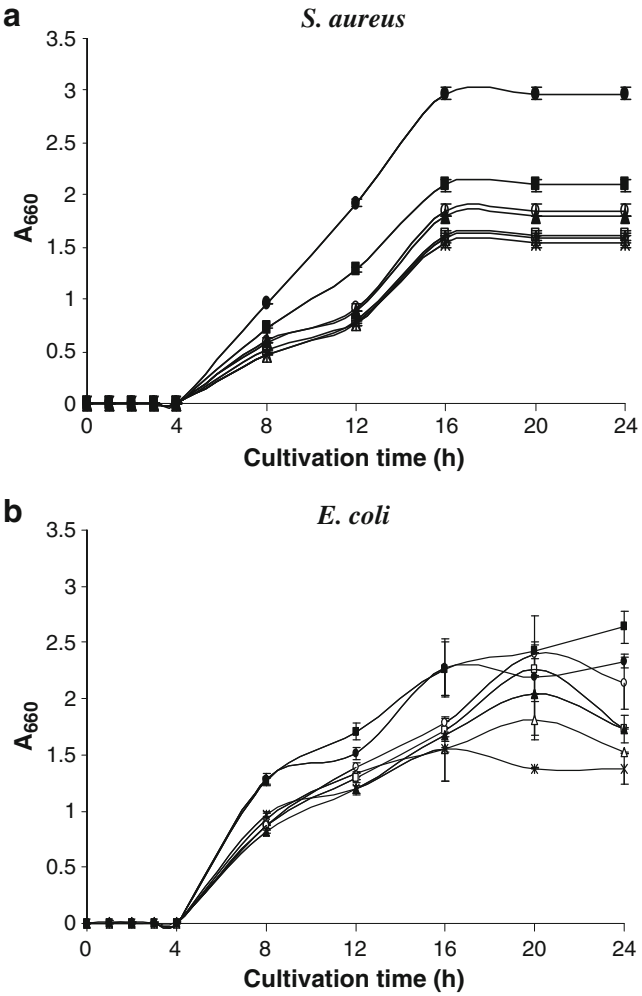

Fig. 1 Growth curve of *S. aureus* (**a**) and *E. coli* (**b**) during cultivation in NB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5% (Δ), 15% (□), 25% (○); HF with DH of 5% (▲), 15% (■), 25% (•), Bacto Peptone (*). *Bars* represent standard deviations from triplicate determinations

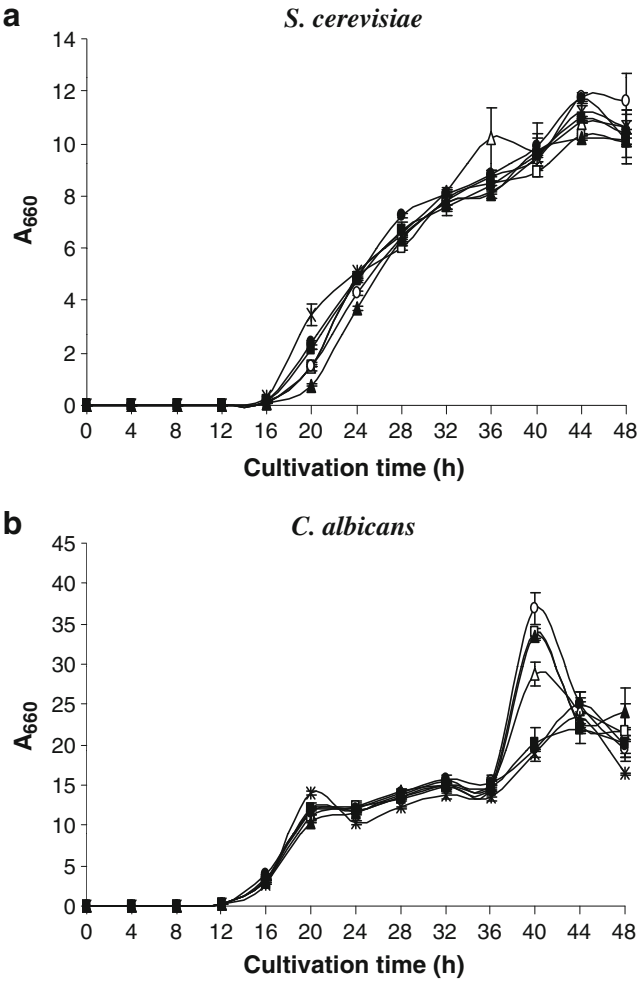

Fig. 2 Growth curve of *S. cerevisiae* (**a**) and *C. albicans* (**b**) during cultivation in YMB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5% (Δ), 15% (□), 25% (○); HF with DH of 5% (▲), 15% (■), 25% (•), Bacto Peptone (*). *Bars* represent standard deviations from triplicate determinations

(HA_{25} and HF_{25}) showed the higher cell density and μ_{max} with a lower t_d than did those with HA or HF at lower DHs ($p<0.05$). At the same DH tested, HF yielded the greater cell density and μ_{max} and lower t_d than did HA, manifesting that the types of enzyme used for hydrolysate preparation affected the growth of *S. aureus*. All media containing HA or HF exhibited the higher cell density and μ_{max} with lower t_d than did that containing Bacto Peptone except for HA_5. The pHs of media containing HF started to drop at hour 4. This was coincidental with the beginning of log phase. The decrease in pH was more intense at hour 8, followed by the gradual increase (data not shown). The medium containing HF_{25} had the lower pH than did others. This was concomitant with the higher cell density. The result suggested that the extraordinary growth of *S. aureus* occurred in the medium containing HF_{25}.

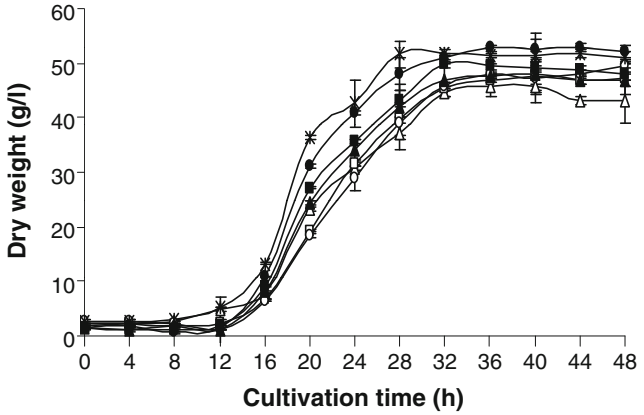

Fig. 3 Growth curve of *A. oryzae* during cultivation in MEB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5% (Δ), 15% (□), 25% (○); HF with DH of 5% (▲), 15% (■), 25% (•), Bacto Peptone (*). *Bars* represent standard deviations from triplicate determinations

Escherichia coli maintained the lag phase up to 4 hours of cultivation period, followed by the log phase and reached the stationary phase at hours 16–24 (Fig. 1b). *E. coli* cultured in media containing HA or HF with various DHs or Bacto Peptone started the log phase at the same time but reached stationary phase and death phase at different times. Although the media containing HF_{25} or HF_{15} showed the greatest cell density, that containing HF_{25} possessed the highest μ_{max} and the lowest t_d ($p<0.05$). Generally, the media containing HA or HF with higher DHs exhibited the higher cell density and μ_{max} with lower t_d. The media containing HF yielded the higher cell density and μ_{max} with the lower t_d than did those containing HA with the same DH. Thus, the types of enzyme used to produce HA and HF also affected the growth of *E. coli*. All media containing HA or HF exhibited the higher cell density and μ_{max} with the lower t_d than did media containing Bacto Peptone except HA_5. The pHs of all media containing HA or HF with all DHs except HF_{25} increased gradually with increasing cultivation time (data not shown). For the medium with HF_{25}, the pH decreased within the first 8 hours of cultivation. The pH drop of medium containing HF_{25} was much more intense than was other media. This was concomitant with the highest cell density and μ_{max} and the lowest t_d ($p<0.05$). However, the continuous increase in pH was observed during 8–24 hours of cultivation. The increase in pH was associated mostly with the increase in populations.

The differences between cell density, μ_{max}, t_d and pH changes of *S. aureus* and *E. coli* cultured in media containing various protein hydrolysates (HA_5, HA_{15}, HA_{25}, HF_5, HF_{15}, HF_{25}) or Bacto Peptone might be due to the differences in size of peptide. HA and HF with different DHs contained peptides with different sizes, in

Table 1 Specific growth rate (μ_{max}, h^{-1}) and generation time (t_d, min) of microorganisms cultured in media containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs compared with Bacto Peptone

Peptones	DH (%)	*S. aureus*		*E. coli*		*S. cerevisiae*		*C. albicans*		*A. oryzae*	
		μ_{max}	t_d	μ_{max}	t_d	μ_{max}	t_d	μ_{max}	t_d	μ_{max}	t_d
HA	5	0.95 ± 0.01^{e}	44 ± 0.26^{e}	1.38 ± 0.05^{b}	30 ± 1.01^{b}	0.30 ± 0.02^{c}	139 ± 7.00^{c}	0.33 ± 0.01^{b}	127 ± 4.43^{bc}	0.26 ± 0.00^{ns}	158 ± 1.17^{ns}
	15	0.98 ± 0.01^{d}	42 ± 0.43^{cd}	1.57 ± 0.10^{ab}	26 ± 1.58^{ab}	0.30 ± 0.00^{c}	138 ± 2.12^{c}	0.31 ± 0.00^{c}	134 ± 1.93^{c}	0.26 ± 0.03^{ns}	158 ± 11.66^{ns}
	25	1.02 ± 0.02^{c}	41 ± 0.64^{c}	1.52 ± 0.12^{ab}	27 ± 2.22^{ab}	0.26 ± 0.01^{d}	161 ± 5.24^{d}	0.31 ± 0.02^{bc}	132 ± 7.27^{bc}	0.26 ± 0.01^{ns}	159 ± 14.85^{ns}
HF	5	1.05 ± 0.02^{bc}	40 ± 0.63^{bc}	1.43 ± 0.12^{b}	29 ± 2.37^{b}	0.39 ± 0.03^{b}	106 ± 6.91^{b}	0.32 ± 0.01^{bc}	131 ± 2.46^{c}	0.27 ± 0.00^{ns}	151 ± 2.37^{ns}
	15	1.07 ± 0.01^{b}	39 ± 0.38^{b}	1.46 ± 0.14^{b}	28 ± 2.78^{ab}	0.20 ± 0.00^{e}	205 ± 0.47^{e}	0.33 ± 0.01^{b}	126 ± 2.19^{b}	0.27 ± 0.01^{ns}	153 ± 6.78^{ns}
	25	1.19 ± 0.00^{a}	35 ± 0.02^{a}	1.72 ± 0.09^{a}	24 ± 1.23^{a}	0.17 ± 0.00^{f}	238 ± 1.33^{f}	0.27 ± 0.01^{d}	152 ± 8.06^{d}	0.27 ± 0.02^{ns}	157 ± 9.40^{ns}
Bacto Peptone		0.97 ± 0.01^{de}	43 ± 0.60^{d}	1.40 ± 0.07^{b}	30 ± 1.56^{b}	0.61 ± 0.03^{a}	68 ± 3.26^{a}	0.41 ± 0.01^{a}	102 ± 1.46^{a}	0.28 ± 0.02^{ns}	149 ± 9.27^{ns}

Means ± standard deviations from triplicate determinations.

Different superscripts in the same column indicate significant differences ($p<0.05$)

ns non-significant difference ($p>0.05$).

which molecular weights lower than 204, 70 and 7 kDa were found with DHs of 5, 15 and 25%, respectively (Klompong et al. 2007b). This could contribute to a different biomass production of bacteria. Reductions of the molecular size of hydrolysate appeared when the DH increased (Klompong et al. 2007b). The results suggested that the DH of hydrolysates correlated with the maximum cell density, μ_{max}, t_d and pH changes in both cultures. Dufosse et al. (2001) also found a correlation between the peptone hydrolysis rate and the biomass production of *E. coli* and *Lactobacillus casei*. Peptides with the molecular weight of 6,500 Da, di-peptides and amino acids from tuna treated with Alcalase were a suitable nitrogenous source in microbial media (Guerard et al. 2001). HF_{25} was prepared by hydrolyzing yellow stripe trevally with Flavourzyme and had MW lower than 7 kDa (Klompong et al. 2007b). Thus, it was a suitable nitrogenous source for culturing bacteria. It could be concluded that HF_{25} with MW lower than 7 kDa might provide the valuable nutrients, which were readily available for growths of both *S. aureus* and *E. coli*.

Differences in maximum cell densities and growth rates of *S. aureus* and *E. coli* were noticeable when different hydrolysates and DHs were used ($p<0.05$). For all DHs, HA showed the inferior growth stimulation to HF. The result suggested the certain differences in hydrolysate composition such as size of peptides, amino acid composition of peptides, vitamins, fatty acids and inhibitory peptides. At the same DH, HA and HF might contain different peptides due to the cleavage of peptides at different positions or amino acids, generating various amino acids and peptides. Owing to exopeptidase activity, HF might contain more free amino acids that were readily available for bacterial growth than did HA. Aspmo et al. (2005) found that proteolytic enzyme clearly affected the growth performance of microorganisms cultured in media containing protein hydrolysate. The results suggested that HA and HF showed the better efficacy for bacterial growth than did commercial peptone (Bacto Peptone) ($p<0.05$).

In fish muscle, glycogen is the main carbohydrate. After capture, postmortem degradation of glycogen to glucose-6-phosphate and glucose occurs via phosphorolytic pathway and via hydrolytic or amylolytic pathway (Eskin 1990). This sugar could be consumed by bacteria as a carbon source (Boyd and Hoerl 1991). After sugar fermentation, organic acids were produced, resulting in the dramatic drop of pH of the medium containing HF_{25} used for culturing *S. aureus* and *E. coli*. The result suggested that medium containing HF_{25} might have the low buffering capacity. pH increases or decreases in culturing system depended on media and buffering capacity (Fernandez-Lopez et al. 2008). Protein and free amino acid are major contributors to pH buffering in the biological system. After 8 hours of cultivation, pH of media culturing *S. aureus* and *E. coli* increased gradually. When protein and amino acid were degraded during microbial growth, ammonia, non-protein nitrogen and amino acid catabolism products were accumulated (Nychas et al. 1998; Parente et al. 1994).

Yeasts

The log phase of *S. cerevisiae* started after cultivation for 16 hours (Fig. 2a). *S. cerevisiae* cultured in media containing HA or HF with various DHs or Bacto Peptone reached the plateau after 44 hours. Similar growth curves were obtained for *S. cerevisiae* cultured in all media. The medium containing HF_5 showed the greater μ_{max} and the lowest t_d than did those added with HA or HF having other DHs. Nevertheless, the medium containing Bacto Peptone possessed the highest μ_{max} and the lowest t_d ($p<0.05$) and had the greatest cell density during 16–24 hours of cultivation. Thereafter, no differences in cell densities in all media were distinguishably observed. Basically, the media containing HA or HF with higher DHs exhibited the lower μ_{max} and higher t_d ($p<0.05$). The pH of all media for culturing *S. cerevisiae* decreased after 12 hours, corresponding to the beginning of log phase at hour 16 (data not shown). As the cell density increased, the pHs slightly decreased in all media. However, a slight increase in pH was noticeable after 24 hours. The lower pH of the medium containing Bacto Peptone was more distinguished than other media, likely associated with the highest cell density and μ_{max} and the lowest t_d, particularly during 16–24 hours of cultivation ($p<0.05$).

The log phase of *C. albicans* began after cultivation for 12 hours and reached stationary phase at hour 20 (Fig. 2b). *C. albicans* cultured in all media began log phase synchronously. Nevertheless, the system containing Bacto Peptone possessed the highest μ_{max} and the lowest t_d ($p<0.05$) and showed the greatest cell density during 12–20 hours of cultivation. Thereafter, no differences in cell density among the media containing HA or HF were observed ($p>0.05$). Generally, the media added with HA or HF having the higher DHs exhibited the lower μ_{max} and higher t_d ($p<0.05$). The pHs of all media decreased at hour 12. This was associated with the multiplying of cells in the late lag phase found at hour 12 (data not shown). In general, the pH gradually increased after 24 hours of cultivation. The pH of the medium containing Bacto Peptone was lower than those of other media, particularly during 12–20 hours ($p<0.05$). *C. albicans* reached stationary phase within 4 hours after the beginning of log phase. After the easily fermentable sugar (dextrose in YMB) was exhausted, *C. albicans* used slow fermenting nutrients (Fernandez-Lopez et al. 2008). *C. albicans* expressed the

Table 2 Growth (A_{660}=0.5) and colony size of microorganisms cultured in media containing protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total nitrogen (HF_{25tn}) and total solid contents (HF_{25ts}) of Bacto Peptone, compared with Bacto Peptone

Microorganisms	Number (log CFU/ml)			Colony diameter (cm)		
	HF_{25tn}	HF_{25ts}	Bacto Peptone	HF_{25tn}	HF_{25ts}	Bacto Peptone
S. aureus	6.57±0.08^{a}	6.40±0.08^{b}	6.39±0.02^{b}	0.11±0.02^{b}	0.22±0.03^{a}	0.19±0.05^{a}
E. coli	7.15±0.05^{b}	7.26±0.03^{a}	7.09±0.06^{b}	0.27±0.07 ^{ns}	0.31±0.05 ^{ns}	0.24±0.04 ^{ns}
S. cerevisiae	5.48±0.02^{b}	5.21±0.03^{c}	5.80±0.02^{a}	0.20±0.02 ^{ns}	0.20±0.01 ^{ns}	0.21±0.03 ^{ns}
C. albicans	5.80±0.01 ^{ns}	5.78±0.15 ^{ns}	5.99±0.19 ^{ns}	0.16±0.04 ^{ns}	0.19±0.02 ^{ns}	0.23±0.04 ^{ns}
A. oryzae	3.77±0.07 ^{ns}	3.78±0.01 ^{ns}	3.81±0.05 ^{ns}	0.62±0.12 ^{ns}	0.64±0.12 ^{ns}	0.70±0.10 ^{ns}

Means ± standard deviations from triplicate determinations for numbers and from 20 determinations for colony diameter

Different superscripts in the same row within the same parameters indicate significant differences ($p<0.05$); *ns* non-significant difference ($p>0.05$).

Diauxie at hours 40–44. Diauxie was observed at low sugar concentration, independent of nitrogen supplementation and the types of sugar (Batistote et al. 2006). When the primary carbon sources are absent or present at concentrations low enough to limit growth, others nitrogen source such as amides, amino acids and peptides can be used as a carbon source. Batistote et al. (2006) reported that the growth of *S. cerevisiae* in medium containing maltose and glucose supplemented with different nitrogen sources exhibited diauxie. *C. albicans* reached the death phase at the period of 40–44 hours.

Mold

Aspergillus oryzae was in the lag phase up to 12 hours of cultivation period before the log phase was started (Fig. 3). *A. oryzae* reached stationary phase synchronously for all media at hour 32 except for the medium containing Bacto Peptone, which reached plateau at hour 28. That might be due to the greater biomass in the medium containing Bacto Peptone during 12–20 hours of cultivation ($p<0.05$), leading to the faster beginning of stationary phase. However, no significant differences of μ_{max} and t_d were found among the media containing HA, HF and Bacto Peptone (Table 1). DHs and enzyme types had no impact on μ_{max} and t_d of *A. oryzae* cultured in media containing HA or HF that might be due to the ability of *A. oryzae* in producing extracellular enzymes. The pHs of all media began to drop at hour 8 and decreased continuously up to 48 hours of cultivation (data not shown). As the dry biomass increased, the pHs decreased in all systems. The medium containing Bacto Peptone exhibited the lowest pH ($p<0.05$), which was concomitant with the greatest growth in this system. The decreased pH observed after growth of *A. Oryzae* is likely to be caused by assimilation of sugars and a metabolic capability (Pitt and Hocking 1999). For all bacteria tested, growth rates and maximum cell densities were higher when HF_{25} was used as a replacer of Bacto Peptone. However, HF_{25} was inferior to Bacto Peptone for yeast cultivation. In addition, no difference was observed between HF_{25} and Bacto Peptone for mold culturing. Each peptone has its own biologic characteristics and could not meet the requirements of all microorganisms during cultivation (Kurbanoglu and Algur 2002). Performances of peptones for production of cell biomass also exhibited large differences, depending on the strain tested (Boyd and Hoerl 1991).

Table 3 Growth and colony size of *S. aureus* cultured in NA and NB containing buffer and protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total nitrogen (HF_{25tn}) and total solid contents (HF_{25ts}) of Bacto Peptone, compared with Bacto Peptone during log phase

Duration (hours)	Number (log CFU/ml)			Colony diameter (cm)		
	4	8	12	4	8	12
HF_{25tn}	7.67±0.03^{a}	7.80±0.04^{a}	7.99±0.02^{a}	0.20±0.03 ^{ns}	0.21±0.03 ^{ns}	0.19±0.02 ^{ns}
HF_{25ts}	7.33±0.08^{b}	7.54±0.01^{b}	7.96±0.03^{a}	0.21±0.05 ^{ns}	0.20±0.02 ^{ns}	0.18±0.02 ^{ns}
Bacto Peptone	7.17±0.07^{c}	7.45±0.09^{b}	7.75±0.07^{b}	0.19±0.02 ^{ns}	0.19±0.01 ^{ns}	0.18±0.02 ^{ns}

Means ± standard deviations from triplicate determinations for numbers and from 20 determinations for colony diameter

Different superscripts in the same column indicate the significant differences ($p<0.05$); *ns* non-significant difference ($p>0.05$)

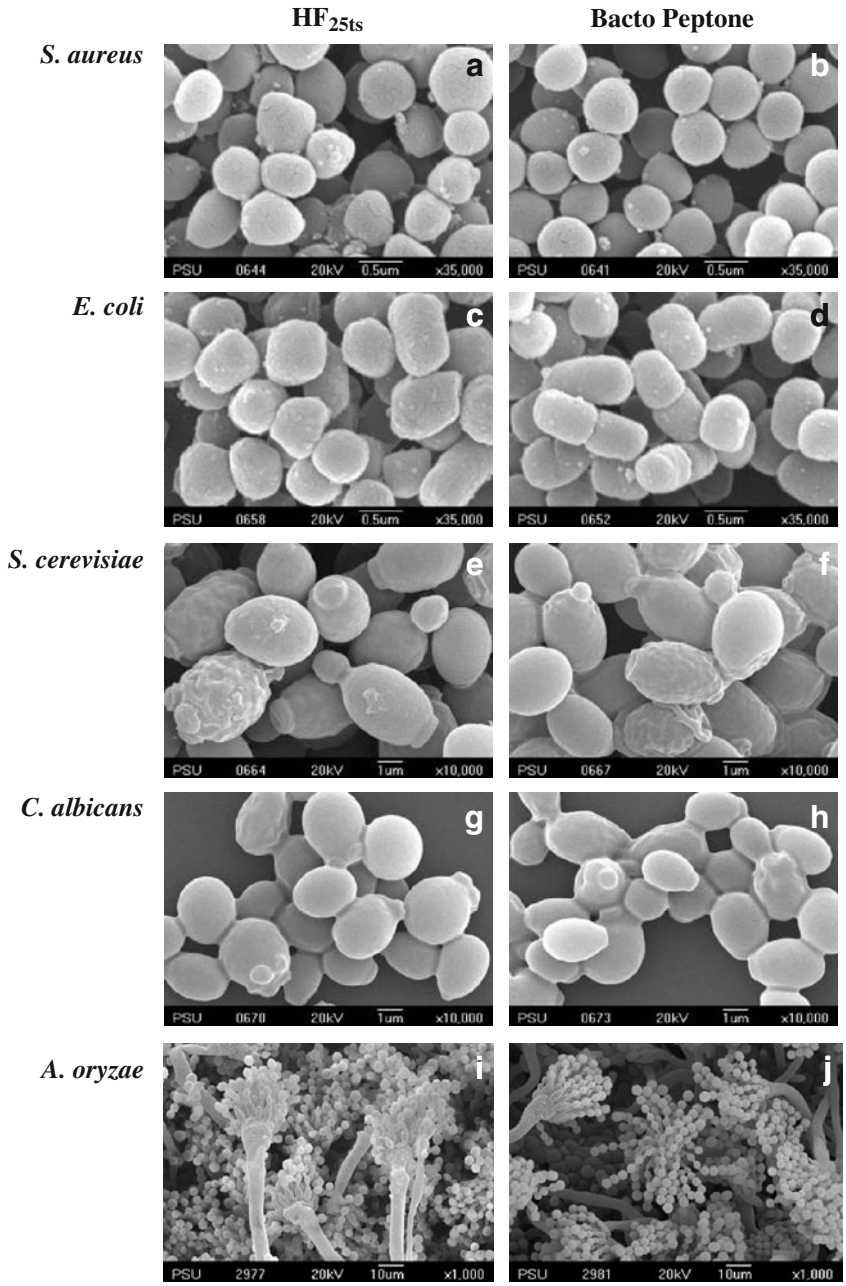

Fig. 4 Scanning electron microscopic photographs of *S. aureus*, *E. coli*, *S. cerevisiae*, *C. albicans* and *A. oryzae* cultured on media containing protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total solid content of Bacto Peptone (HF_{25ts}) and those cultured on Bacto Peptone

Effect of Protein Hydrolysate from Yellow Stripe Trevally Prepared Using Flavourzyme on Microbial Growth and Colony Size

Growth and colony size of microorganisms cultured in agar medium containing HF_{25} as a replacer of Bacto Peptone (based on total nitrogen and solid contents) are shown in Table 2.

Staphylococcus aureus

Although the cell density was adjusted to 0.5 of A_{660} to obtain a similar cell density, total viable count of *S. aureus* cultured on NB containing HF_{25tn} was greater than that observed in NB with HF_{25ts} or Bacto Peptone ($p<0.05$). As a consequence, *S. aureus* cultured on NA comprising HF_{25tn} exhibited the smaller colony than that found on NA containing Bacto Peptone and HF_{25ts} ($p<0.05$). The smaller size of colony cultured on medium comprising HF_{25tn} might be affected by the decrease in pH during culturing.

Escherichia coli

Escherichia coli cultured in NB comprising HF_{25ts} had the greater number than that found in NB containing HF_{25tn} or Bacto Peptone ($p<0.05$). NB containing HF_{25ts} possibly

Table 4 Size of microorganisms cultured on media containing protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total solid contents of Bacto Peptone (HF_{25ts}), compared with Bacto Peptone

Microorganisms		Cell size (μm)	
		HF_{25ts}	Bacto Peptone
S. aureus	Diameter	0.67±0.09 ^{ns}	0.66±0.05 ^{ns}
E. coli	Length	0.84±0.12 ^{ns}	0.79±0.10 ^{ns}
	Width	0.58±0.04 ^{ns}	0.54±0.03 ^{ns}
S. cerevisiae	Length	3.68±0.70 ^{ns}	3.50±0.57 ^{ns}
	Width	2.73±0.32 ^{ns}	2.70±0.22 ^{ns}
C. albicans	Length	3.01±0.39 ^{ns}	2.81±0.33 ^{ns}
	Width	2.34±0.31^{ns}	2.26±0.29 ^{ns}
A. oryzae	Spore diameter	6.30±0.48 ^{ns}	6.01±0.43 ^{ns}

Means ± standard deviations from 20 determinations based on SEM photomicrograph

ns non-significant difference ($p>0.05$)

contained a suitable amount of nitrogen source for the growth of *E. coli* than did NB containing HF_{25tn}. However, no significant difference in colony size of *E. coli* cultured on NA containing HF_{25tn}, HF_{25ts} and Bacto Peptone was observed (p>0.05).

An excessive amount of nitrogen did not increase the microbial growth (Lynch and Poole 1979). The lower growth of *E. coli* was found in the medium containing HF_{25tn} than that comprising HF_{25ts}. The inhibition might be due to the differences in nutrient composition, unsuitable carbon and nitrogen ratio (C/N ratio) and some toxic materials (Dufosse et al. 2001). Kurbanoglu and Algur (2002) found that ram horn hydrolysates at a level of 4% were found to be optimal for bacterial growth and applications higher than 4% had an inhibitory effect on growth.

Saccharomyces cerevisiae

A great number of *S. cerevisiae* cultured in YMB comprising Bacto Peptone was found, when compared with that cultured in YMB containing HF_{25tn} or HF_{25ts} ($p<0.05$). YMB comprising Bacto Peptone might contain a suitable peptide size for the growth of *S. cerevisiae* under the condition used. However, a similar colony size was observed among *S. cerevisiae* cultured on YMA containing HF_{25tn}, HF_{25ts} and Bacto Peptone ($p>0.05$).

Candida albicans

No differences in the number and colony size of *C. albicans* cultured on YMA containing HF_{25tn}, HF_{25ts} and Bacto Peptone were observed ($p>0.05$).

Aspergillus oryzae

Similar number and colony size of *A. oryzae* cultured in MEB containing HF_{25tn}, HF_{25ts} or Bacto Peptone were obtained ($p>0.05$). It might be due to the fact that all protein hydrolysates were available substrates as a nitrogen source for *A. oryzae*.

The media containing different amounts of nitrogen sources from yellow stripe trevally protein hydrolysate (HF_{25tn}, HF_{25ts}) showed no effect on the quantitative and qualitative growth of *C. albicans* and *A. oryzae*. Differences in growth of *S. aureus*, *E. coli* and *S. cerevisiae* observed when culturing in HF_{25} and Bacto Peptone could be related to the differences in the amino acid composition of peptides in protein hydrolysates. In addition, the amount of hydrolysate used could exhibit either stimulating or inhibitory effects on microbial growth, depending upon the types of microorganisms. The amounts of nitrogen sources affected the growth of *S. aureus* markedly for both positive and negative aspects. The growth of *S. aureus* in this system was most likely associated with the decrease in pH. Thus, the use of buffer to maintain the pH of medium could be a promising means to solve the problem about colony size.

Effect of Buffer on Growth and Colony Size of *S. aureus*

When 0.2% K_2HPO_4 was supplemented in all media to maintain the pH during microbial cultivation, total viable count of *S. aureus* cultured in the medium containing HF_{25tn} was higher than that found in media containing HF_{25ts} or Bacto Peptone ($p<0.05$) (Table 3). When the cultivation time increased, the growth of *S. aureus* in all media increased throughout the log phase (4–12 hours) (data not shown). *S. aureus* cultured in NB containing HF_{25tn} exhibited the higher cell density than did NB containing HF_{25ts} or Bacto Peptone, respectively, up to 24 hours of cultivation time ($p<0.05$). *S. aureus* started the log phase at hour 3, and the stationary phase was observed at hour 16. However, the pH of the medium containing HF_{25tn} showed the greater decrease than did the media containing HF_{25ts} or Bacto Peptone ($p<0.05$). The more decrease in pH was related with the higher cell density of *S. aureus*. Nevertheless, the decrease in pH of NB supplemented with buffer was not as much as that of NB without buffer (data not shown). In addition, no difference in colony size of *S. aureus* was observed among the media containing HF_{25tn}, HF_{25ts} and Bacto Peptone supplemented with 0.2 % K_2HPO_4 ($p>0.05$). K_2HPO_4 acts as a potential buffer in culturing system. The result reconfirmed that acidity might affect colony size as previously mentioned (Table 2). In the presence of buffer, *S. aureus* in the medium containing HF_{25tn} showed the higher growth than that cultured in the

medium containing HF_{25ts} ($p<0.05$). This might be associated with the higher amount of nitrogen source for the growth. However, in the late log phase at hour 12 of cultivation, no difference between the number of *S. aureus* culturing in media containing HF_{25tn} and HF_{25ts} was observed ($p>0.05$). Normally used as inoculants, cells in the late log phase are important. Thus, the medium containing HF_{25ts} was selected for further study.

Effect of Protein Hydrolysate from Yellow Stripe Trevally on Microbial Morphology

Microstructures and size of microbial cells are shown in Fig. 4 and Table 4. Sizes of cells of all microorganisms cultured on NA containing HF_{25ts} were similar to those of microorganisms cultured on NA with Bacto Peptone ($p>0.05$). Cells of *S. aureus* were round. Nevertheless, the cells cultured on NA comprising Bacto Peptone had a more regular spherical shape and finer surface than those cultured on NA containing HF_{25ts} (Fig. 4a, b). *E. coli* was short rod in shape. The cells cultured on NA containing HF_{25ts} were similar in length and width to those cultured on NA containing Bacto Peptone ($p>0.05$) (Fig. 4c, d). However, the cells cultured on Bacto Peptone had a more regular shape and smoother surface than that cultured in NA containing HF_{25ts}. Microscopic features of *S. cerevisiae* were unicellular and ellipsoidal in shape and showed the doubling by budding (Fig. 4e, f). *C. albicans* were single ellipsoidal cells (Fig. 4g, h). Both *S. cerevisiae* and *C. albicans* cultured on YMA containing HF_{25ts} had the similar size and shape, in comparison with those cultured on YMA containing Bacto Peptone. For mold, *A. oryzae* cultured on MEA containing HF_{25ts} or Bacto Peptone produced upright conidiophores (Fig. 4i, j), which are simple and terminate in a globose or clavate swelling (Tortora et al. 1997). No difference in size and shape of the conidia, which were I-celled and globose in shape, were observed on MEA containing HF_{25ts} and Bacto Peptone ($p>0.05$). However, *A. oryzae* started producing spore at hour 38 and hour 40 when cultured on MEA containing HF_{25ts} and Bacto Peptone, respectively (data not shown). The differences in starting production of conidiospores of *A. oryzae* culturing on MEA containing between HF_{25ts} and Bacto Peptone might be governed by peptide composition.

Conclusions

Efficacy of protein hydrolysate from yellow stripe trevally as microbial nutrient varied with DH, enzyme and amount of hydrolysate used. Microbial kinetics of bacteria, yeasts and mold cultured in media containing protein hydrolysate from yellow stripe trevally produced by Flavourzyme with 25% DH were comparable to those of commercial Bacto Peptone. Therefore, production of fish protein hydrolysate for upgrading low market value species as a high-value nitrogenous substrate for microbial growth can be achieved.

Acknowledgements This research was supported by Staff Development Project from the Ministry of Education, the Grant for dissertation from Graduate School, Prince of Songkla University, Thailand and Thailand Research Fund Senior Research Scholar program. We thank East Asiatic Company (Thailand) Ltd. and Novo Nordisk (Bagsvaerd, Denmark) for donating Flavourzyme and Alcalase, respectively.

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