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## Yeasts as the Novel Attractant of *Pomacea canaliculata*

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### Abstract

*Pomacea canaliculata* (golden apple snail, GAS) has become a major pest, which threatens rice cultivation especially in Asia. To control this pest, many farmers use extensively synthetic molluscicides that are not only expensive but of broad spectrum, therefore affecting non-target organisms including human beings. This study aimed to explore new biological attractant that could be used to attract the snails as an essential aspect of integrated pest control. The novel potential of isolated yeasts, identified as *Candida ethanolica* and *Pichia kudriavzevii* based on the 18S rRNA identification, as microbial-based bait for attraction of GAS was elucidated. Both the male and female GAS showed the highest moving rates towards *C. ethanolica* strain M2 activated in 5% molasses. This is the first report on the potential of yeasts as bait or lure for GAS which can be used in integrated pest management.

**Keywords:** Attractant; *Candida ethanolica*; golden apple snail; pest management; *Pichia kudriavzevii*

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## 1. Introduction

Since 2001, *Pomacea canaliculata* (golden apple snail, GAS) has been a major rice pest in Asia [1]. In Malaysia, infestation by GAS has been prioritized as one of the major challenges in rice cultivation. The snail was initially brought in from Taiwan to boost the earning of rural poor through backyard rearing and to supplement protein in diets [1]. Eventually, the market demand was poor and many snail farming projects were abandoned, resulting in the escape of snails [1]. The snail devours young rice seedlings speedily resulting in extensive damage to both transplanted rice seedlings up to 15 days and direct-seeded rice from 4 to 30 days after sowing [1-3]. Molluscicides and insecticides have been used with limited success. A few molluscicides have been known to be broad in spectrum and could affect most life forms including human beings [4]. Additionally, a few varieties of ducks and fishes had been studied as biological control for the snails in rice cultivation [1, 5]. Yet, feasible pest control alternatives remain lacking.

Integrated pest management without the use of chemicals has been practiced in certain parts of Asia. Handpicking is now easier with the use of herbage attractants. Leaves of papaya, sweet potato, tapioca and gliricidia were potent snail attractants [6]. To-date, there is no report on the application of microorganisms for bioattraction of the snails. In this study, yeast strains were isolated from fermenting pineapple and a local preparation of effective microorganisms were tested for their potential as bait in bioattraction of GAS. The potential strains that could attract GAS were identified by 18S rRNA identification. This is the first report to discuss the novel application of the two yeasts - *Candida ethanolica* strain M2 and *Pichia kudriavzevii* strain M12 in bioattraction of GAS.

## 2. Materials and Methods

### 2.1. Sampling of golden apple snails (GAS)

The adult GAS with an average length of  $4.5 \pm 0.5$  cm were randomly sampled from the lakes in the vicinity of Universiti Teknologi Malaysia (Johor, Malaysia). The snails were reared in an aquarium of 60 x 30 x 30 cm<sup>3</sup> and fed with fresh lettuce daily.

### 2.2. Isolation and screening of yeast isolates as attractant

Serial dilutions were done on samples of a 5-day fermenting pineapple and a local preparation of effective microorganisms, followed by spread plate on potato dextrose agar (PDA) with overnight incubation at 30°C. Different types of colonies were distinguished. Colonies were then isolated and sub-cultured on PDA and incubated for 2 d at 30°C. The plate with the colonies (attractant) was placed on a raised platform in an aquarium (shown in Fig. 1). Three snails were randomly chosen and being placed 10 cm away from the plates containing microbial isolates. The response by these snails was observed within 30 min. Two potential yeast isolates, designated M2 and M12, were isolated and maintained on PDA and stored at 4°C.

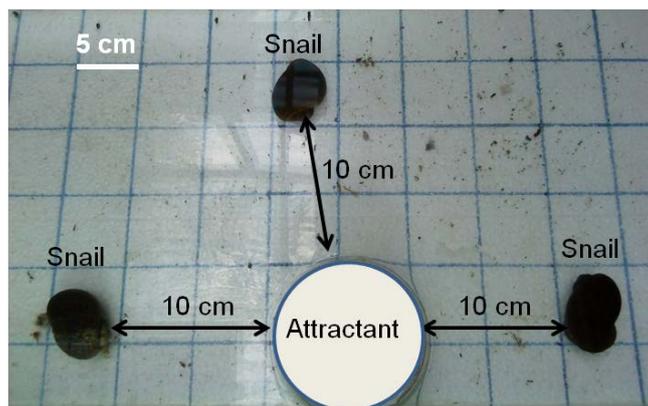


Fig. 1. Experimental design in the aquarium for bioattraction of golden apple snails to yeast isolates.

### 2.3. Molecular identification of potential yeasts

DNA isolation from strains M2 and M12 was performed using Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instruction. The isolated genomic DNA was viewed under UV transillumination in 1% (w/v) agarose. The amplification of 18S rRNA gene was performed using NS1 and NS8 primers [7], following standard procedures (25 cycles of 30 s at 94°C, 30 s at 50°C and 1.5 min at 72°C) with GoTaq Flexi DNA polymerase (Promega). The amplified 18S rRNA gene was purified using QIAquick PCR Purification Kit (QIAGEN) and sequenced using NS1 and NS8 primers [7]. The sequence was assembled and submitted to online BLASTn analysis (<http://ncbi.nlm.nih.gov/BLAST>) [8]. The sequence was aligned with sequences from related yeasts available at GenBank database by using ClustalW. MEGA version 5 was used for construction of Neighbor-Joining phylogenetic tree with bootstrap values calculated based on 1000 replicates [9].

### 2.3. Comparison of golden apple snail response to potential yeasts

The yeast isolates were cultured separately in either 5% molasses or potato dextrose broth (PDB) up to 5 d at 30°C with shaking at 100 rpm. The culture containing cells at  $1 \times 10^{10}$  cfu in 10 ml volume was filtered and allowed to saturate a 125 mm filter paper (Whatman) for bioattraction of GAS. Growth medium (5% molasses or PDB) without yeast culture was used as control. Thirty GAS, which were randomly sampled to reflect natural snail population in the environment, were used in the comparison. These snails were categorized according to their sex. The study was carried out in an aquarium of 60 x 30 x 30 cm<sup>3</sup> with a raised platform. For each sampling of thirty snails, a cluster of three snails were being placed 10 cm away from the attractant (Fig. 1). The movement of the snail towards the attractant was monitored and the time recorded for a snail to reach the attractant is considered the response by the snail. Maximum time given for the snail to move 10 cm was 30 min. The actual respond time recorded was when the snail started to climb the platform to reach for the attractant. The response was calculated in term of moving rate in cm min<sup>-1</sup>. Statistical analyses were done using Excel 2010. One-way ANOVA test was performed to compare the response between the yeast strains with  $P < 0.05$ .

### 3. Results

#### 3.1. Isolation and screening of bioattraction potential yeasts

Microbial isolation from a 5-day fermenting pineapple and a local preparation of effective microorganisms revealed two major distinguishable isolates that resulted in the isolation of two potential yeast strains, designated as strains M2 and M12. The yeast strains were observed to possess peculiar odour. The yeast strains were tested as bait for the snails by placing the PDA plates with streaked colonies of either M2 or M12 isolates in the middle of the aquarium. Fig. 2a and Fig. 2b show the golden apple snails (GAS) that were attracted to the yeast colonies of M2 and M12 streaked on the PDA plates. In the control experiment, there was no snail attracted to the PDA plates. On an average, thirty snails took 10.9 min and 14.2 min to move from a distance of 10 cm to M2 and M12 yeast isolates, respectively. The preliminary screening was done regardless of the sex of the snails.

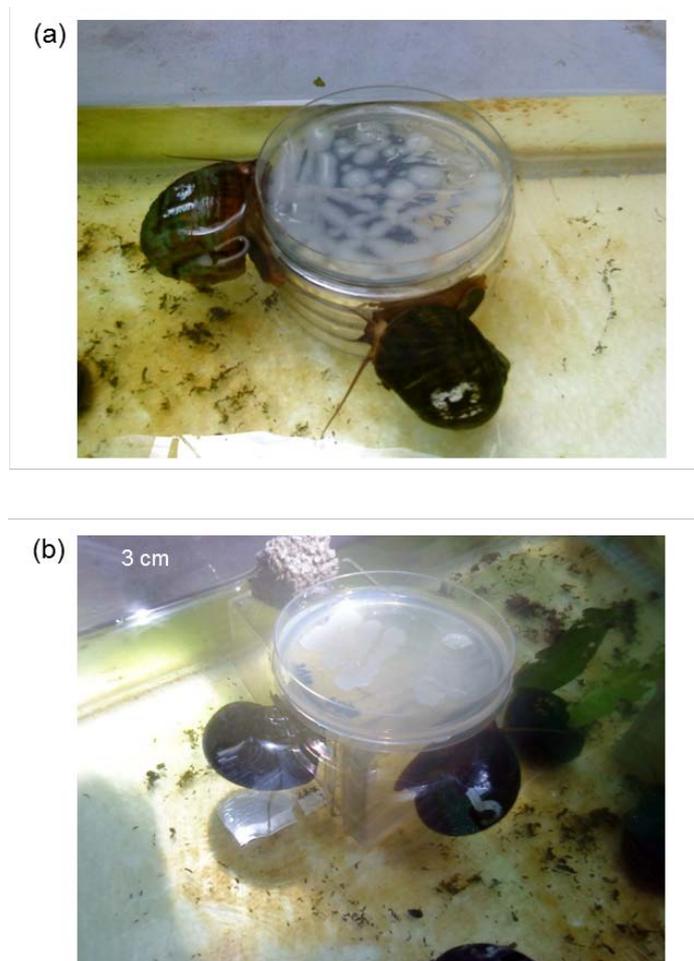


Fig. 2. Preliminary screening of bioattraction of golden apple snails by (a) *Candida ethanolica* strain M2, and (b) *Pichia kudriavzevii* strain M12.

### 3.2. Molecular identification of potential yeasts

From the phylogenetic tree shown in Fig. 3, strain M2 is in the cluster of *Candida ethanolica* and strain M12 is in the cluster of *Pichia kudriavzevii*. Hence, molecular identification based on the 18S rRNA gene sequences revealed that strains M2 and M12 are closely related to *C. ethanolica* and *P. kudriavzevii*, respectively. The 18S rRNA gene sequences of *C. ethanolica* strain M2 and *P. kudriavzevii* strain M12 are available in GenBank database under accession numbers JF274496 and JF274497, respectively.

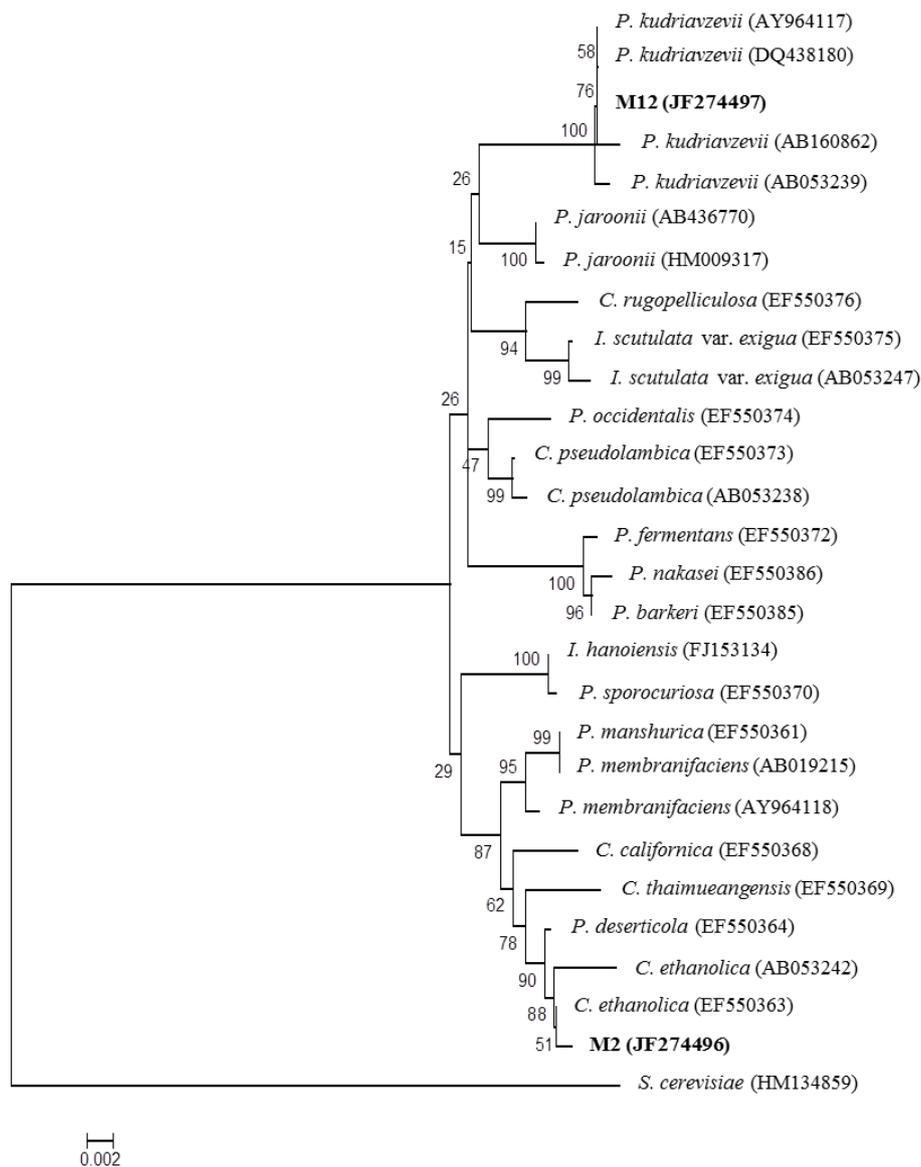


Fig. 3. Phylogenetic tree based on 18S rRNA gene sequence comparison showing the relationship between yeasts of genus *Pichia* spp., *Candida* spp., *Candida ethanolica* strain M2 and *Pichia kudriavzevii* strain M12.

Numbers in parentheses indicate the GenBank accession numbers.

### 3.3. Comparison of Golden Apple Snail Response to Potential Yeasts

From the sampling, it was observed that almost 33% of the randomly sampled GAS was male snails. Hence, 30 golden apple snails (20 females and 10 males) were randomly chosen to determine their response, in term of the moving rate, to *C. ethanolica* strain M2 (cultured in either 5% molasses or PDB) and *P. kudriavzevii* strain M12 (cultured in either 5% molasses or PDB). The responses of the 30 snails were compared and the mean of moving rates towards the yeast attractants according to the sexual category of the snails is shown in Table 1. A higher moving rate indicates a faster response and movement of the snails towards microbial attractants. From Table 1, the male snails showed a relatively faster response than the female snails toward all the microbial attractants used. This could probably due to the size of male snails, which are relatively smaller than the female snails. Female snails are generally bigger in size and are more sluggish in movement.

Table 1. Comparison of response of golden apple snails (GAS) towards *Candida ethanolica* strain M2 and *Pichia kudriavzevii* strain M12. Values are means  $\pm$  standard error of the mean.

Microbial attractants	Mean of moving rates (cm min <sup>-1</sup> )	
	Female GAS	Male GAS
<i>C. ethanolica</i> M2 (in 5% molasses)	1.586 $\pm$ 0.975	1.806 $\pm$ 1.908
<i>C. ethanolica</i> M2 (in potato dextrose broth)	1.102 $\pm$ 0.767	1.228 $\pm$ 0.729
<i>P. kudriavzevii</i> M12 (in 5% molasses)	0.895 $\pm$ 0.530	1.145 $\pm$ 0.749
<i>P. kudriavzevii</i> M12 (in potato dextrose broth)	0.953 $\pm$ 0.681	1.216 $\pm$ 1.330

In addition, as shown in Table 1, the means of response were significantly higher towards pure culture attractants of *C. ethanolica* strain M2 compared to *P. kudriavzevii* strain M12. Both the male and female snails showed the highest moving rate towards *C. ethanolica* strain M2 grown in 5% molasses. One-way ANOVA test revealed the significance with ( $P > 0.05$ ) in response by GAS towards the yeast attractants either grown in 5% molasses or PBD. In all the control experiments, there was no snail attracted to 5% molasses or PDB. Fig. 4 shows the response of individual GAS towards *C. ethanolica* strain M2 and *P. kudriavzevii* strain M12. From the result, *C. ethanolica* strain M2 cultured in 5% molasses could be the best and optimal yeast strain for bioattraction of GAS.

## 4. Discussion

This study reveals a new application of fermenting yeasts in sustainable agriculture. Use of beneficial microorganisms is an essential approach in eliminating problems associated with the long-term applications of chemical pest control agents, and this is mainly applied in nature and organic agriculture.

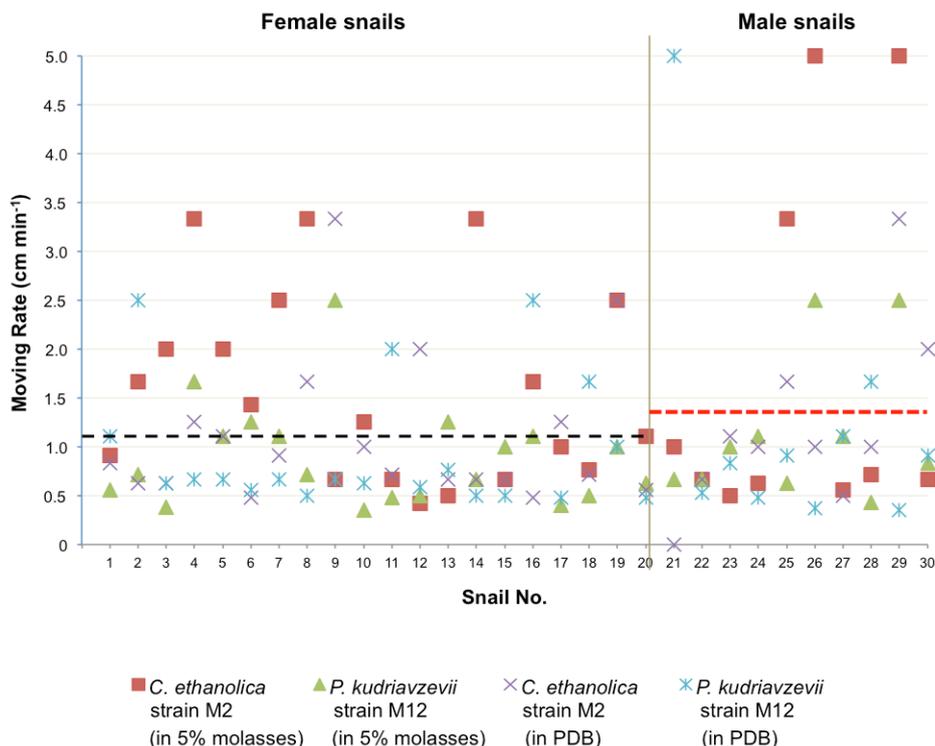


Fig. 4. The response of 30 golden apple snails towards *Candida ethanolica* strain M2 and *Pichia kudriavzevii* strain M12. The black and red dashed lines represent the mean of response by female and male snails, respectively. The higher moving rates indicate better responses of golden apple snails towards the microbial attractants.

The novel potential of yeasts as bioattractant of golden apple snails is reported here with the elucidation of the role of yeasts from *C. ethanolica* strain M2 as bioattractant of GAS. The snails were probably attracted to the yeast volatile metabolites and moved towards the microbial attractants. The yeasts were hypothesized to produce volatiles such as alcohols, carboxylic acids and esters that could attract the snails. In this case, since *C. ethanolica* strain M2 had shown a good potential as bioattractant, it will be chosen rather than *P. kudriavzevii* strain M12, for further optimization as bait for the GAS as part of a sustainable and integrated pest management.

Back in 1980, *C. ethanolica* was firstly isolated from industrial fodder yeast and was cultivated on synthetic ethanol as the only source of carbon. This species differs from other accepted *Candida* species as it is not nitrate-assimilating, non-urease producing and non-sugar fermenting [10]. Until now, there is very limited study published on *C. ethanolica*. On the other hand, *P. kudriavzevii* formerly known as *Issatchenkia orientalis* is an anamorph of *C. krusei* [11, 12]. *P. kudriavzevii* was reported from fruit and food sources including non-fermented carrot pomace [13], fermented pineapple juice [14], orange juice [15], grape [16], sourdoughs [17, 18], fermented butter-like *mashita* [19], starter culture of fermented *togwa* [20], fermented cassava [21], and fermented cocoa bean heap [22]. Though mostly associated with food fermentation, Shemer et al. [23] reported

on *P. kudriavzevii* as an opportunistic pathogen and a potential source of fungemia. Hence, this finding suggests the cautionary step to exclude *P. kudriavzevii* from future field experiments for bioattraction of golden apple snails, to avoid indiscriminate and uncontrolled release of the yeast strain into the environment. It is much easier to monitor the movement of a single yeast strain. *C. ethanolica* will make an excellent choice of snail attractant. Future work will include field trials at paddy fields to estimate the potential of the *C. ethanolica* in bioattraction and the development of a support material for slow release of the volatiles.

## 5. Conclusion

Our findings clearly revealed the capability of *C. ethanolica* which is a local yeast strain isolated from fermenting pineapple in bioattraction of golden apple snails. This study also revealed the best response of golden apple snails was derived from the exposure of the snails to the 5-day culture of *C. ethanolica* strain M2 in 5% molasses.

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