International Journal of Advanced Research in Biological Sciences
www.ijarbs.com

Research Article

Production of angiotensin converting enzyme inhibitor from marine yeast *Candida utilis* SSK04

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Abstract

Yeast strains were isolated from marine water samples using YEPD medium. Density of the yeast in the marine water samples were $2.7x10^3$ - $1.6x10^5$ CFU/ml. Four isolated yeast strains namely MY01, MY02, MY03 and MY04 showed an ACE inhibitory percentage of 48.9, 66.4, 21.6 and 79.1 respectively. Potent strain MY04 was identified and designated as *Candida utilis* SSK04. The optimized growth parameters of *C. utilis* SSK04 were pH - 8, temperature - 35°C, salinity - 1%, sucrose -1% and yeast extract - 0.5% and incubation time 48hrs were found to be ideal. The total protein concentration of the purified protein was 422mg/ml and the IC_{50}value of the peptide was calculated as 0.38mg/ml. The purified protein showed a major protein band of 12 kDa on SDS-PAGE with an ACE inhibitory activity of 69.8%.

Keywords: Marine Yeast, *Candida utilis*, Proteolytic activity, ACE inhibitor, Bioactive proteins.

Introduction

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxpeptidase, EC 3.4.15.1) plays an important role in the regulation of blood pressure as well as cardiovascular function. ACE converts the inactive decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II and also inactivates vasodilator, bradykinin (Hyun and Shin, 2000).

Marine organisms are rich sources of structurally diverse bioactive compounds. Hence, a great interest has been developed nowadays to isolate bioactive compounds, which act as ACE inhibitors from marine resources due their proven beneficial health effects. Marine-derived bioactive peptides have a potential use as functional ingredients in nutraceuticals and pharmaceuticals due to their effectiveness in both prevention and treatment of hypertension in addition to the nutritive value.

Marine microbes have been studied and developed as materials for the production of novel bioactive substances but many of these studies were focused on marine bacteria and marine *Actinomycetes* for

utilization of products as antibiotics and antitumor materials only a very few studies are available on the isolation and utilization of marine yeast (Taga *et al.,* 2002).

Yeast is one of most important microbes in the fermentation industry and could be a good source of proteases. Research has shown that marine yeasts also have highly potential uses in food, feed, and medical industries as well as marine biotechnology (Chi et al. 2009). Yeast has some industrial advantages, including rapid growth, ease of cultivation and the capacity to be grown in a cheap medium containing agricultural by-products (Kim et al., 2004). Bioactive compounds such as an antihypertensive angiotensin I-converting enzyme (ACE) inhibitor (Kim et al., 2004), ribonucleotides (Kim et al., 2002 and Lee et al., 2004) chitosan (Kim et al., 2002), an anti-angiogenic compound (Jeong et al., 2006) and an antidementia β-secretase inhibitor (Lee et al., 2006) have been produced and characterized from yeasts including *Saccharomyces cerevisiae.* The present study aimed to produce ACE inhibitor proteins from skimmed milk digested by yeast isolated from marine origin.

Materials and Methods

Isolation and identification of yeast

For the isolation of marine yeast, 10 marine water samples were collected from Vellar estuary, Parangipettai. Samples were serially diluted and 0.1ml from 10^{-3}, 10^{-4}, 10^{-5} dilutions were spread on YEPD medium (0.5% yeast extract, 3.0% peptone and 2.0% glucose) supplemented with 50µg/ml of chloramphenical plates using spread plate technique and incubated for 3 days at room temperature. Numbers of yeast colonies were counted and the results were expressed as CFU/ml.

Preparation of hydrolysate for ACE inhibitory activity

The well isolated yeast colonies on YEPD plates were aseptically transferred into 10ml of YEPD broth supplemented with 5% skimmed milk as protein substrate and were incubated at room temperature for 48-72 hrs. After incubation the cell free extracts (broth) of each strain were separated by centrifugation at 7000 rpm for 10min. The cell free extracts collected were used for ACE inhibitory activity assay.

Assay for ACE inhibitory activity

The ACE-inhibition activity of the filtrates was measured according to (Cushman and Cheung, 1971) with some modification. Briefly, 180 µl of HHL buffer (5 mM Hip-His-Leu in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) were mixed with 100µl of sample solution and pre-incubated at 37^{o}C for 10 min. Both ACE and substrate HHL were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The reaction was initiated by adding 20 µl of ACE (dissolved in distilled water, 0.1 units/ml), and the mixture was incubated at 37^{o}C for 30 min. The reaction was stopped by adding 250 µl of 1.0 N HCl and mixed with 1.0 ml of ethyl acetate. The liberated hippuric acid was extracted with ethyl acetate, and 1.0 ml of ethyl acetate was removed from the ethyl acetate layer after centrifugation (10000 rpm, 10 min) and evaporated at room temperature for 20 min. in a rotary vacuum evaporator. The hippuric acid was diluted in 1.0ml sterile distilled water and the absorbance was measured at 228 nm using a spectrophotometer to measure the ACE activity. The average value from three determinations at each concentration was used to calculate the IC_{50} value and ACE-inhibitor rate. The extent of inhibition was calculated as follows:

ACE- Inhibitor rate (%) = [(B-A) / (B-C)] x 100

Where A is the absorbance in the presence of ACE and with the ACE-inhibitory component, B is the absorbance with ACE and without the ACE-inhibitory component, C is the absorbance without ACE or ACE inhibitor component. The concentration of an ACE inhibitor needed to inhibit 50% of the ACE activity was defined as IC_{50} under these conditions. The cell free extract showed highest inhibition rate was selected as a potent strain and further growth optimization studies of the yeast strain was performed.

Calculation of IC_{50}

The IC_{50} value was defined as the concentration of the inhibitor that is required to inhibit 50% of the ACE inhibitory activity. The protein concentration required to inhibit the 50% inhibitory activity was calculated.

Identification of the potential strain

The potent yeast isolate was selected based on their ACE inhibitory activity and it was identified based on colony color, shape, texture, microscopic morphology, physiological, biochemical tests and various sugar assimilations (Wickerham, 1951; Sanni and lonner, 1993; and Barnett *et al*., 2000).

Growth optimization of potential strain

The factors like pH, temperature, salinity and substrate concentration which were expected to influence the production of ACE inhibitory peptide of the selected strain was optimized by selecting one parameter at a time.

Parameters like different pH viz., 6, 7, 8, 9, 10 and 11, different temperatures 25, 30, 35 and 40^{o}C, different salinity 0.5, 1.0, 1.5, 2.0 and 2.5% were maintained in the medium in separate flasks. Carbon sources like maltose, sucrose, glucose, fructose and cellulose were incorporated separately at the rate of 1% in YEPD broth were maintained in the medium. Likewise nitrogen sources like peptone, ammonium nitrate, beef extract, yeast extract, potassium nitrate and casein were added at the rate of 0.5% in YEPD medium. Growth was measured at 600nm for every 12hrs using a spectrophotometer.

Mass scale production in shake flask

The optimized growth conditions like pH – 8, temperature- 35ºC, salinity-1.0%, sucrose- 1.0% and yeast extract – 0.5% for *C. utilis* SSK04 were maintained in the medium 1L flask. The flask was kept for incubation at 35^{o}C in a water bath shaker at 150 rpm for 72 hrs.

Purification of ACE inhibitor from marine yeast

The crude cell free extracts were ultrafilterated with 10kDa MWCO (PALL –MacrocepCentifugal Device) filters. The filtrates were collected into a new tube and it was further filtered on 5kDa MWCO (PALL –MacrocepCentifugal Device) filters. The retentate was washed with sodium borate buffer pH 8 and further purified on Sephadex G -25column. Active fractions were pooled together and were stored at -20^{o}C until further experiment.

Determination of protein concentration

Protein concentration of both crude cell free extract and purified sample was determined by the method of Lowry *et al.,* (1951).

Molecular weight determination ACE inhibitor protein

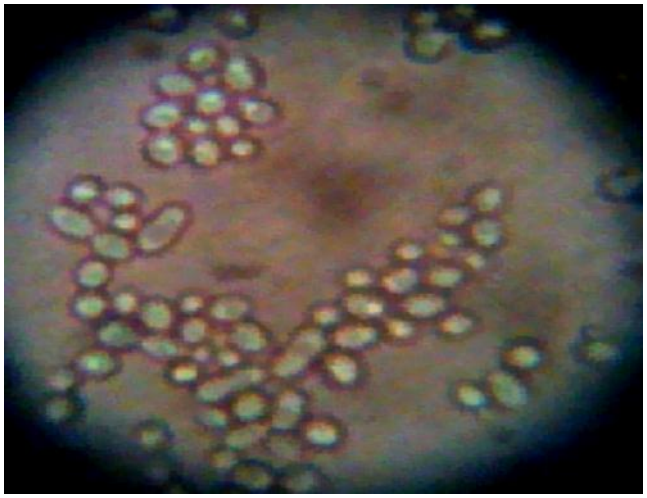
Molecular weight of the purified ACE inhibitor was determined by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was stained with coomassie brilliant blue (Laemmli, 1970).

Results

Yeast strains were isolated from marine water samples using YEPD broth supplemented with 50µg/ml of chloramphenical. In the present study, collected marine water sample was plated on YEPD agar plate. Yeast density in the water sample was $2.7x10^{3}$ - $1.6x10^{5}$ CFU/ml. The colonies were creamy white and smooth round and raised (Fig. 1 and 2). Four isolated yeast strains namely MY01, MY02, MY03 and MY04 showed an ACE inhibitory percentage of 48.9, 66.4, 21.6 and 79.1 respectively. Among four strains, MY04 was selected as the most potent strain and it was identified as *Candida utilis* based on its morphological, physiological and biochemical characteristics. It showed positive reaction for fermentation in glucose, maltose, sucrose and

Fig. 1 Isolation of *Candida utilis* SSK04 from marine water on YEPD agar plate

Fig. 2: Light Microscopic (100X magnification) view of *Candida utilis* SSK04



cellobiose and mannitol. The potent strain was designated as *Candida utilis* SSK04

Culture conditions like temperature and pH, nutrients like carbon and nitrogen sources were found influence on growth of *C. utilis* SSK04. The optimized growth parameters of *C.* utilis were as follows, pH-8, temperature 35°C, salinity 1%, sucrose-1% and yeast extract -0.5% and incubation time 48hrs were found to be ideal conditions for the yeast strain used in the present study (Fig: 3-7).

Among four strains screened the higher inhibitory activity shown strain was selected as a potential strain. The inhibitory activity of the potent strain was 79.1%. The crude cell free extracts showed an ACE inhibitory activity percentage of 79.1% whereas the purified protein showed 69.8% activity. The total protein concentration of the purified protein was 422mg/ml and the IC_{50}value of the peptide was calculated as 0.38mg/ml.

The purified protein sample was separated on SDS-PAGE (Fig. 8) showed a major protein band of 12 kDa. The purified protein was assayed for ACE inhibitory activity, it showed an inhibition of 69.8%.

Discussion

Yeasts are ubiquitous in their distribution and populations mainly depend on the type and concentration of organic materials. Marine yeasts were first discovered from the Atlantic Ocean and following this discovery, yeasts were isolated from

Fig. 3: Effect of pH on growth of *Candida utilis* SSK04

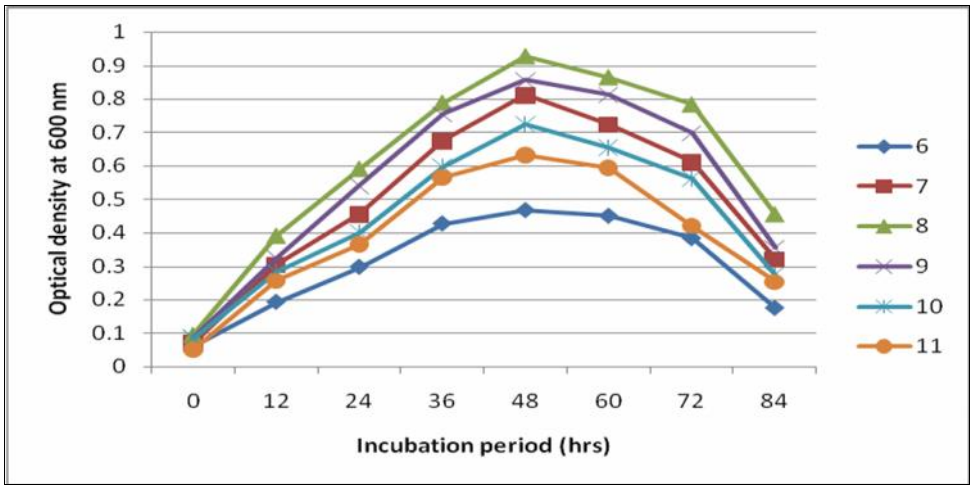


Fig. 4: Effect of temperature on growth of *Candida utilis* SSK04

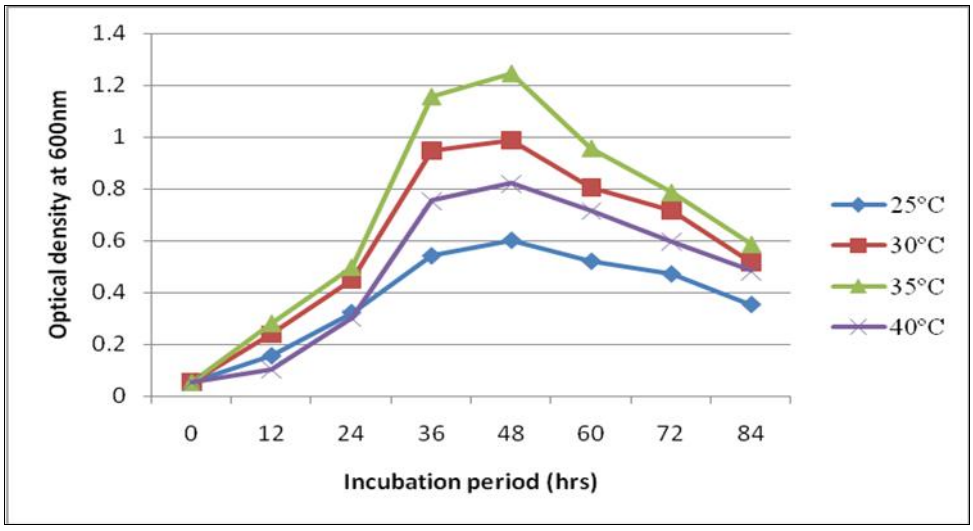


Fig. 5: Effect of salinity on growth of *Candida utilis* SSK04

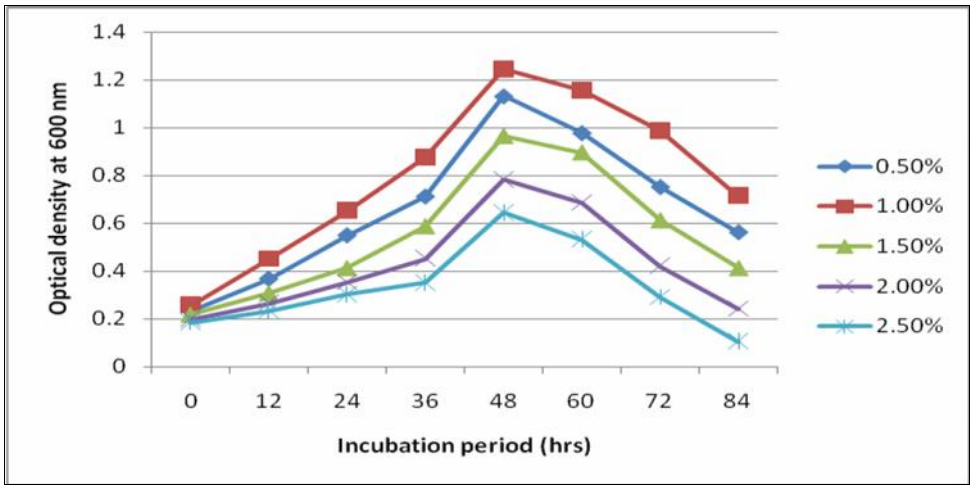


Fig. 6: Effect of Carbon source on growth of *Candida utilis* SSK04

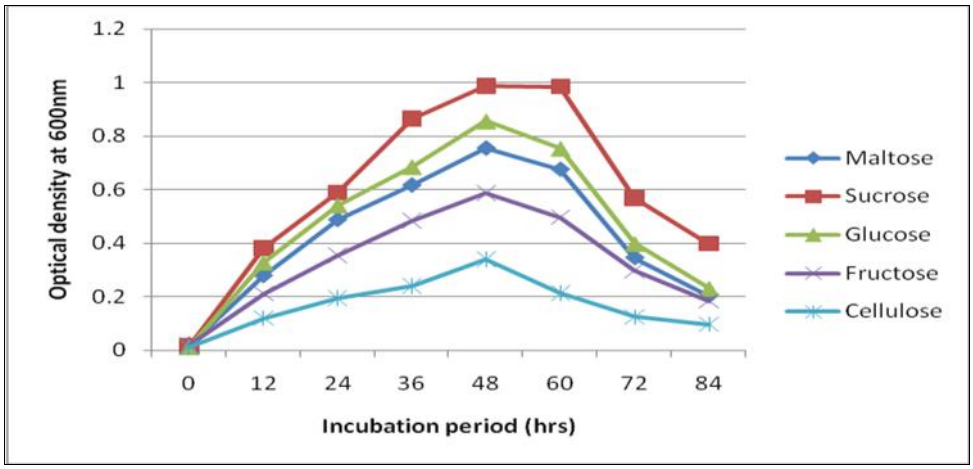


Fig. 7: Effect of nitrogen sources on growth of *Candida utilis* SSK04

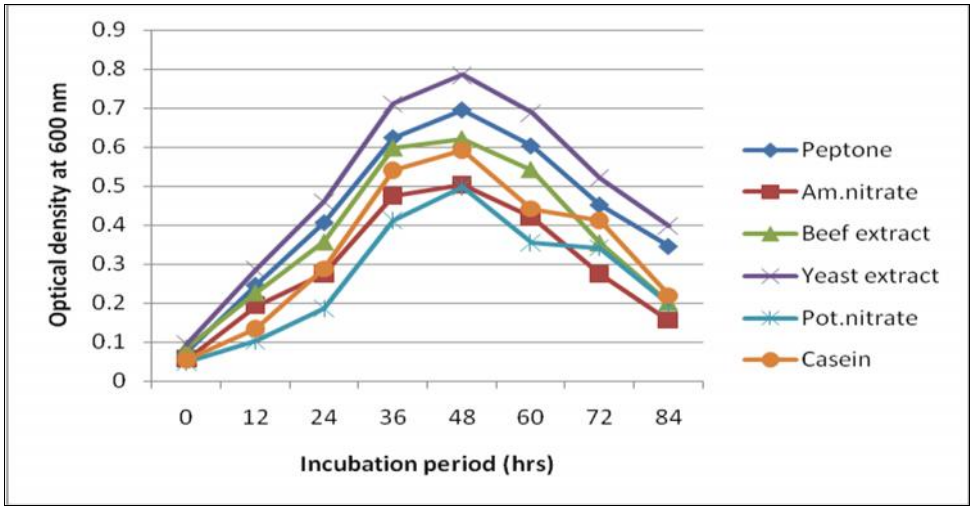
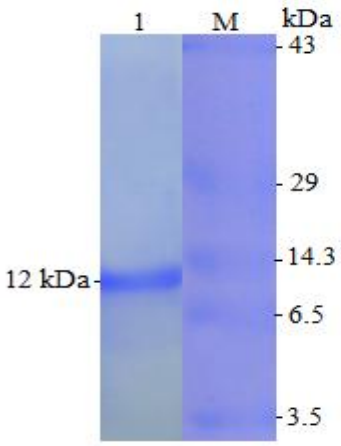


Fig. 8: SDS-PAGE profile of purified protein of *Candida utilis* SSK04



Lane 1: Purified protein sample
Lane M: Standard protein molecular weight marker

different sources, viz. seawater, marine deposits, seaweeds, fish, marine mammals and sea birds. Near shore environments are usually inhabited by tens to thousands of cells per litre of water, whereas low organic surface to deep-sea oceanic regions contain 10 or fewer cells/litre. Major genera isolated in this study were *Candida, Cryptococcus, Debaryomyces* and *Rhodotorula* (Sreedevi *et al.*, 2008).

The most important genera of true marine yeasts are *Metchnikowia, Kluyveromyces, Rhodosporidium, Candida, Cryptococcus, Rhodotorula* and *Torulopsis.* From these studies it was found that marine yeasts do not belong to a specific genus or group, but that they are distributed among a wide variety of well-known genera, such as *Candida, Cryptococcus, Debaryomyces, Pichia, Hansenula, Rhodotorula, Saccharomyces,Trichosporon* and *Torulopsis*. Loureiro *et al*., 2005 isolated and identified 292 yeasts strains from sand and seawater collected from two beaches of Olinda, Pernambuco state, Brazil were belonging to four genera and 31 species, among which *Candida* was the most prevalent genus.

In the present study Yeast strains were isolated from marine water samples using YEPD showed a density range in the water sample was $2.7x10^3$ - $1.6x10^5$ CFU/ml.

The optimized growth parameters of *C. utilis* SSK04 were as follows, pH-8, temperature 35°C, salinity 1%, sucrose-1% and yeast extract - 0.5% and incubation time 48 hrs were found to be ideal conditions for the yeast strain used in the present study (Fig: 3-7). Jeong *et al*., 2005 produced a maximal extracellular ACE inhibitor from *M. pachydermatis* G-14 with an optimized culture condition in YEPD medium containing 0.5% yeast extract, 3.0% peptone and 2.0% glucose at 30^oC for 24 hrs.

Jeong *et al*., 2005 found an ACE inhibitory activity of 48.9% with extracellular ACE inhibitor from *M. pachydermatis* G-14. Whereas the present study *C. utilis* SSK04 isolated from marine water showed an inhibitory activity of 79.1%. The crude cell free extracts showed an ACE inhibitory activity percentage of 79.1% whereas the purified protein showed 69.8% activity.

Roy *et al.,* 2000 purified ACE inhibitory protein from skimmed milk digested with cell-free extract of yeast *Saccharomyces cerevisiae* using DEAE-Sephacel, chromatography showed a molecular weight of 33 kDa with an IC_{50} value of 0.42 mg where as in the present study the purified protein sample from *C. utilis* SSK04 separated on SDS-PAGE (Fig. 8) showed a major protein band of 12 kDa. The purified protein was assayed for ACE inhibitory activity, it showed an inhibition percentage of 69.8%. The total protein concentration of the purified protein was 422mg/ml and the IC_{50}value of the peptide was calculated as 0.38mg/ml.

Conclusion

The present study revealed that the yeast strain isolated from marine origin have the ability to inhibit ACE. The marine yeast may have better potential than their terrestrial counterparts. Yeasts are rich with proteins, lipids and vitamins. Yeast can be produced very efficiently and economically because of their shorter generation time and use of inexpensive culture media. Proteins and enzymes from yeasts are extracellular metabolites of pharmaceutical and commercial importance. The present study concluded that the isolated marine Yeast *C. utilis* SSK04 have the ability to produce ACE inhibitory peptides.

Acknowledgments

The author thankful to The Director, Smart BioSolutions, Research Institute, Chidambaram, Tamilnadu for providing facilities.

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