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# ACE-inhibitory peptide from rohu fish waste: Optimisation of ultrasound and microwave assisted enzymatic extraction using response surface methodology

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## ABSTRACT

Anti-hypertensive [angiotensin-I converting enzyme (ACE) inhibitory (ACE*i*) peptides are enzymatically extracted from biological material. The high cost of enzymes, low peptide yield and long hydrolysis time in enzymatic extraction paved the way for alternative ultrasound assisted enzymatic extraction (UAEE) and microwave-assisted enzymatic extraction (MAEE). In this study, rohu (*Labeo rohita*) fish wastes were homogenised and treated with ultrasound (40 kHz) and microwave (2450 MHz) before enzymatic hydrolysis using optimised concentration of alcalase® for the extraction of ACE*i*. The UAEE (sonication temperature: 30-70; optimum: 57.88ºC and time: 10-50; optimum: 63.4 min) and MAEE (microwave power: 180-900, optimum: 335.23 W and time 5-25, optimum: 15 min) were studied for their effect on degree of hydrolysis (DH), ACE inhibition (ACE*i*) and peptide yield (PY). The efficiency obtained by UAEE (5%) was higher than MAEE (4%) in terms of ACE*i*. In contrast, MAEE showed higher efficiency with respect to DH and PY (7 and 6.5%, respectively) than UAEE (2.5 and 4%, respectively). The study indicated that UAEE and MAEE are efficient methods for extraction of ACE-inhibitory peptides from rohu fish waste. However, UAEE peptides shows better ACE*i* than MAEE.

Keywords: ACE-inhibitory peptide, Alcalase, Degree of hydrolysis, Enzymatic extraction, Peptide yield, Rohu processing waste

## Introduction

Cardiovascular disease (CVD) incidences are increasing recently because of reduced physical activities, stressful lifestyle and obesity (Saleh *et al.,* 2016). The CVD is one of the major causes of global death (WHO, 2021). Hypertension is the biggest contributor of CVD and the mortality is estimated as 10 million deaths per annum (Poulter *et al.,* 2015). Globally more than 1.13 billion people suffer from hypertension, which estimates one in four men and one in five women (WHO, 2021). It is a condition when systolic blood pressure (SBP) reaches to ≥140 mm Hg or/and diastolic blood pressure (DBP) to 90 mm Hg or both while the normal SBP/DBP is <120/<80 (Poulter *et al.,* 2015).

Blood pressure in human is controlled by renin-angiotensin system (RAS), which play the critical role of ACE (Ngo *et al.,* 2016). ACE (EC 3.4.15.1) is a membrane bound enzyme found at pulmonary and renal endothelium surfaces, which converts angiotensin I into angiotensin II by removing a dipeptide (His-Leu) from C-terminus (Gao *et al.,* 2018) which results in high BP. Therefore, any ACE-inhibitory biomolecule can reduce BP and minimise hypertension. Many pharmaceutical drugs (*e.g.* enalapril, captopril, lisnopril, cilazapril andramipril) lowers BP by targeting ACE, but can lead to drug-associated side-effects such as renal impairment, dry cough and angioedema and chest pain (Wu *et al*., 2017). Therefore, peptides having potential therapeutic benefits need to be identified from various food products including fish or their byproducts (Saleh *et al.,* 2016, Yathisha *et al.,* 2018).

Global fish production now stands at 178.5 million t (FAO, 2020) and India is the second largest producer of freshwater fish in the world, accounting for 14.16 million t in 2019-20 (DoF, 2020). About 70-75% of total freshwater fish produced is Indian major carps (IMC) *viz.* catla, rohu and mrigal followed by silver carp, grass carp, common carp and catfishes (Kudre *et al.,* 2017). Among the IMCs, rohu (*Labeo rohita*) is one of the commonly and extensively cultured freshwater fish with huge consumer preference. Rohu is also the most sold and processed fish in the Indian fish market, which leads to generation of huge quantities of byproducts as a waste. The total waste generation from rohu fish in India was estimated to be approximately 0.9 million t per annum (Kudre *et al.,* 2017). Fish processing industries generates >60% of processed fish biomass as waste in the form of skin, head viscera, trimmings, liver, frames, bones and roes (Chalamaiah *et al.,* 2012; Halim *et al.,* 2016). The generation of considerable amount of fish waste imposes a cost burden on the industry for its

disposal (He *et al.,* 2013). Traditionally, the waste is used to produce low value products such as fish silage, fish meal and fish sauce to minimise the environmental pollution (Halim *et al.,* 2016). As fish waste contains a considerable amount of protein (Chalamaiah *et al.,* 2012), it can be effectively and efficiently be utilised for the development of high value products such as fish protein hydrolysate (FPH). Fish protein, upon enzymatic hydrolysis, yields ACE-inhibitory peptides that exhibit anti-hypertensive effects (Yathisha *et al.,* 2018). Large quantities of raw materials, high protein content and good amino acid balance make fish unique for the discovery of new bioactivities.

Conventionally bioactive peptides are extracted by enzymatic hydrolysis, microbial fermentation, or chemical hydrolysis and among which enzymatic hydrolysis is mostly preferred. However, due to the high cost of enzymes, low peptide yield and long hydrolysis time, industry is finding alternative methods such as ultrasound, microwave, high hydrostatic pressure, ohmic heating, pulsed electric field and sub-critical water hydrolysis for assisting extraction (Ulug *et al.,* 2021). Ultrasound-assisted enzymatic extraction (UAEE) is an eco-friendly technology which uses ultrasonic waves (>20 kHz) for the hydrolysis of a number of food proteins (whey, milk, rapeseed, corn) to produce food peptides. However, ultrasound alone cannot break peptide bonds and therefore it is applied in combination with enzymatic hydrolysis to enhance the production of bioactive peptides (Ulug *et al.,* 2021). Microwave-assisted enzymatic extraction (MAEE) is used in recent years to prepare bioactive peptides from cricket and rainbow trout frames. Microwave in combination with enzymatic hydrolysis is used for preparing peptides from sea cucumber collagen and bighead carp (Ulug *et al.,* 2021). Pre-treatment by either US or MW tends to enhance the enzymatic hydrolysis of proteins leading to a higher yield in releasing small molecular weight bioactive peptides. Microwave irradiation (50-250 W) has enhanced DH of black soybean protein (Li *et al.,* 2018). These technologies mainly assist to unfold, denature, or aggregate proteins by breaking down weak molecular interactions with little or no effect on the covalent bonds (Ulug *et al.,* 2021). Limited studies are available on the effect of US and MW in the extraction of bioactive peptides from fish waste. Therefore, this study was carried out for optimisation of UAEE and MAEE using RSM in enhancing degree of hydrolysis (DH), ACE inhibition (ACE*i*) and peptide yield (PY) of the peptides derived from rohu fish waste.

## Materials and methods

Rohu (*Labeo rohita*) fish (18 kg) procured from fish market near Gowsala, Hambran Road, Ludhiana, India were brought to the Transfer of Technology (TOT) Laboratory, ICAR-Central Institute of Post Harvest Engineering and Technology (ICAR-CIPHET), Ludhiana in chilled condition (1:1, fish: ice) in an insulated container. Fish was dressed to collect non-edible waste portion comprising of fins, scales, swim bladder and head. Fish waste was mixed in their natural proportion to get a homogenised rohu fish waste protein (RFWP).

All the chemicals, organic solvents and enzymes used for the experiment were of analytical grade procured from HiMedia, Mumbai, India; MP Biomedicals Mumbai, India and Sigma, USA. Alcalase® from *Bacillus licheniformis* was obtained from Sigma-Aldrich, USA. Molecular weight cut-off (MWCO) filters of 3, 5 and 10 kDa were purchased from GE Healthcare, UK and Sartorius, UK for ultrafiltration. Angiotensin-I converting enzyme (ACE) and Captopril were procured from Sigma, USA while AR grade N[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) was obtained from M/s MP Biomedicals, Mumbai, India.

*Non-assisted enzymatic extraction (NAEE)*

Homogenised RFWP was hydrolysed enzymatically without any assistance under the optimised condition (at 1.08% v/w alcalase® concentration; 52.10°C temperature; 129.18 min hydrolysis time and 0.8:1 solid liquid ratio) to obtain non-assisted enzymatic extraction (NAEE) peptides. The DH, ACE*i* and PY of NAEE peptides were 19.27, 54.98 and 51.37% respectively (Kumar *et al.,* 2021).

*Ultrasound assisted enzymatic extraction (UAEE)*

One batch (6 kg) of homogenised RFWP was subjected to ultrasound (40 kHz) treatment using a bench top bath type sonicator (Make: ACZET, India, Model: CUB10) operated at 240 W power for 30 min. Five levels of sonication temperatures (30, 40, 50, 60 and 70°C for 30 min) and time (10, 20, 30, 40 and 50 min at 50°C) were given and the samples were then hydrolysed enzymatically.

*Microwave assisted enzymatic extraction (MAEE)*

Another batch (6 kg) of homogenised RFWP was subjected to microwave (2450 MHz) treatment in a microwave oven (LG Electronics, India). Five levels of microwave power (180, 360, 540, 720 and 900 W; at 15 min) and treatment time (5, 10, 15, 20, 25 min at 360 W) were given and the samples were then hydrolysed enzymatically.

*Enzymatic hydrolysis*

Ultrasound assisted and microwave assisted samples were subjected to enzymatic hydrolysis as per the method described by Kumar *et al.,* (2021) similar to non-assisted enzymatic extraction peptides, *i.e.* concentration 1.08% v/w alcalase®; temperature 52.10°C; hydrolysis time 129.18 min and solid liquid ratio 0.8:1.

After hydrolysis, enzyme was inactivated by heating at 90□ (15 min) and then cooled immediately on ice. The content was centrifuged at 10,000 rpm for 20 min at 4ºC. The supernatant was dried in a lyophilizer (MAC, Delhi) and stored at -15°C until used for the estimation of DH, ACE*i* and PY. The protein content in the supernatant was determined by Bradford Assay using bovine serum albumin (BSA) as standard protein (Bradford, 1976) and the absorbance was measured at 595 nm in a UV-VIS spectrophotometer (UV3200, Labindia).

*Ultrafiltration*

RFWP hydrolysate was filtered twice through qualitative filter paper (No. 4; 12.5 cm dia; Hi-media, India) and then passed through a syringe filter (Polyethersulfone, PES 0.45 µm and subsequently 0.2 µm filter). Filtrate obtained was then passed through MWCO filters of sizes 10 kDa and 3 kDa. Filtrate having molecular mass <3kDa was used for assessment of ACE-inhibitory activity (ACE*i*).

*Degree of hydrolysis*

DH was determined by the method of Adler-Nissen (1979). RWFP hydrolysate (0.25 ml) was added to 2 ml phosphate buffered solution (0.2125 M, pH 8.2) and 2 ml of 2, 4, 6-Trinitrobenzene sulfonic acid (TNBS) (0.1%) and incubated in the dark at 50°C for 1 h. The reaction was terminated using HCl (0.1 M, 5 ml), the absorbance was measured at 340 nm and expressed as α-free amino acids in terms of L-Leucine.

$$DH\ (\%) = \frac{(Lt - Lo) \times 100}{Lmax - Lo}$$

where, Lt: Amount of α-amino acids released at time‘t’; Lo: Amount of α-amino acids released in acid-solubilised substrate and Lmax: amount of maximum α-amino acids found.

*ACE-inhibitory assay*

N[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG), a synthetic substrate was used following the method of Murray *et al.* (2014) and Theodore and Kristinsson (2007). Peptide (100 µl, 0.2% protein w/v), ACE (50 µl, 15 mU) and substrate (1 ml, 0.5 mM FAPGG) were mixed and incubated at 25°C for 60 min. After incubation, the absorbance was measured at 340 nm. FAPGG with ACE enzyme served as control. Hydrolysis of FAPGG by ACE resulted in a decrease in absorbance at 340 nm. Therefore, ACE*i* is measured by its ability to decrease the hydrolysis of FAPGG. A complete inhibition of the enzyme is indicated by a 100% ACE*i.*

$$\text{ACE inhibition (ACEi) (\%)} = \left(1 - \frac{\text{Slope of sample curve}}{\text{Slope of control curve}}\right) \times 100$$

*Peptide yield*

PY was determined as per the method of Wu *et al.* (2019). Peptide (5 ml) was dissolved in trichloroacetic acid (10%, 1 ml), kept at room temperature for 20 min and then centrifuged at 10,000 *g* for 10 min. Supernatant was collected to determine the peptide content by Biuret method using BSA as a standard (Savory *et al*., 1968). Peptide yield was calculated in percentage considering conversion coefficient of nitrogen to protein as 6.25.

$$\text{Peptide yield (PY) (\%)} = \frac{\text{TCA - Soluble peptide content}}{\text{Total protein nitrogen} \times 6.25 \times 100}$$

*Statistical analysis*

Single factor tests were performed to check the effect of two independent factors *viz.* temperature and time of UAEE (sonication temperature and time) and MAEE (microwave power and time) on 3 dependent parameters (DH, ACE*i*, PY). Response surface methodology with Central composite design (RSM-CCD) (13 runs each for UAEE and MAEE) was used to optimise independent variables (sonication temperature and time of UAEE; microwave power and time of MAEE) for the highest DH, ACE*i* and PY. Extraction efficiencies of NAEE, UAEE and MAEE were compared by Tukey *post hoc* test. Design-Expert version 12 was used for design of experiments, optimisation of enzymatic extraction process and statistical calculation. Experiments were performed in triplicate and expressed as the mean±standard deviation (n=3). Differences between variables were tested for significance by one-way ANOVA for single factor experiments and two way ANOVA for their effects on DH, ACE*i* and PY. When p value <0.05, the difference was considered significant.

## Results and discussion

*Effect of UAEE on DH, ACEi and PY*

*Effect of UAEE on DH*

Enzymatic hydrolysis of a protein is measured by DH which should be controlled to obtain optimum peptide size distribution, when the enzymatic hydrolysis is applied to produce potent bioactive peptides. Fig. 1a presents 2D contour and 3D response surface of interaction effects of sonication temperature and time on DH. In our study, DH increased significantly ($p<0.05$) from 4.1±1.2 to 22.2±1.4%, When sonication temperature increased from 30 to 60°C (Table 1). This is probably due to acceleration of molecular thermal motion and increase in solubility with the increase in temperature which means sonication alone did not increase DH but the temperature was more effective for the action of enzyme to get the highest DH

at 60°C. This coincided with the optimum temperature of 55°C for alcalase®. Beyond 60°C, the DH declined with the increase in sonication temperature due to the thermal effect and the added heat from acoustic cavitation phenomenon of ultrasound that resulted in partial inactivation of enzyme. Wali *et al.* (2017a) also found a significant ($p<.05$) increase in DH (19.12%) of rapeseed protein hydrolysate by ultrasound pre-treatment (24/28 kHz) compared to untreated control. In our study, DH increased significantly ($p<0.05$) (Table 1) as sonication time increased from 10 to 30 min of sonication time and thereafter remained more or less similar between 30 and 40 min and later reduced. The increase in DH achieved could be attributed to the faster diffusion of enzyme into RFWP at the early stages of hydrolysis reaching the plateau at around 30-40 min. A prolonged sonication time had probably degraded the enzyme resulting in the reduction of DH.

*Effect of UAEE on ACE*i

Fig. 1b depicts 2D contour and 3D response surface of interaction effects of sonication temperature and time on ACE*i*. ACE*i* increased significantly ($p<0.05$) from 15.4±1.3% (30ºC) to 63.9±0.2% (60ºC) with the increase in sonication temperature until 60ºC (Table 1) and thereafter showed only marginal increase ($p>0.05$) from 63.9±0.2% (60ºC) to 65.2±1.3% (70ºC). As compared to that of NAEE (54.98% ACE*i*), UAEE peptides resulted in 18.58% increase in ACE*i*. Jia *et al.* (2010) found 21.0-40.7% increase in ACE*i* of defatted wheat germ protein hydrolysate prepared with ultrasonic pre-treatment and also indicated that ultrasound during enzyme treatments had less effect on ACE*i*. Wali *et al.* (2017a) reported 35.08% increase in ACE*i* in rapeseed protein hydrolysate prepared by combined fixed frequency 24/28 kHz of ultrasound. With respect to sonication time, ACE*i* increased significantly ($p<0.05$) during 10-30 min (Fig. 2). Sonication followed by enzymatic hydrolysis enhances surface hydrophobicity, loosens protein tissues and thereby releases hydrophobic amino acids (Wali *et al.,* 2017b), which were the probable reasons for the increase in ACE*i*.

Table 1. Effect of sonication temperature and time on degree of hydrolysis (DH), ACE inhibition (ACE*i*) and peptide yield (PY). Results expressed as the mean±standard deviation (n=3)

| | | DH (%) | ACE*i* (%) | PY (%) |
|---|---|---|---|---|
| Sonication temperature (°C) | 30 | 4.1±1.2 | 15.4±1.3 | 25.2±0.6 |
| | 40 | 7.9±0.8 | 19.7±0.7 | 36.1±1.4 |
| | 50 | 15.4±1.1 | 54.9±0.4 | 42.2±1.5 |
| | 60 | 22.2±1.4 | 63.9±0.2 | 43.7±0.6 |
| | 70 | 18.7±2.1 | 65.2±1.3 | 42.5±0.9 |
| Sonication time (min) | 10 | 6.8±1.7 | 13.8±1.3 | 17.6±0.9 |
| | 20 | 13.2±1.5 | 28.9±1.3 | 26.5±0.8 |
| | 30 | 22.4±0.7 | 57.1±1.1 | 41.8±1.1 |
| | 40 | 23.7±1.2 | 55.2±0.8 | 42.5±2.1 |
| | 50 | 21.5±0.9 | 58.4±1.1 | 45.7±1.8 |

*Effect of UAEE on PY*

Peptide yield is the amount of peptides (g) produced per 1 g crude protein. Fig. 1c shows 2D contour and 3D response surface of interaction effects of sonication temperature and time on PY, which increased significantly ($p<0.05$) with the increase in temperature until 50ºC (Table 1). At subsequent higher temperature of 70ºC, the PY remained almost similar. The PY increased significantly ($p<0.05$) with the increase in sonication time from 10 to 40 min (Table 1) and remained almost similar at sonication time between 30 and 40 min but increased at 50 min. The faster diffusion of enzyme into RFWP at early stages of hydrolysis might be the reason for the increase in peptide yield and then reaching a plateau at around 30-40 min. The extension of sonication time occurred due to degradation of the enzyme. Siewe *et al.* (2021) attributed higher extraction yield at more sonication time due to cavitation and enhanced mass transfer rate across cell membrane and also ultrasound waves cause destruction and unfolding of proteins resulting in release of more peptides.

*Optimisation of UAEE conditions*

The combined effect of two factors (X1: sonication temperature and time) on three responses (DH, Y1; ACE*i*, Y2 and PY, Y3) (Fig. 1a-c) was studied using RSM-CCD. A regression analysis using RSM was performed to determine all the coefficients of linear (X1, X2), quadratic (X11, X22) and interaction (X1 X2) terms to fit a full response surface model of the responses. In our study, the linear term was suitable for Y1 and Y2, while interaction term was suitable for all the responses. The linear model term, sonication temperature (X1) was found significant ($p<0.05$) for two responses *viz.* DH and ACE*i* while interaction term (X1 X2) was significant ($p<0.05$) for all three responses. Our study revealed a significant ($p<0.05$) effect of sonication temperature on DH and ACE*i* and not on PY. Additionally sonication time had no influence on any of the responses. The final response surface regression equation obtained by RSM were:

$$Y = Y1 + Y2 + Y3$$

where, $Y1 = -20.28 + 5.47\,X1 - 8.32\,X11$

$Y2 = -58.9 + 11.16X1 - 24.025\,X11$

$Y3 = -54.1 - 14.71X11$

$Y = -133.28 + 16.63\,X1 - 47.055\,X11$

Different independent variables were optimised by desirability profile, assigning the highest value as 1.0 to the highest DH observed and the lowest desirability of

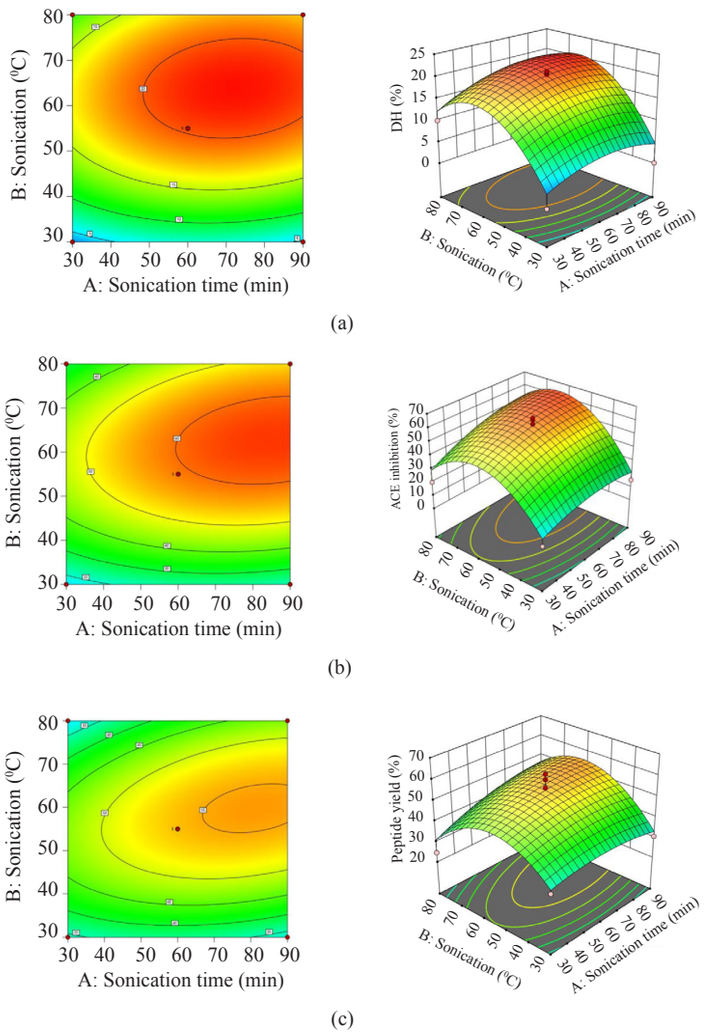


Fig. 1. 2D contour plot and 3D response surface of the relative effects of sonication temperature (°C) and time (min) on (a) Degree of hydrolysis (%), (b) ACE inhibition (%) and (c) Peptide yield (%) of rohu fish waste ACE-inhibitory peptides extracted using Ultrasound assisted enzymatic extraction

0.0 to the lowest DH found. Similarly the desirability values were assigned for ACE*i* and peptide yield also. In our study, the desirability profile (0.678) indicated that an optimum DH (20.97%), ACE*i* (60.69%) and PY (54.65%) could be achieved with 57.88°C sonication temperature and 63.4 min sonication time. The experiments conducted using optimised conditions gave 21.5% DH, 60% ACE*i* and 55% PY.

*Effect of MAEE on DH, ACEi and PY*

*Effect of MAEE on DH*

The effect of MAEE on DH was dependent on microwave power and time (Table 2). Fig. 2a shows 2D contour and 3D response surface of interaction effects of microwave temperature and time on DH. With an increase in microwave power (at 15 min), DH increased significantly ($p<0.05$) initially reaching the peak (31.6%) at 360 W, slightly decreased and finally increased. Similar increase and a little drop in DH was also found in black soybean protein hydrolysate (Li *et al.,* 2018) as the microwave power increased and the highest DH achieved was at 227.5 W, which was closer to our result. In this study, DH increased significantly ($p<0.05$) from 24.3±1.4 to 35.1±1.2% as the time increased from 5 to 15 min (at 360 W) and then gradually decreased with the increase in time. Similar to our results, in fresh rainbowtrout hydrolysed using alcalase® (3%, w/w), the DH increased from 14 to 50%, when microwave pre-treatment time increased from 2 to 10 min (Ketnawa and Liceaga, 2017). The increase is mainly due to microwave heating induced modification on

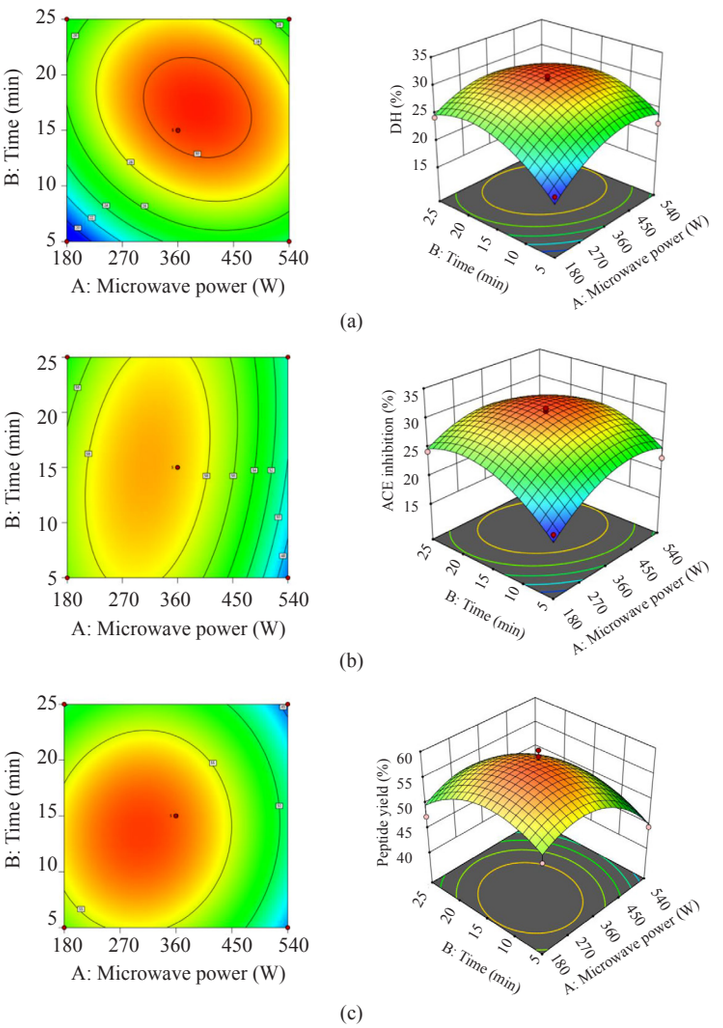


Fig. 2. 2D contour plot and 3D response surface plots of the relative effects of microwave power (W) and time (min) on (a) Degree of hydrolysis (%), (b) ACE inhibition (%) and (c) Peptide yield (%) of rohu fish waste ACE-inhibitory peptides extracted using Microwave assisted enzymatic extraction

Table 2. Effect of microwave temperature and time on degree of hydrolysis (DH), ACE inhibition (ACE*i*) and peptide yield (PY). Results expressed as the mean±standard deviation (n=3)

| | | DH (%) | ACE*i* (%) | PY (%) |
|---|---|---|---|---|
| Microwave power (W) | 180 | 24.3±1.1 | 48.3±1.4 | 32.4±1.5 |
| | 360 | 31.6±0.9 | 62.6±1.8 | 39.5±1.2 |
| | 540 | 27.9±1.2 | 46.9±1.3 | 44.6±0.8 |
| | 720 | 29.1±0.7 | 47.1±0.9 | 43.7±1.1 |
| | 900 | 29.4±1.0 | 41.4±1.2 | 41.1±1.0 |
| Microwave time (min.) | 5 | 24.3±1.4 | 35.5±1.5 | 24.2±1.1 |
| | 10 | 29.7±0.7 | 51.9±1.7 | 32.5±1.3 |
| | 15 | 35.1±1.2 | 61.9±1.2 | 44.6±1.5 |
| | 20 | 29.9±1.3 | 47.7±1.4 | 48.7±0.9 |
| | 25 | 29.4±0.9 | 40.5±1.3 | 49.9±1.2 |

the flexibility of enzyme, which consequently accelerated the enzymatic action (Ketnawa and Liceaga, 2017). The highest DH recorded was at 15 min, which was closer to 20 min reported in fresh rainbow trout (Ketnawa and Liceaga, 2017). Authors observed a similar trend with microwave hydrolysis time which showed an increase in DH with the increase in time reaching a plateau at 20 min followed by a decline. The reduction is due to loss in the enzymatic activity over time. Ketnawa and Liceaga (2017) reported that hydrolysis time affected DH significantly than that of microwave pre-treatment time. Nevertheless, microwave treatment itself had influenced DH by lessening the time required for enzymatic hydrolysis. This is mainly attributed to the increased irradiative power of microwave that stimulates the catalytic reaction.

*Effect of MAEE on ACE*i

ACEi increased significantly ($p<0.05$) with the increase in microwave power and time (Table 2). Fig. 2b presents 2D contour and 3D response surface of interaction effects of microwave temperature and time on ACE*i*. Initially ACE*i* increased and later declined sharply beyond 360 W and again gradually increased as the power increased. The ACE*i* (62.6%) was the highest at 360 W. ACEi increased by 13.86% as soybean hydrolysate showing the maximum at 800 W (Li *et al.*, 2018). They reported 14.18% higher ACEi in MAEE of black soybean hydrolysate than those prepared by conventional hydrolysis. In our study, ACEi gradually increased with the increase in microwave time and the highest (61.9%) was recorded at 15 min.

*Effect of MAEE on PY*

Fig. 2c gives 2D contour and 3D response surface of interaction effects of microwave temperature and time on PY which increased significantly ($p<0.05$) as the microwave power increased to 540 W and later dropped gradually. Microwave assisted hydrolysis improved protein solubility, resulting in increased protein recovery compared to that of NAEE (Ketnawa and Liceaga, 2017). They reported that microwave treatment at 90°C followed by conventional enzymatic hydrolysis improved protein solubility better than microwave assisted enzymatic hydrolysis. With microwave pre-treatment, direct energy transfer from the electromagnetic field to the polar protein domains could induce modifications on the flexibility of protein structure and consequently changes its structure and allows access of enzyme to the target sites (Ponne and Bartels, 1995). The presence of an electric field can influence biochemical reaction by changing molecular spacing and increasing inter-chain reaction (El Mecherfi *et al.,* 2011). In our study, PY increased significantly ($p<0.05$) with the increase in microwave time and the highest yield (49.9%) was recorded at 25 min possibly due to improvement in protein solubility, resulting in increased protein recovery.

*Optimisation of MAEE conditions*

The combined effect of 2 factors (X1: Microwave Power and Time) on three responses (DH, Y1; ACE*i*, Y2 and PY, Y3) is shown in Fig. 2a to c. A regression analysis was performed using RSM to determine all the coefficients of linear (X1, X2), quadratic (X11, X22) and interaction (X1 X2) terms to fit a full response surface model for the responses. In our experiments, the linear term was suitable for Y2 and Y3 while quadratic term (X11) was suitable to all the responses of Y and X22 was suitable for DH and PY and not for ACE*i*. The linear model term, microwave power (X1) was found significant ($p<0.05$) for two responses *viz*. ACE*i* and PY while quadratic terms, X11 was significant ($p<0.05$) for all three responses and X22 for DH and PY only. Our study revealed a significant ($p<0.05$) effect of microwave power on ACE*i* and PY and not on DH. Additionally microwave time had no influence on any of the responses. The final response surface regression equation obtained by RSM were:

$$Y = Y1 + Y2 + Y3$$

where, $Y1 = -30.46 - 3.49X11 - 4.06\ X22$

$Y2 = -59.4 - 3.36X1 - 6.17X11$

$Y3 = -58.08 - 3.44X1 - 5.65X11 - 4.48\ X22$

$Y = -147.94 - 6.88X1 - 15.31X11 - 4.06\ X22$

In this study, the desirability profile (0.881) indicated that an optimum DH (30.18%), ACE*i* (59.74%) and PY (58.44%) could be achieved with 335.23 W microwave power in 15 min. The experiments undertaken with the optimised conditions later proved 26.4% DH, 59.1% ACE*i* and 57.5% PY.

Both ultrasound and microwave treatments assisted in enzymatic extraction efficiency of ACE*i*peptides. The optimised sonication temperature (57.88ºC) and time (63.4 min) yielded UAEE peptides with DH (21.5±1.3%), ACE*i* (60.0±1.4%) and PY (55.0±1.2%). The optimised microwave power (335.23 W) and time (15 min) of MAEE peptides exhibited DH (26.0±1.8%), ACE*i* (59.0±1.1) and PY (57.5±1.4%). This clearly shows the assistance rendered by ultrasound (40 kHz) and microwave (2450 MHz) compared to NAEE (DH: 19.0±1.0; ACE*i*: 55.0±1.2 and PY: 51.5±0.9) peptides from rohu (*L. rohita*) fish wastes. Thus, UAEE and MAEE improved all three dependent variables (DH, ACEi and PY) compared to NAEE. ACE*i* was higher in UAEE peptides (5%) compared to MAEE peptides (4%). Conversely, DH (7%) and PY (6.5) were higher for MAEE peptides than UAEE (2.5 and 4% respectively) (Table 3). This may be due to higher heat generated in microwave compared to

Table 3. Comparison of DH, ACE*i* and PY obtained by UAEE, MAEE and NAEE

| Depending responses | NAEE[1] (Control) (%) | UAEE[2] (%) | MAEE[3] (%) |
|---|---|---|---|
| DH | 19.0±1.0[a] | 21.5 ±1.3[a] (13.15)*** | 26.0±1.8[b] (36.84) *** |
| ACE*i* | 55.0±1.2[a] | 60.0 ±1.4[b] (9.09)*** | 59.0±1.1[b] (7.27)*** |
| PY | 51.5±0.9[a] | 55.0 ±1.2[b] (6.79)*** | 57.5±1.4[c] (11.65)*** |

[1]Non-assisted enzymatic extraction (NAEE); [2]Ultrasound assisted enzymatic extraction (UAEE); [3]Microwave assisted enzymatic extraction (MAEE). ***Indicates % of extraction improvement in comparison with NAEE

ultrasound which would have reduced ACE*i* but not the DH and PY. Therefore, this study indicates the potential of ultrasound and microwave in assisting enzymatic extraction of ACEi peptides from rohu fish waste Future studies need to be done in terms of validation of this assistance in pilot or industry scale, to check its economic viability and to assess the *in vivo* efficacy and safety of the peptides.

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