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THE ROLE OF ANTIMICROBIAL PEPTIDES IN A YEAST-BEETLE ASSOCIATION

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THE ROLE OF ANTIMICROBIAL PEPTIDES IN A YEAST-BEETLE ASSOCIATION

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Abstract

Each species of Nitidulid beetle in the genus *Conotelus* is associated with a specific yeast species in the genus *Metschnikowia*. However, the reason for this selectivity is still unknown. Insects produce antimicrobial peptides (AMPs) that inhibit the growth of microorganisms. I hypothesized that *Conotelus obscurus* would produce specific AMPs that select in favor of its associated *Metschnikowia* species and that peptides produced by a particular *Conotelus* species can be induced by other yeast species. I investigated the antifungal activity of *Conotelus obscurus* larval extracts and compared their effect on three yeast species, *Metschnikowia borealis*, which is normally found in association with the beetle, and *Metschnikowia reukaufii* and *Debaryomyces maramus*, which are not commonly present. Although the hypothesis was rejected, differential inhibition was observed. I also demonstrated that adult beetles carry the yeasts in their digestive tract and release them during excretion.

Keywords:

Yeast, beetle, bindweed, antimicrobial peptides, innate immunity, symbiosis, *Conotelus obscurus*, *Metschnikowia borealis*, *Metschnikowia reukaufii*, *Debaryomyces maramus*, inhibition, stimulation.

Co-Authorship Statement

The methods of protein separation and assays conducted in this project were provided by Dr. Mark Bernards. Part of this work was performed together with Anica Bjelica in Mark Bernards's lab. Dr. Mark Bernards contributed to the protein analysis part of this thesis. Beetle collection and experiments of localization of yeasts were performed together with Tanya Berkers.

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List of Abbreviations

AMPs	Antimicrobial peptides
CM	Corn Meal (1.7% corn meal powder)
CTAB	Cetyl trimethylammonium bromide
DMSO	Dimethyl sulfoxide
IEF	Isoelectric focusing
LE	Larval extract
LPS	Lipopolysaccharides
OD	Optical density
PBS	Phosphate buffered saline (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.2)
PI	Isoelectric points
PO	Phenoloxidase
SEM	Scanning electron microscope
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
YCB	Yeast Carbon Base (0.17% YCB powder)
YNB	Yeast Nitrogen Base (0.67% YNB powder)
YM	Yeast Malt (0.5% peptone, 1% glucose, 0.3% malt extract, 0.3% yeast extract)

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CHAPTER 1

INTRODUCTION

1.1 Interactions between fungi, insects, and flowers

1.1.1 The yeast-insect-flower symbiosis system

Yeasts have been found in association with many different species of insects over a large geographical region (Benda et al., 2008, Lachance et al., 2001a). These interactions feature various degrees of specificity. One of the most specific associations is that involving a certain group of yeasts in the genus *Metschnikowia* and beetles of the genus *Conotelus*. This remarkable insect-yeast-flower interaction was first discovered during a study of yeasts associated with flowers and Hawaiian *Drosophila* species (Lachance et al., 1989). A *Metschnikowia* species shared by morning glory (*Ipomoea acuminata*) and two of its visiting drosophilid species was isolated and later described as *Metschnikowia hawaiiensis* (Lachance et al., 1990). Direct streak plating of inner corolla scrapings from different aged morning glories, as well as flower bagging experiments, clearly showed that the drosophilid flies are vectors of the yeasts (Lachance et al., 1989). Subsequent investigations demonstrated several similar symbiotic systems of insect-yeast-ephemeral flowers all over the world and identified nitidulid beetles as the principal insects that perpetuate *Metschnikowia* species. For instance, *Metschnikowia borealis* is associated with the nitidulid beetle, *Conotelus obscurus*, and bindweeds (*Calystegia* or *Convolvulus* spp.) in the Great Lakes region of North America (Lachance et al., 2001a). Nearly every single beetle in that ecosystem contains *M. borealis* and no other yeast species. *Kodamaea kakaduensis* is carried by sap beetles visiting *Hibiscus* flowers in Australia (Lachance et al., 1999).

C. mexicanus is a vector of *M. lochheadii* in Costa Rica (Lachance et al., 2001b) and *M. cerradonensis* is associated with *Conotelus* sp. and *Ipomoea* spp. in Brazil (Rosa et al., 2007).

There is a strong biogeographic pattern of insects visiting ephemeral flowers and their associated yeasts species (Lachance et al., 2001a; Lachance et al., 2003a). Insects living in a particular area always carry specific species of yeasts. The only exception is *M. lochheadii*, which is associated with *C. mexicanus* in Hawaii and in Costa Rica (Lachance et al., 2001b). It is thought that *M. lochheadii* together with its beetle host was introduced to Hawaii through human activities and may slowly displace the Hawaiian endemic species *M. hawaiiensis* (Lachance et al., 2005).

1.1.2 The *Conotelus-Metschnikowia*-ephemeral flower symbiosis system

The insect-yeast-flower symbioses involve different species of nitidulid beetles, *Drosophila*, and yeasts (Lachance et al., 1998a; Lachance et al., 2001a).

Beetles of the genus *Conotelus* (Coleoptera: Nitidulidae) only occur in the New World and primarily visit morning glories and hedge bindweeds (Fickle et al., 1987). Beetles in the genus *Conotelus* are small (usually 3-4 mm) and cone-shaped (White, 1983). It is easy to identify the sex of the beetles by observation of the posterior segment with the naked eye, as males have a longer slender pygidium. *Conotelus obscurus* is found in the north eastern United States and Canada, from Nova Scotia to Manitoba, and from Maine to Tennessee (Parsons, 1943; Majka and Cline, 2006). This species of *Conotelus* has been reported on morning glories (*Ipomoea* spp.,

Convolvulus sp., *Calystegia* sp.; Lachance et al., 1998b), southern magnolia (*Magnolia grandiflora*; Thien, 1974), wild carrot (*Daucus carota*; Lago and Mann, 1987), and milkweed (*Asclepias syriaca*; Dailey et al., 1978). The egg of *C. obscurus* is milk-white and sausage-shaped, and takes 2 days to give rise to a larva at 24 °C. The larva stage lasts 1 to 3 weeks and the pupa stage for 5 to 6 days at the same condition (Unpublished data by T.E. Berkers). Larvae of this species may be mycophagous (Lachance et al., 2003a).

The ascomycetous yeast genus *Metschnikowia* contains 51 species (Lachance, 2010) and comprises several clades, including the large-spored clade, which contains all species associated with beetles and flowers (Marinoni and Lachance, 2004). This genus is characterized by multilateral budding of vegetative cells and production of no more than two needle-shaped ascospores per ascus. It produces pseudohyphae which are the result of a sort of incomplete budding where the cells remain attached after division. Species in the large-spored clade form unusually large (100-250 µm) needle-shaped ascospores (Marinoni and Lachance, 2004), in contrast to the normal size (20-30 µm) ascospores made by the rest of the genus (Lachance and Bowles, 2002). The asci of large-spored *Metschnikowia* species usually contain ascospores which are covered with helically arranged barbs that point posteriorly (Lachance et al., 1998b). Interestingly, only species within the large-spored clade have been found in association with beetles in the genus *Conotelus* (Lachance et al., 2001a) (Table 1.1). *C. obscurus* is highly specific, carrying the species *Metschnikowia borealis* (Lachance et al., 1998b). The availability of the *Conotelus obscurus-Metschnikowia borealis-Calystegia sepium* (hedge bindweed)

Table 1.1 Species within the large-spored clade of *Metschnikowia* that have been found in association with *Conotelus* spp. of beetles in the New World.

The code under each species name is the strain number of type culture.

Strain	Locality	Plant Species	Reference
M. continentails (AF034126)	Minas Gerais, Brazil	*Ipomoea carnea*	(Lachance et al., 1998b)
M. borealis (AF034127)	Great Lakes area of North America	*Convolvulus arvensis* *Calystegia sepium* *Ipomoea* spp.	(Lachance et al., 1998b) (Lachance et al., 2001a) (Wardlaw et al., 2009)
M. lochheadii (AF279304)	Hawaii and Sector Santa Rosa, Costa Rica	*Ipomoea* spp. *Brugsmansia candida* *Merremia* spp. *Hibiscus* sp. *Tabebuia rosea*	(Lachance et al., 2001b)
M. dekortorum (AY056036)	Near Santa Cecilia, Costa Rica	*Ipomoea* spp.	(Lachance and Bowles, 2002)
M. hawaiiensis (DQ 641239)	Hawaii	*Ipomoea* spp.	(Lachance et al., 2003a)
M. santaceciliae (AF514292)	Santa Cecilia, Costa Rica	*Ipomoea indica*	(Lachance et al., 2003b)
M. similis (AY313962)	El Gavilán and Sangregado，Costa Rica	*Ipomoea batatas*	(Lachance and Bowles, 2004)
M. colocasiae (AY313960)	Santa Cecilia, Costa Rica	*Merremia tuberosa*	(Lachance and Bowles, 2004)
M. cerradonensis (DQ641237)	Jalapão, Brazil	*Ipomoea carnea*	(Rosa C. A et al., 2007)
Metschnikowia. sp. (DQ641241)	Dos Ríos, Costa Rica	*Merremia tuberosa*	(Fidalgo-Jiménez et al., 2008)
M. cubensis (MUCL 51011)	Sancí Spiritus, Cuba	*Talipariti elatum* *Gesneria humilis* *Bidens alba* *Ipomoea acuminata*	(Fidalgo-Jiménez et al., 2008)

symbiosis system in Ontario made it a good model for studying yeast-insect-flower interactions.

1.2 Fungus-insect parasitism and the defense of insect

Fungi are ubiquitous and play an essential role in nutrient cycling in most natural and man-made ecosystems. This may be accomplished through symbiotic relationships with organisms in most, if not all, kingdoms (Campbell and Reece, 2005), which may be mutualistic, parasitic, or commensalistic (Das and Varma, 2009). A great number of fungus-insect associations fit within these three forms of symbiosis.

1.2.1 Fungus-insect parasitism

Parasitism is one of the most widespread forms of fungus-insect interaction. In the 18th century, Agostino Bassi experimentally demonstrated that *Beauveria bassiana* was the cause of the white muscardine disease, which devastated the European silkworm industry, and brought the entomopathogenic fungi to the attention of scientists of the western world (Rehner, 2005). More than 700 species of entomopathogenic fungi have now been described (St. Leger et al., 1997). Their modes of infection, as well as their impact on their hosts, have been well studied because many have the potential for use as biological-control agents of insect pests.

Fungi may infect their hosts not only following ingestion, but also have the ability to penetrate the cuticle using a series of physical and enzymatic mechanisms (Ferron, 1978). For instance, *Metarhizium anisopliae* has at least six different stages that can

infect wireworms: external infective spores that produce a germ tube, appressorial cells which produce penetrant pegs that can penetrate cuticle, penetrant plates which lead to penetrant hyphae, penetrant hyphal bodies that penetrate the procuticle to the coelom, yeast-like hyphal bodies that circulate in the hemolymph and spreadind the fungus in the body cavity, and chlamydospores, which are produced after death of the host and maintain the fungus in a viable state (Ferron, 1978).

Some fungi maintain biotrophic relationships with their hosts with little or no saprophytism, while others kill their hosts in a short period and live on the dead body (Pell and Shah, 2003). Entomopathogenic fungi may modify behaviors or physiological processes of their host that optimize transmission from one host to another, including defense activity, elevation seeking, reduced or increased activity, reduced response to semiochemicals (chemical substances or mixtures that carry messages), and changes in reproductive behavior (Roy et al., 2006).

1.2.2 Insect immune responses

Insects colonize nearly all ecological niches on earth except the high seas (Hoffmann, 1997) and are thus exposed to a wide range of potential pathogens. The first line of defense of insects against microbes is physical, the cuticle, which is an extracellular structure covering the total outer surface (Bulet et al., 1999). However, once the cuticle has been breached, the immune system is activated. Unlike vertebrates, insects do not have T cells, B cells, or even an "immunity memory", and so they must rely totally on innate immunity as self defense (Wilson et al., 1999).

The first step is the recognition of foreign particles that induce a cascade of different defense mechanisms, which include localized blood clotting (coagulation; Hoffmann, 1995), phagocytosis of bacteria, the encapsulation of larger parasites by "blood" cells, and melanization (Gillespie et al., 1997). These occur both in tissues and the haemocoel (Bulet et al., 1999). Melanization is an immediate localized blackening reaction caused by phenoloxidase (PO) which can help to wall off invaders (Pham and Schneider, 2008) and also stimulate the production of cytotoxic molecules (Hoffmann, 1995). Antimicrobial peptides are also produced in the fat body, a structure equivalent to the mammalian liver, as well as in haemolymph cells (Hoffmann, 1997). It is thought that certain surface elements of microbes can be recognized, for example lipopolysaccharides (LPS) of the membrane of gram-negative bacteria. The different recognition mechanisms have not been well studied (Schmid-Hempel, 2005), but it is clear that the responses are elicited differently depending on the agent causing the infection and the host species being infected. For example, in *Drosophila*, the "Toll" pathway is triggered primarily through infection by gram-positive bacteria and fungi, while the "Imd" pathway, which is predominantly implicated in the regulation of the genes encoding AMPs, is triggered primarily by gram-negative bacteria (Hoffmann and Reichhart, 2002).

1.2.3 Antimicrobial peptides of multicellular organisms

Every organism has evolved mechanisms to defend itself against attack from various potentially harmful microbes (Aerts et al., 2008). The innate immune system is the most effective barrier (Kamysz et al., 2003). Antimicrobial peptides (AMPs) are an

important part of the innate immune system of all eukaryotes (Gabay, 1994), as they exhibit activity against bacteria, yeasts and other fungi, as well as certain enveloped viruses and protozoa (Ganz, 2003b).

Defensins are the only class of AMPs that seem to be conserved between plants, invertebrates, and vertebrates (Thomma et al., 2002). They are small (3-5kDa), highly basic, cysteine-rich cationic peptides that share a common three-dimensional structure (Thevissen et al., 2004). There are two subfamilies, α-defensins and β-defensins, which differ in their pairing of cysteine (Ganz, 2003a), although the two subfamilies are structurally very similar (Ganz and Lehrer, 1995). Because of their wide distribution and conserved properties, defensins are believed to be ancient molecules with a common ancestor dating over a billion years ago (Aerts et al., 2008).

There is a great diversity of AMPs (Zasloff, 2002). Some are amphipathic or hydrophobic α-helical peptides, some contain β-sheet structures, and some are rich in certain amino acids such as histidine, proline, arginine and tryptophan (Epand and Vogel, 1999). Antimicrobial peptides are generally polypeptides with fewer than 100 amino acids and therefore have low molecular weights (Ganz, 2003a). Most antimicrobial peptides attack microbial cell membranes directly (Ganz, 2003b). Some larger antimicrobial peptides containing more than 100 amino acids act as lytic enzymes or nutrient-binding proteins. Among the most studied are the cationic amphipathic peptides, with 12 to 50 amino acids with 2–9 positively charged lysine or arginine residues (Brown and Hancock, 2006). The positive charges carried by the cationic

amphipathic peptides effect electrostatic interactions with negatively charged components of microbial cell membranes (Andreu and Rivas, 1998) and the amphipathic structure allows the peptides to bind to the membrane (Epand and Vogel, 1999). In the end, the peptides will lead to membrane permeabilization and an increase of the membrane permeability, and thus kill the microbe. A number of different models have been proposed to explain membrane permeabilization such as the barrel-stave model, the carpet model, and the toroidal model (Brogden, 2005). Some antimicrobial peptides are cytotoxic; they are able to pass through the membrane and attack targets inside the cell (Epand and Vogel, 1999).

1.2.4 Antimicrobial peptides of insects

Any given insect species may produce a number of different AMPs, and genome analysis suggested that *Drosophila melanogaster* may have at least 30 (Pham and Schneider, 2008). This diversity of AMPs could serve to maximize defenses against different pathogens, as each one may only be effective against certain microbes. For instance, insect defensins act against a very broad range of gram-positive bacteria, but less so against gram-negative bacteria, yeasts, and other fungi (Bulet et al., 1999). Although there are large numbers of AMPs in insects, little information is available on antifungal AMPs. The activity spectrum of three different insect AMPs has been studied (Lamberty et al., 2001). It was shown that the termicin of *Pseudacanthotermes spiniger* (termite), the heliomicin of *Heliothis virescens* (tobacco budworm), and the defensin of *Phormia terranovae* (fly) have different antimicrobial activities against various species of

bacteria, yeasts, and other fungi (Table 1.2). Antifungal AMPs of various insects may have different action mechanisms from antibacterial AMPs. Compared to the broad activity spectrum of antibacterial AMPs, which can affect both bacteria and fungi, antifungal AMP activity is restricted to fungi (Bulet and Stöcklin, 2005). For example, high concentrations (> 10 μM) of drosomycin produced by *Drosophila* completely inhibited spore germination while lower concentrations caused delayed growth of hyphae and abnormal morphology in eight tested fungi strains (Fehlbaum et al., 1994).

The induction and production of most insect AMPs happens in a short time. For instance, after infection by microbes, the transcription of drosomycin, an AMP of *Drosophila,* peaks within 6 to 12 hours (Lemaitre et al., 1997), and may remain in the hemolymph 3 weeks after infection (Uttenweiler-Joseph et al., 1998). Most of the insect AMPs are produced at high molar concentrations, at levels of milligrams per liter (Bulet and Stöcklin, 2005).

1.3 Fungus-insect mutualism

There are many cases of mutualistic relationships between insects and fungi. In some cases fungi may help in the degradation of lignin and enhance the digestibility of cellulose by insects (Hyodo et al., 2000), while insects may play a role in the dispersal of fungal spores and even the fertilization of some fungi (Bultman et al., 1998). .

One of the best studied fungus-insect mutualisms involves the fungus-growing ants of the tribe Attini (subfamily *Myrmicinae*), where the ants cultivate fungus gardens,

Table 1.2 Antimicrobial activity spectrum of termicin of *Pseudacanthotermes spiniger*, heliomicin *of Heliothis virescens* and defensin of *Phormia terranovae* (modified from Lamberty, et al. 2001).

Activity not detected at the highest concentration tested (100 µM) is indicated in the table by ND. The minimal inhibitory concentration is expressed as final concentration in µM.

Microorganisms	Minimal inhibitory concentration		
	Termicin (*P. spiniger*)	Heliomicin (*H. virescens*)	Defensin (*P. terranovae*)
Gram-positive bacteria		µM	
A. viridians	ND	ND	0.05–0.1
B. megaterium	50–100	ND	0.05–0.1
M. luteus	50–100	ND	0.4–0.8
S. pyogenes	25–50	ND	1.5–3
Gram-negative bacteria			
E. coli SBS363	ND	ND	25–50
P. aeruginosa	ND	ND	ND
Filamentous fungi			
A. fumigates	ND	6–12	ND
F. culmorum	0.2–0.4	0.2–0.4	1.5–3
F. oxysporum	0.8–1.5	1.5–3	3–6
N. crassa	0.2–0.4	0.1–0.2	3–6
T. viride	6–12	1.5–3	6–12
Yeast			
C. albicans	6–12	2.5–5	ND
C. glabrata	ND	ND	ND
C. neoformans	6–12	2.5–5	ND
S. cerevisiae	6–12	ND	ND

which has been referred to as "insect agriculture" (Weber, 1966). In this case, the cultivated fungi are the only source of nutrition for the larvae and the principal source of nutrition for the adults (Schultz et al., 2005). The attine fungi are dispersed only by the queen and transmitted vertically from queen to daughters. In this way, the fungi increase their geographic distribution due to dispersal by foundress queens and are protected from parasites and pathogens (e.g., fungi of the genus *Escovopsis* sp.) as a result of various activities of the ants (Schultz et al., 2005).

Another well-studied mutualistic interaction involves the use of fungi by bark beetles (Curculionidae: Scolytinae). The fungi are carried by the beetles in special spore-containing structures on the body surface, called mycangia (Blackwell and Vega, 2005) and released into the brood galleries when the host beetles attack a tree. The fungal growth may play an active role in overcoming the tree defense (Paine et al., 1997) and serve as food for the developing beetle larvae (Ayres et al., 2000).

1.4 Fungus-insect commensalism

Few commensal interactions between fungi and insects have been described, but some have been well documented. For example, Trichomycetes live obligately in the guts of many insects (Robert, 2004) and utilize food within the digestive tract of their hosts. However no detectable harm or benefit to most of the hosts has been observed (Moss, 1977).

1.5 Objectives and hypotheses

The ultimate aim of this project is to study the basis for the specificity in the yeast-insect-flower symbiosis system. Several questions were asked, as follows. Does the immune system of the insect play a role in the high degree of specificity seen in these yeast-beetle-flower interactions? One could suppose that the beetles produce AMPs that inhibit growth of all but their associated yeast. Symbiotic yeasts have been found in the gut (Suh et al., 2005) and mycangia (Tanahashi et al., 2010) of insects. Where does the *Conotelus* beetle carry the yeast cells? How does the beetle transmit the yeasts? My hypotheses were that: (1) the nitidulid beetle *Conotelus obscurus* produces specific antimicrobial peptides that select in favor of *Metschnikowia borealis*; (2) the peptides produced by *C. obscurus* can be induced by yeast species other than *M. borealis*; and (3) transmission of the yeast cells occurs through the guts of beetles and the corolla of flowers. If it can be shown that there is a specific mechanism that favours the growth of one particular yeast species, it will follow that the relationship between *Conotelus* spp. and *Metschnikowia* spp., should be regarded as mutualistic or commensalistic, but not an example of parasitism.

CHAPTER 2

MATERIALS AND METHODS

2.1. Antifungal activity of AMPs produced by *Conotelus obscurus*

My experiments were carried out using the larval extracts collected from later instar larvae. I hypothesized that *Conotelus obscurus* produces specific antimicrobial peptides that select in favor of certain *Metschnikowia* species. To test my hypotheses, a number of experiments were conducted with the larvae of *C. obscurus*. Larvae of the red flour beetle (*Tribolium castaneum*; from United States Department of Agriculture), which have similar body sizes as *C. obscurus,* were also used to develop and adapt methods of isolation and purification of larval extracts.

2.1.1. Beetle collection and rearing of larvae

The specimens of adult *C. obscurus* were collected during June and September 2009 from bindweed flowers (*Calystegia sepium*) at three locations: roadsides of Long Point Road (Norfolk, ON; 42'35"00 N, 80'23"45 W); a shrubby marsh behind Long Point Bird Observatory (Norfolk, ON; 42'35"02 N, 80'26"26 W); a bicycle path near the University of Western Ontario (London, ON; 43'00"11 N, 81'16"10 W). Flowers with beetles deep in the corolla were collected and sealed immediately in Whirl-Pak sterile sampling bags to prevent microbial contamination during transport. Later, one male and multiple female beetles were transferred to 0.5% Malt agar plates (0.5% malt powder, 1.5% agar) with 0.1g/L chloramphenicol (antibiotic) and left overnight at room temperature. Beetles were moved to new plates every day until the first generation all died. After three to five days, eggs and larvae were transferred to fresh plates and allowed to develop free of molds. This also prevented the loss of eggs due to predation

by adult beetles or the larvae.

Eggs and larvae died quickly when held in Petri dishes plates, even in the absence of adults. For this reason, the larval rearing method was later improved by keeping the adult beetles and the host flowers in sampling bags for 7 to 10 days. Larvae grew quickly while feeding on the decaying flower tissue and infection by bacteria or mold during transfer of larvae was minimized. Large sized larvae were maintained on 0.5% Malt plates with 0.1 g/L chloramphenicol and 0.2 g/L nystatin (antimycotic) for two days to remove endogenous microorganisms. The larvae were then moved to 0.5% malt plates and left overnight to allow elimination of the residual antibiotic and antimycotic.

The larvae were divided into three groups and moved to plates with three different species of yeasts: *Metschnikowia borealis* (strain 08-153.1), *Metschnikowia reukaufii* (strain 06-29.1), and *Debaryomyces maramus* (strain 06-101.1). *M. borealis* has already been introduced as a large-spored species carried by *Conotelus obscurus* in the Great Lakes area (Section 1.1.2). *M. reukaufii* is also associated with flower-visiting insects but does not belong to the large-spored clade. It is one of the most frequent species carried by bumblebees in Central Europe (Brysch-Herzberg, 2004) and is not normally carried by *Conotelus* species. *Debaryomyces maramus*, formerly named "*Debaryomyces marama*", was also used in this study. It has been isolated from cider (Martorell et al., 2005) and ham (Simoncini et al., 2007), but the strain used here came from *C. obscurus* in Ontario.

All three species were routinely maintained and grown at 24 °C on Yeast Malt (YM)

agar plates (0.5% peptone, 1% glucose, 0.3% malt extract, 0.3% yeast extract, 2% agar). The larvae were fed yeast colonies for two days. The fed larvae were stored in Nalgene 2 mL cryovials, frozen at -20 °C for 3 hours and stored in liquid nitrogen until used.

2.1.2. Collection of larval extracts

Extraction of beetles followed a modification of the procedure reported by Lamberty et al. (1999). Between 20 and 40 frozen *C. obscurus* larvae were quickly transferred to cold Eppendorf 2 mL tubes and pulverized with a plastic pestle. Phosphate buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.2) was added at the rate of 10 µL per larva. Final concentrations of 3 mM aprotinin (protease inhibitor) and 20 mM phenylthiourea (antioxidant) were added to the extract, the latter to inhibit melanization of larval extracts. The material was centrifuged at 12,000 × g for 5, 10, and 15 minutes sequences to remove the hemocytes and other larval tissues, before the supernatant was collected. Three different larval extracts were prepared based on the yeast fed to the beetle larvae. They are individually named Larval Extracts (LE) 1, 2 and 3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus*. In later experiments, larval extracts were centrifuged at 12,000 × g for 3 minutes through Millipore Ultrafree-MC centrifugal filters (pore size 0.22 µm) to remove microorganisms that may have been present.

2.1.3. Antifungal activity testing of larval extracts

2.1.3.1. Densitometric assays

The antifungal activity of different larval extracts was first tested in

densitometric assays. Three yeast species suspended in fermentation liquid medium (2% glucose, 0.5% yeast extract) were individually added to 96-well plates. The suspension of one yeast species was treated with either 1, 5 or 10 µL of the three larval extracts both at the beginning (treatment) and the end (control) of 4 hour incubation at 24°C. This was replicated so that there were 6 replicates for each concentration of the three larval extracts. The total volume of each well was adjusted to 100 µL and the optical densities (OD) of the mixtures were monitored in a VERSAmax microtitre plate reader at 600 nm. The final ODs were compared to find out whether the larval extracts could inhibit the growth of the different yeasts over 4 hours. The method was later modified to include multiple readings and a different control. A suspension of each yeast species was treated with different volumes (1, 5, and 10 µL) of the three larval extracts, as well as PBS buffer as a control. The total volume of each well was also adjusted to 100 µL with 6 replicates for each concentration and the ODs were monitored every half hour at 600 nm over 4 hours at 24°C. The plate was shaken for 1 minute before each reading. The growth curves of each species of yeast mixed with different concentrations of larval extracts were calculated using SoftMax Pro. These growth curves were compared to the control to determine if there was any inhibitory activity by the different larval extracts.

2.1.3.2. Viable counts

As the possibility existed that larval extracts could act through damage to the cell envelope, thus requiring prolonged contact with the yeast cells, the viability of cells was examined following such a treatement. Each of yeast species was mixed with

sterilized water and diluted to a concentration of approximately 200 cells per 100 µL. The number of yeast cells in each suspension was counted twice with a hemocytometer (Hausser Scientific) under the microscope. Then 50 µL of a given suspension of known cell density was incubated at room temperature for 1 hour with 50 µL of larval extract. The same volume of PBS buffer was added to a given suspension as control. Each mixture was then plated and incubated at 24 °C overnight. The colonies were counted the next day. All combinations of yeasts and larval extracts were tested.

2.1.3.3. Agar diffusion assays

The agar diffusion approach was used to determine if there was any inhibition. The three species of yeasts were suspended in sterilized water and spread on Corn Meal (CM) agar plates (1.7% corn meal powder, 1.5% agar). Ten or 20 µL of LE1, LE2, LE3, and PBS buffer, respectively, were added to sterilized Bacto concentration disks and the disks placed on the surface of CM agar plates with each species of yeast after air drying for 5 minutes. The plates were maintained at 24 °C for two days. This experiment was repeated 6 times. Yeast Carbon Base (YCB) agar plates (0.17% YCB powder, 1.5% agar) and Yeast Nitrogen Base (YNB) agar plates (0.67% YNB powder, 1.5% agar) without amino acids were also evaluated in this experiment. The plates were examined with the naked eye for the presence of inhibition and under the 20 × objective lens of a microscope to assess morphological changes. The antifungal activity of different concentrations of nystatin dissolved in dimethyl sulfoxide (DMSO), aprotinin, and phenylthiourea were also tested as positive controls. To find out whether the inhibitors might be lipids, 100 µL of

each larval extract were mixed with an equal amount of chloroform. After centrifugation at 12,000 × g for 3 minutes, the bottom layer (lipid fraction) was collected and analyzed by the agar diffusion assay, as described above, with PBS buffer as a control.

2.1.4. Fractionation of antifungal peptides

I also fractionated the larval extracts in an attempt to separate out the specific molecules that may have antifungal activity.

2.1.4.1. Collection of fractions

Larval extracts were fractionated according to molecular size using Amicon Ultra-0.5 centrifugal filter devices. The extracts were sequentially processed with devices with cut-off points of 100, 50, 30, 10, and 3 kDa, respectively. In each case, the extract was placed in the first device (100 kDa) and centrifuged at 14,000 × g at 4 °C. The centrifugation time varied according to the pore size of filter used. The retentate was collected by inverting the filter into a collection tube, centrifuging at 1,000 × g for 2 min, and restoring the original volume by addition of PBS. The filtrate was transferred to the next device and the process repeated until six fractions were obtained: < 3 kDa fraction, 3 kDa-10 kDa fraction, 10 kDa-30 kDa fraction, 30 kDa-50 kDa fraction, 50 kDa-100 kDa fraction, and > 100 kDa fraction.

2.1.4.2. Agar diffusion assays

The different fractions from each of the three larval extracts were tested using agar diffusion assays on Corn Meal plates. Twenty microlitre fractions were applied to filter paper disks which were then placed on the agar. The same amount of PBS buffer

was used as control. The plates were maintained at 24 °C for 2 days. The antibacterial activity of the < 3 kDa fraction and > 100 kDa fraction was also tested on 0.1% peptone agar plates with four unidentified bacteria that grew as contaminants of plates used to recover beetle larvae.

2.1.4.3. Protein concentration measurement

An adequate and standardized protein concentration is essential for the success of isoelectric focusing. The protein concentration of the fractions containing molecules larger than 100 kDa was determined using the Pierce BCA protein assay kit. A series of dilutions of each fraction (from 10^{-1} to 10^{-3}) were prepared. Ten microlitres of each protein standard, fraction, and dilutions were duplicated into microplate wells. After addition of 200 µL BCA working reagent, the plate was shaken in a VERSAmax microplate reader for 30 seconds. The plate was then incubated at 37 °C in the reader for 30 minutes. The samples were cooled at room temperature and their absorbance was measured at 562 nm. The absorbances of fraction samples were compared to the absorbance of protein standards of nine different concentrations from 0 to 2000 µg/mL.

2.1.4.4. Isoelectric focusing

Isoelectric focusing is used to separate protein molecules based on their electric charge differences (Robertson et al., 1987). The proteins align as bands at their isoelectric points (PI) in a gel with a pH gradient (Matthew et al., 1957).

The experiment was conducted using a regular vertical SDS-PAGE electrophoresis system. Gels were prepared using 3.8 mL water, 1.1 mL Bio-Rad 30%

acrylamide and bis-acrylamide solution (37.5:1), 1.3 mL 50% glycerol, and 0.3 mL ampholyte (pH range 3.5-9.5). The mixture was degassed for 30 min, at which time 27.1 µL 10% ammonium persulfate and 10.9 µL N,N,N',N'-Tetramethylethylenediamine (TEMED) were added, and a 1.0 mm gel was cast with a 10-well comb. The gel was allowed to polymerize for 1 hour.

The cathode and anode solutions, both prechilled to 4 °C were 25 mM NaOH and 20 mM acetic acid respectively. Fraction samples and the marker (IEF mix 3.6-9.3 from Sigma-Aldrich) were mixed with an equal volume of 60% glycerol and 4% ampholyte (pH range 3.5-9.5). Ten microlitres of the marker was added to the gel as the indicator of pH gradient. Different amounts of fraction samples were applied to the gels for different assays, namely 10 or 15 µL for staining and 20 µL for antifungal activity assays. The electrophoresis was carried out for 1.5 hours at 200 V, followed by 1.5 hours at 400 V constant voltage, at room temperature.

The gels were then carefully rinsed with Mili-Q water, placed into 10% trichloroacetic acid (TCA) for 10 minutes, then transferred to 1% TCA for 2 hours to remove the ampholytes. The TCA fixed the proteins in the gel for further staining. Other gels were treated with PBS buffer instead of TCA for 1 hour and the PBS buffer was changed every 15 minutes. The PBS buffer removed the ampholytes remaining in the gel and preserved the activity of proteins. After a brief rinse with Mili-Q water, one gel was used for protein blotting, and the other was stained with Coomassie blue staining solution (0.25% Coomasie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid) for

a half hour with gentle shaking. The Coomassie blue staining solution was then replaced with de-staining solution (50% methanol, 10% glacial acetic acid) and the gel was shaken until the background of the gel was clear. The pI values of proteins in > 100 kDa fractions were estimated by comparison with the protein standard (IEF mix 3.6-9.3 from Sigma-Aldrich). The published pI values for the standard proteins are as follows: amyloglucosidase from *Aspergillus niger*, 3.6; trypsin Inhibitor from soybean, 4.6; β-lactoglobulin A from bovine milk, 5.1; carbonic anhydrase II from bovine erythrocytes, 5.9; carbonic anhydrase I from human erythrocytes, 6.6; myoglobin from horse heart, 6.8, 7.2; lectin from *Lens culinaris*, 8.2, 8.6, 8.8; trypsinogen from bovine pancreas, 9.3.

2.1.4.5. Protein blotting

Protein blotting was used to transfer the separated proteins of the > 100 kDa fraction from electrophoresis gels to immobilizing membranes (Gravel, 2002). The method allows proteins to be fixed to a membrane without loss of activity. The transfer buffer for protein blotting contained 10% 10 x Tris-glycine buffer (3.03% Tris, 14.4% glycine) and 20% methanol. The solution was pre-cooled to 4 °C before use. The gel was placed on a nitrocellulose membrane that has been treated with Mili-Q water. These were then sandwiched between two filter papers and two pieces of sponge in transfer buffer. After the bubbles between each layer were removed, the system was subjected to a 75 V constant voltage for 1 hour with ice around the transfer chamber. The membrane was then taken out, stained with 0.1% Ponceau S in 5% acetic acid, and photographed before rinsed in PBS buffer.

2.1.4.6. Antifungal activity assays

One colony each of one day-old cultures of *Metschnikowia borealis* and *Debaryomyces maramus* was suspended in 1 mL sterilized water. The yeast suspensions were each added to 49 mL Basal agar medium (0.67% YNB, 1% glucose, 1.5% agar) cooled to 60 °C, and 3-5 mm thick agar plates were prepared. After setting, a slab of medium was placed on top of the nitrocellulose membrane to which the proteins had been transferred. Two pieces of medium, each with a different yeast species, were put on the same membrane, each covering one replicate of the protein bands of markers and fractions. The membrane and the slabs were sealed in a large Petri dish and maintained at 24 °C for two days. The membrane was then discarded and the media observed under the microscope for changes in yeast morphology.

In later experiments, the membrane was replaced by the gel itself. A piece of isoelectric focusing gel was put directly on top of two slabs of medium and observed under the microscope after incubation for two days at 24 °C. In another experiment, the fraction was concentrated fivefold prior to electrophoresis. The gels were cut into 1 x 0.6 cm pieces containing the protein bands. The small pieces were crushed lightly with a plastic rod and immersed in 40 µL PBS buffer overnight at 4 °C. After centrifugation at 1,000 x g for 2 minutes, the supernatant was collected and tested by agar diffusion assay on Basal agar medium as described in section 2.1.4.2.

2.2. Mechanism of yeast transfer between beetle and flower

Although a number of associations involving yeasts, beetles, and flowers have been described (Lachance et al., 2001a), very little is known about the manner in which the yeast is transmitted.

2.2.1. Localization of yeasts in beetles

Examination of *C. obscurus* adults in the scanning electron microscope demonstrated the absence of yeast cells on the exoskeleton of beetles, suggesting that this was not the normal means of transport (Berkers, 2010). We therefore tested the hypothesis that the yeast is present in the beetle gut and transferred via the feces.

Twenty four field collected adults were placed on ice and once immobilized the anus of 16 was individually sealed using Instant Krazy Glue®, while the remaining 8 served as controls. Each beetle was examined with a stereomicroscope and placed on an individual YM agar plate with 0.1 g/L chloramphenicol and 0.01 g/L cetyl trimethylammonium bromide (CTAB), which is a toxic to many fungi but not *Metschnikowia borealis* (Lachance et al., 2001a). After five minutes, the beetles were removed and the plates were sealed and left at room temperature for three days, at which time the number of *M. borealis* colonies were counted.

2.2.2. Microorganisms in bindweed seeds

A previous study showed that symbiotic fungus can be contained in the seeds and thus transferred from one plant generation to the next (Sacks et al., 2006). To determine whether yeasts are transmitted through seeds, eight seeds were collected

from bindweed plants (*Calystegia* sp.) on campus of the University of Western Ontario in May 2009. The surface of these seeds was sterilized in 70% ethanol for 10 seconds and rinsed in distilled water. After air drying, the seeds were then cut into two halves. Ten half seeds were incubated in fermentation liquid medium (2% glucose, 0.5% yeast extract) at 24 °C for 3 days, while the remains were placed on YM agar plates under the same conditions.

2.2.3. Scanning electron microscopy of the corolla of bindweed

To determine if yeast was present on the bindweed, five flowers of unknown age were collected at the bicycle path near the University of Western Ontario (London, ON) in July, 2009. The flowers were sealed in individual sterilized boxes and immediately taken to the lab. Two pieces of inner corolla (1 cm x 0.8 cm) were excised and fixed in glutaraldehyde buffered with 0.3 M cacodylate in a Nunc cryovial overnight (Lachance et al., 1998b). The samples were transferred to another cryovial and rinsed in 0.3 M cacodylate buffer prior to being dehydrated sequentially in 35%, 50%, 70%, and 95% ethanol, each for 10 minutes. The samples were then soaked in 100% ethanol for 1 hour with a solvent change every 10 minutes. After dehydration, the samples were dried in a Samdri PVT-3B Critical Point Dryer and coated on specimen stubs with a Hummer VI Sputter Coat Unit. The samples were later observed in a Hitachi 3400-N Variable Pressure Scanning Electron Microscope (VP-SEM) with an accelerating voltage of 15000 V.

CHAPTER 3

Results

3.1. Antifungal activity of AMPs produced by *Conotelus obscurus*

3.1.1. Antifungal activity of larval extracts

3.1.1.1. Growth yield of yeasts mixed with larval extracts

No statistically significant difference in growth yields, based on ODs, was observed when 1 µL (Figure 3.1), 5 µL (Figure 3.2) or 10 µL (Figure 3.3) larval extracts were added to yeast cultures. The data were analyzed with a two tailed t-test ($P < 0.05$). Readings where OD values were larger than 1 were excluded from the analysis to prevent problems of non-linearity. No inhibitory activity of larval extracts was observed in this experiment.

3.1.1.2. Growth curves of yeasts with or without larval extracts

The time course of growth was followed over 4 hours for *Metschnikowia borealis* (Figure 3.4), *Metschnikowia reukaufii* (Figure 3.5), and *Debaryomyces maramus* (Figure 3.6) treated with three concentrations (1, 5, and 10 %) of larval extracts. Addition of extracts appears to cause faster growth than in the controls. The rate of yeast growth was determined by fitting each curve to an exponential model. This result was unexpected as the larval extracts stimulated the growth of yeasts instead of inhibiting the growth.

3.1.1.3. Viable counts

The viability of yeasts following treatment with larval extracts was determined by plating. PBS buffer was used as a control. Fewer colonies were formed compared to the number of yeast cells regardless of treatment (Appendix 1). Viable counts

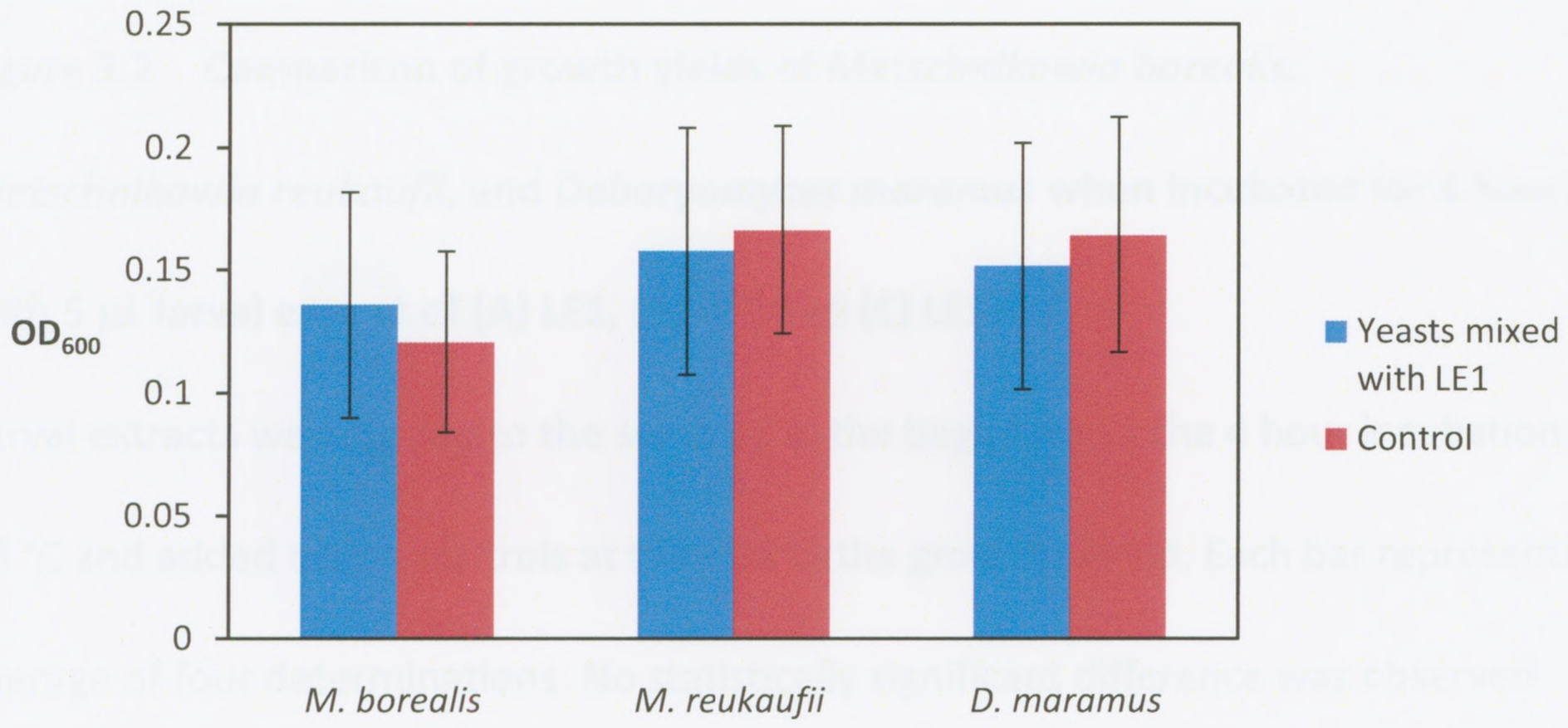
Figure 3.1 Comparison of growth yields of *Metschnikowia borealis*, *Metschnikowia reukaufii*, and *Debaryomyces maramus* when incubated for 4 hours with 1 µL larval extract of (A) LE1, (B) LE2 and (C) LE3.

Larval extracts were added to the samples at the beginning of the 4 hour incubation at 24 °C and added to the controls at the end of the growth period. Each bar represents the average of four determinations. No statistically significant difference was observed between the growth yields of yeasts mixed with larval extract and the controls. Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae. The error bars are standard deviations.

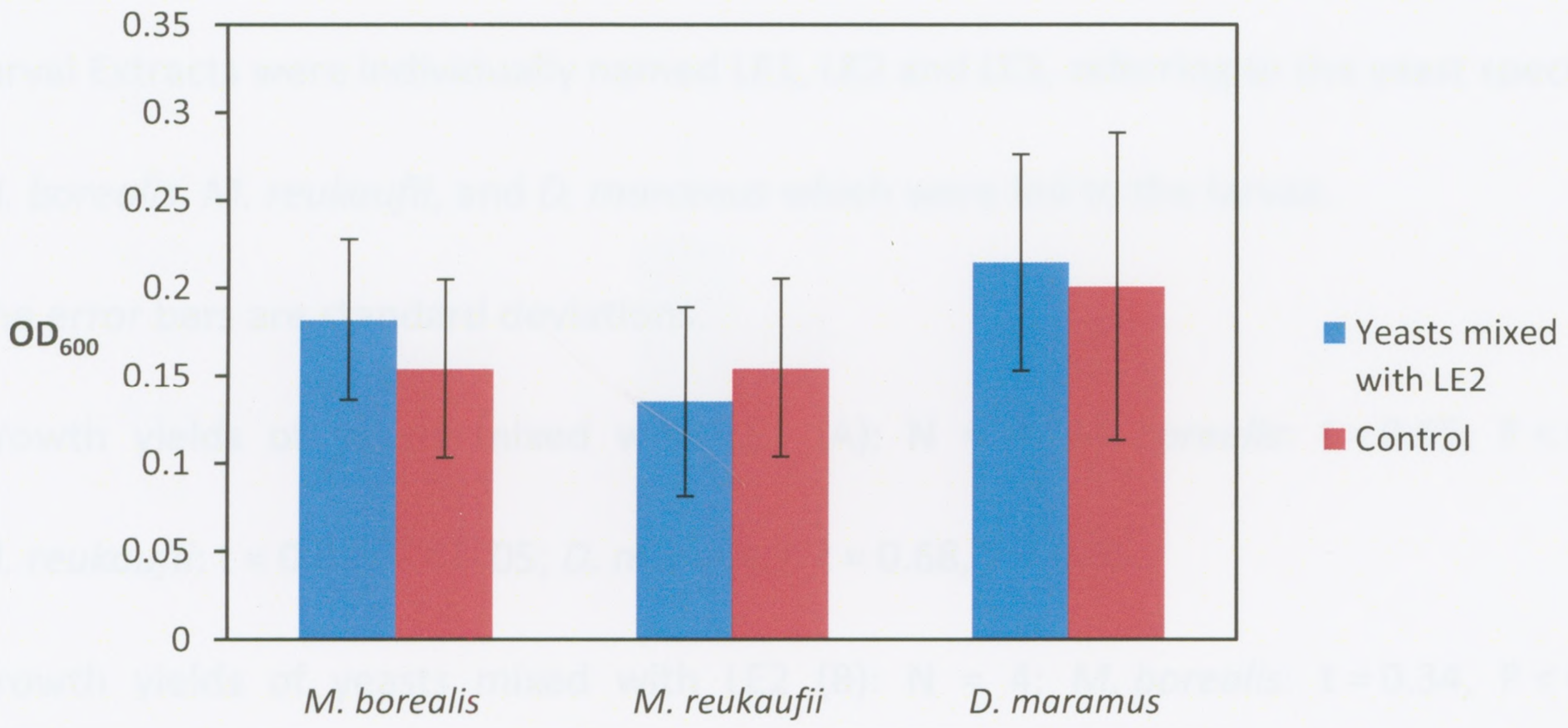
Growth yields of yeasts mixed with LE1 (A): N = 4; *M. borealis*: t = 0.59, $P < 0.05$; *M. reukaufii*: t = 0.79, $P < 0.05$; *D. maramus*: t = 0.75, $P < 0.05$.

Growth yields of yeasts mixed with LE2 (B) N = 4; *M. borealis*: t = 0.45, $P < 0.05$; *M. reukaufii*: t = 0.62, $P < 0.05$; *D. maramus*: t = 0.80, $P < 0.05$.

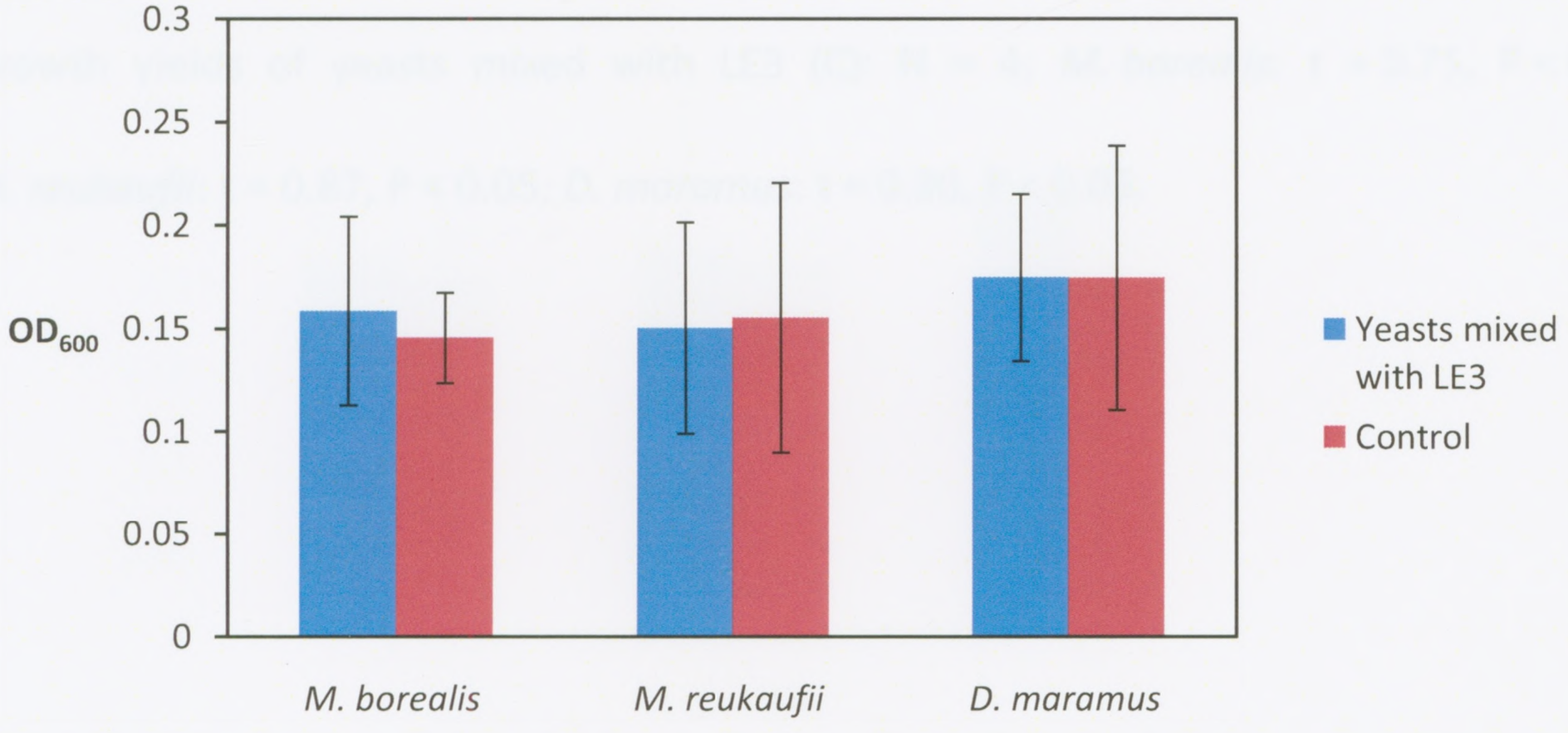
Growth yields of yeasts mixed with LE3 (C): N = 4; *M. borealis*: t = 0.63, $P < 0.05$; *M. reukaufii*: t = 0.91, $P < 0.05$; *D. maramus*: t = 0.99, $P < 0.05$.



(A) Growth yields of yeasts mixed with LE1



(B) Growth yields of yeasts mixed with LE2



(C) Growth yields of yeasts mixed with LE3

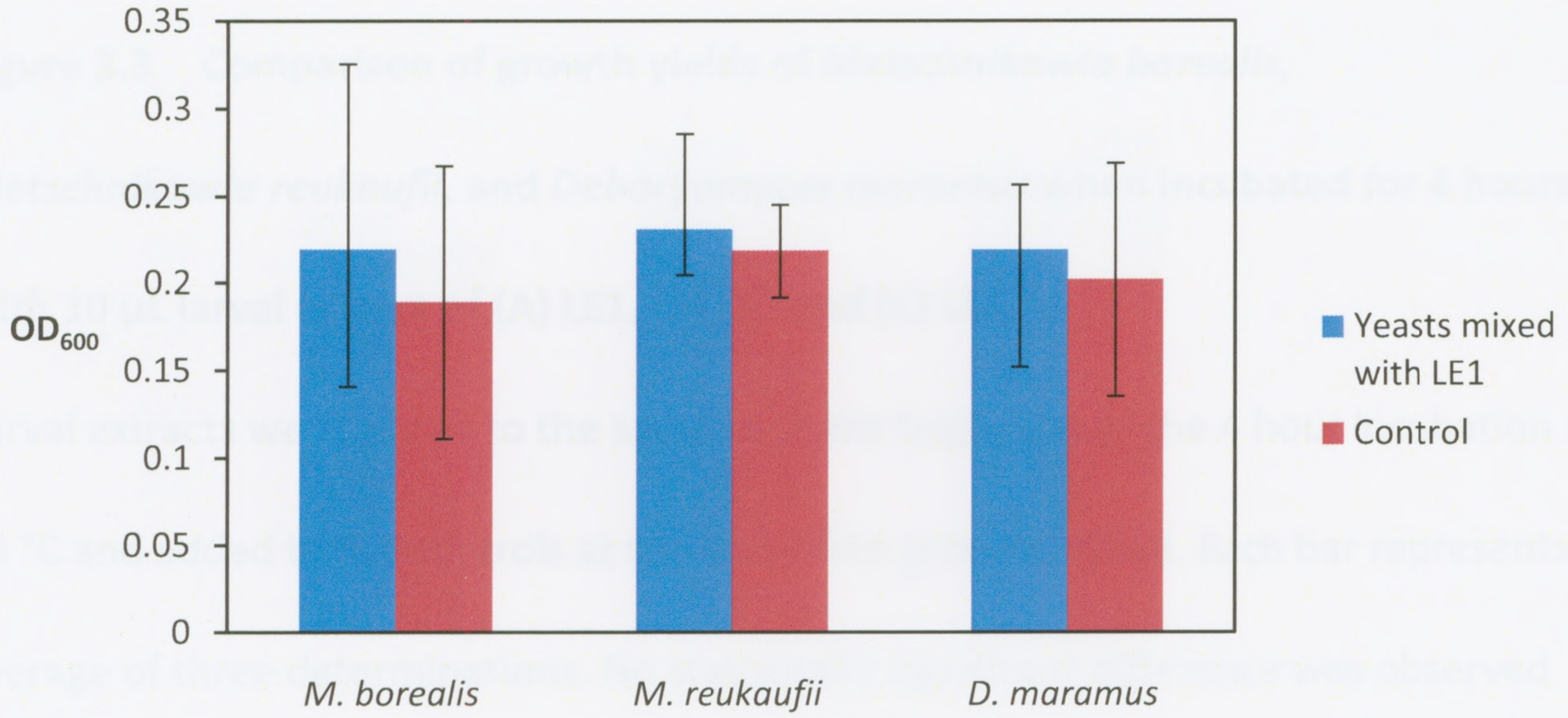
Figure 3.2 Comparison of growth yields of *Metschnikowia borealis*, *Metschnikowia reukaufii*, and *Debaryomyces maramus* when incubated for 4 hours with 5 µL larval extract of (A) LE1, (B) LE2 and (C) LE3.

Larval extracts were added to the samples at the beginning of the 4 hour incubation at 24 °C and added to the controls at the end of the growth period. Each bar represents the average of four determinations. No statistically significant difference was observed between the growth yields of yeasts mixed with larval extract and the controls. Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae. The error bars are standard deviations.

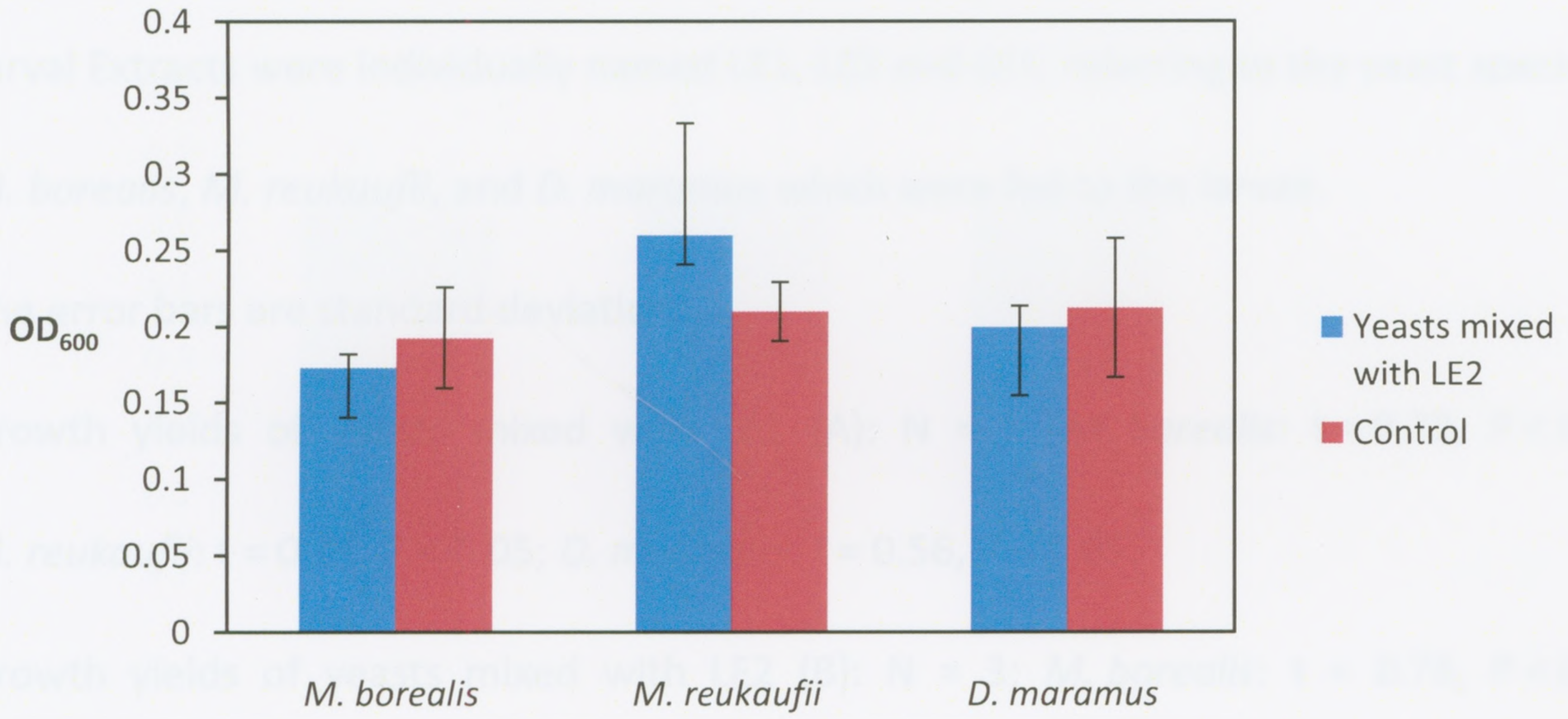
Growth yields of yeasts mixed with LE1 (A): N = 4; *M. borealis*: t = 0.65, P < 0.05; *M. reukaufii*: t = 0.69, P < 0.05; *D. maramus*: t = 0.68, P < 0.05.

Growth yields of yeasts mixed with LE2 (B): N = 4; *M. borealis*: t = 0.34, P < 0.05; *M. reukaufii*: t = 0.27, P < 0.05; *D. maramus*: t = 0.63, P < 0.05.

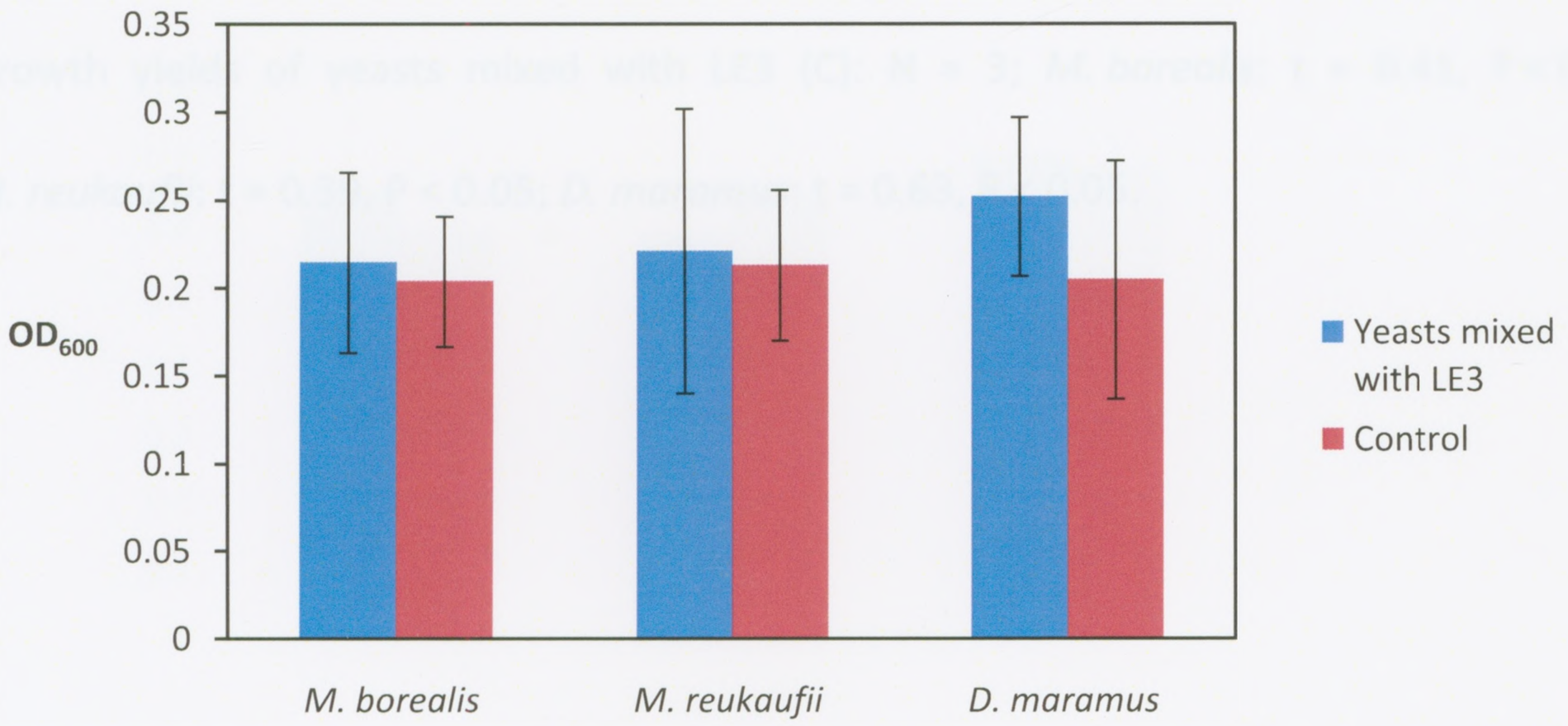
Growth yields of yeasts mixed with LE3 (C): N = 4; *M. borealis*: t = 0.75, P < 0.05; *M. reukaufii*: t = 0.87, P < 0.05; *D. maramus*: t = 0.30, P < 0.05.



(A) Growth yields of yeasts mixed with LE1



(B) Growth yields of yeasts mixed with LE2



(C) Growth yields of yeasts mixed with LE3

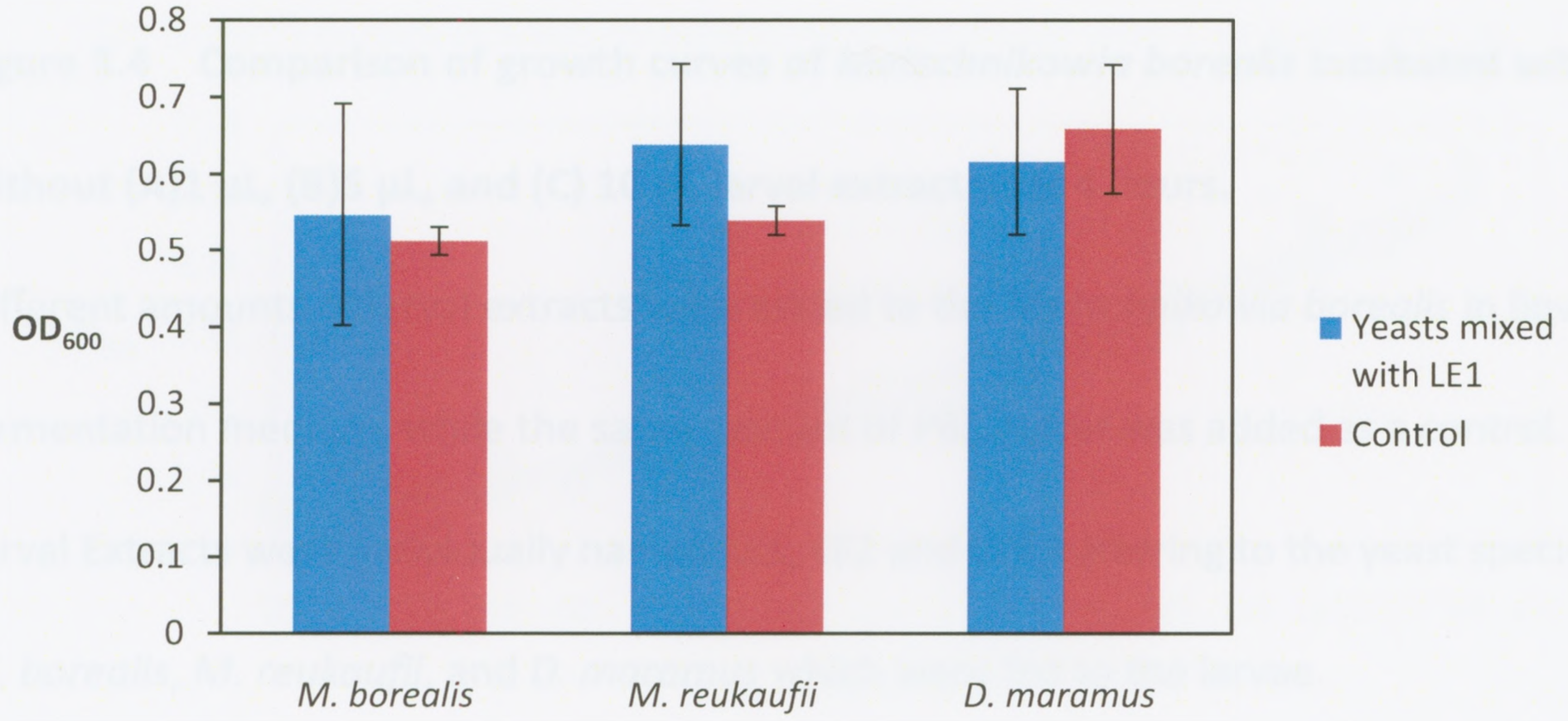
Figure 3.3 Comparison of growth yields of *Metschnikowia borealis*, *Metschnikowia reukaufii*, and *Debaryomyces maramus* when incubated for 4 hours with 10 µL larval extract of (A) LE1, (B) LE2 and (C) LE3.

Larval extracts were added to the samples at the beginning of the 4 hour incubation at 24 °C and added to the controls at the end of the growth period. Each bar represents the average of three determinations. No statistically significant difference was observed between the growth yields of yeasts mixed with larval extract and the controls. Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae. The error bars are standard deviations.

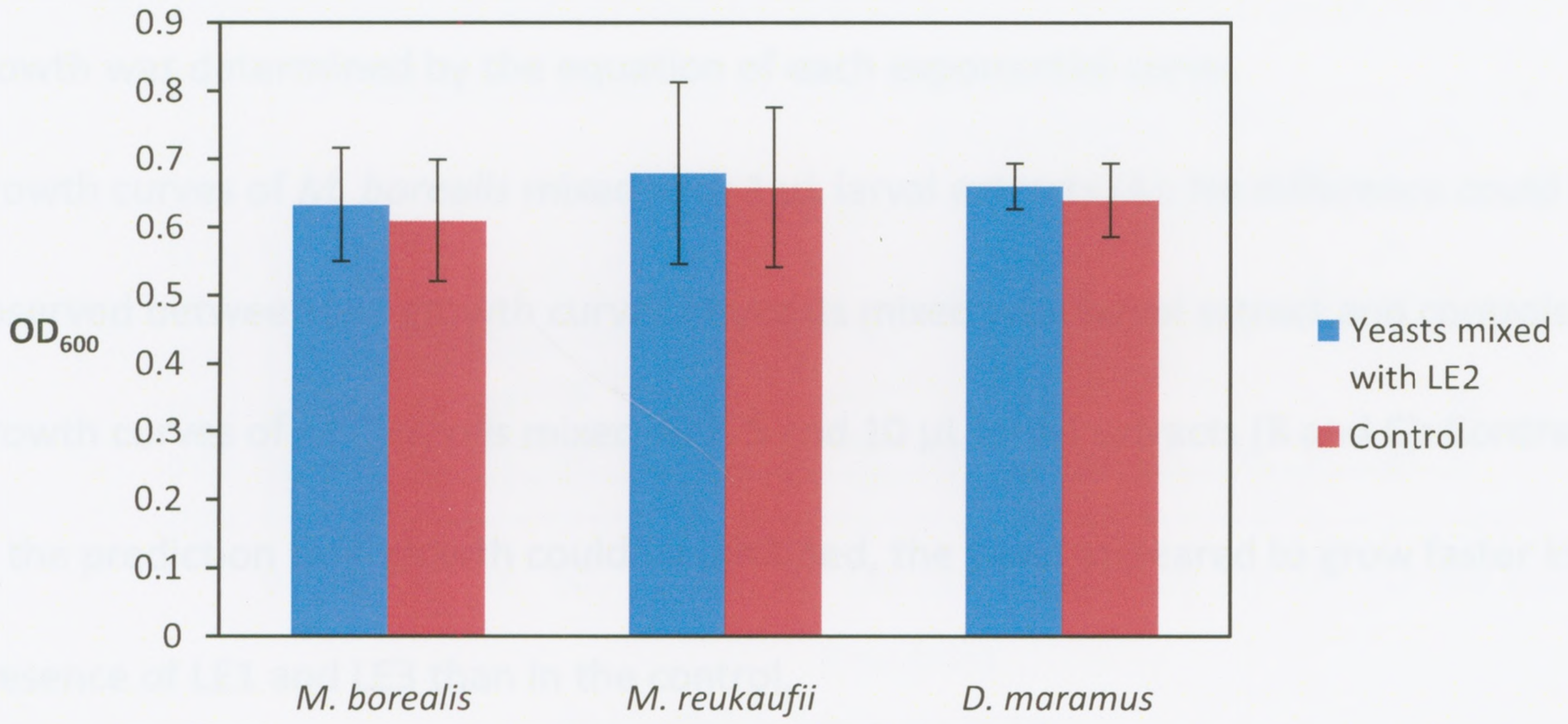
Growth yields of yeasts mixed with LE1 (A): N = 3; *M. borealis*: t = 0.72, P < 0.05; *M. reukaufii*: t = 0.25, P < 0.05; *D. maramus*: t = 0.56, P < 0.05.

Growth yields of yeasts mixed with LE2 (B): N = 3; *M. borealis*: t = 0.76, P < 0.05; *M. reukaufii*: t = 0.85, P < 0.05; *D. maramus*: t = 0.61, P < 0.05.

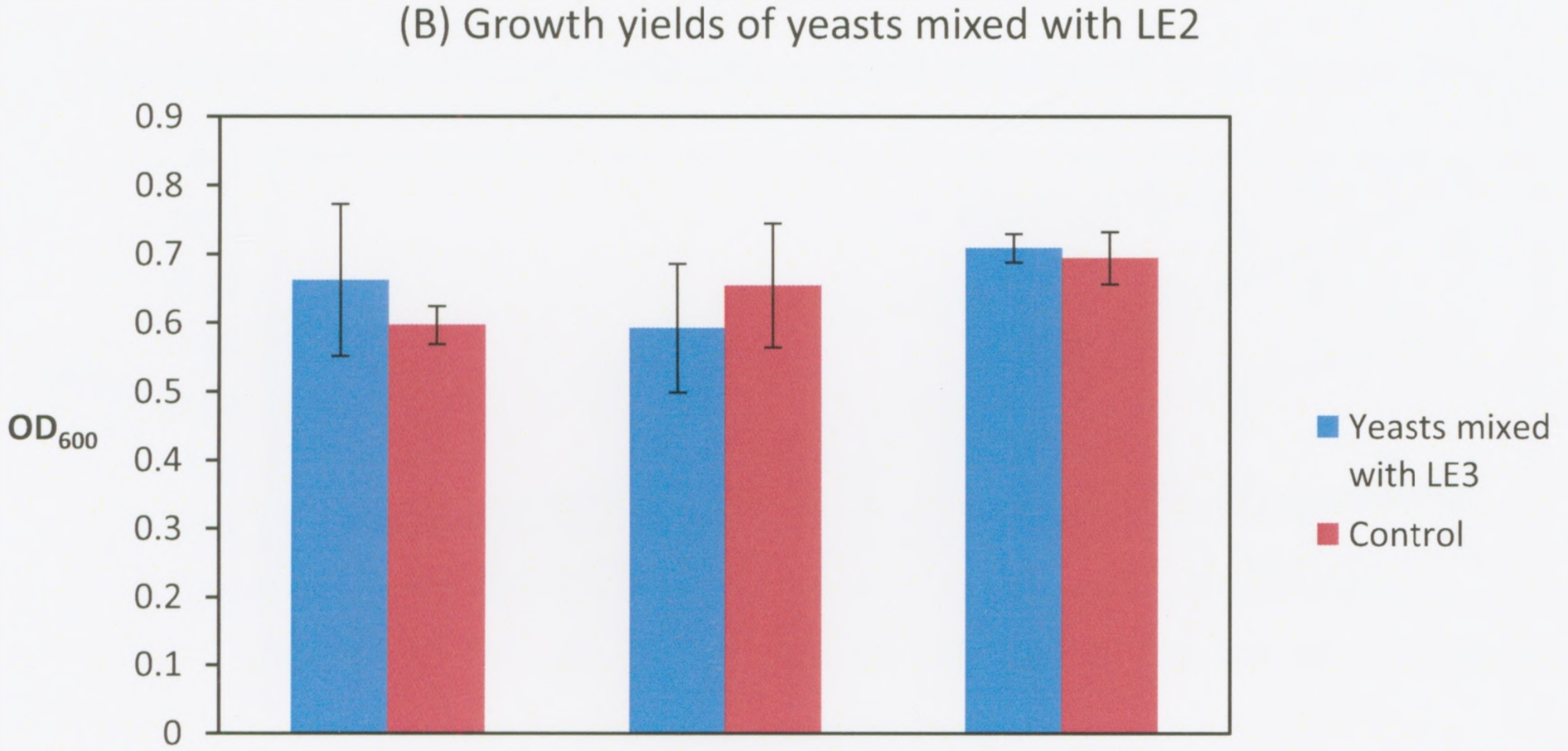
Growth yields of yeasts mixed with LE3 (C): N = 3; *M. borealis*: t = 0.41, P < 0.05; *M. reukaufii*: t = 0.39, P < 0.05; *D. maramus*: t = 0.63, P < 0.05.



(A) Growth yields of yeasts mixed with LE1



(B) Growth yields of yeasts mixed with LE2



(B) Growth yields of yeasts mixed with LE3

Figure 3.4 Comparison of growth curves of *Metschnikowia borealis* incubated with or without (A)1 µL, (B)5 µL, and (C) 10 µL larval extract over 4 hours.

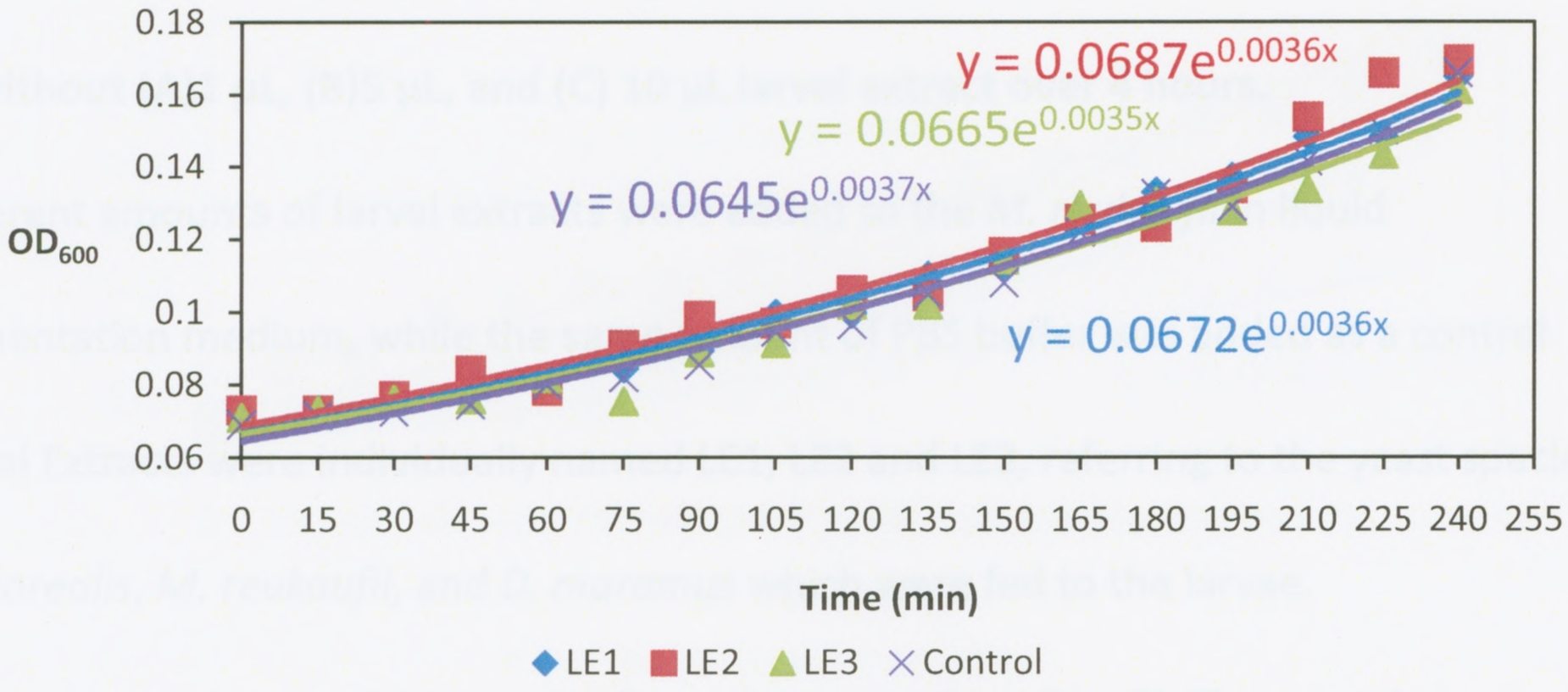
Different amounts of larval extracts were added to the *Metschnikowia borealis* in liquid fermentation medium, while the same amount of PBS buffer was added as a control. Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae.

Each point represents the average of two determinations (N = 2). The rate of yeast growth was determined by the equation of each exponential curve.

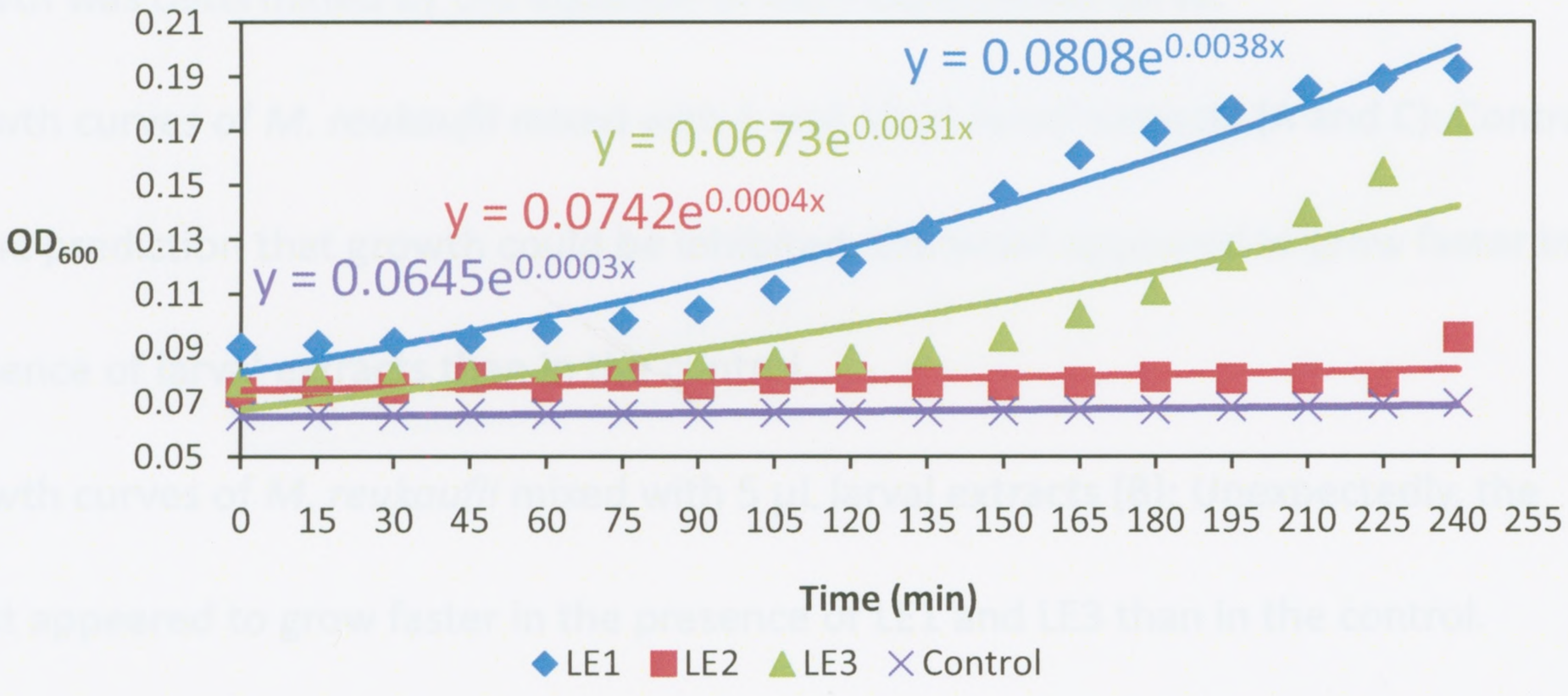
Growth curves of *M. borealis* mixed with 1 µL larval extracts (A): No difference could be observed between the growth curves of yeasts mixed with larval extract and controls.

Growth curves of *M. borealis* mixed with 5 and 10 µL larval extracts (B and C): Contrary to the prediction that growth could be inhibited, the yeast appeared to grow faster in the presence of LE1 and LE3 than in the control.

(A) Growth curves of *Metschnikowia borealis* mixed with 1 µL larval extract



(B) Growth curves of *Metschnikowia borealis* mixed with 5 µL larval extract



(C) Growth curves of *Metschnikowia borealis* mixed with 10 µL larval extract

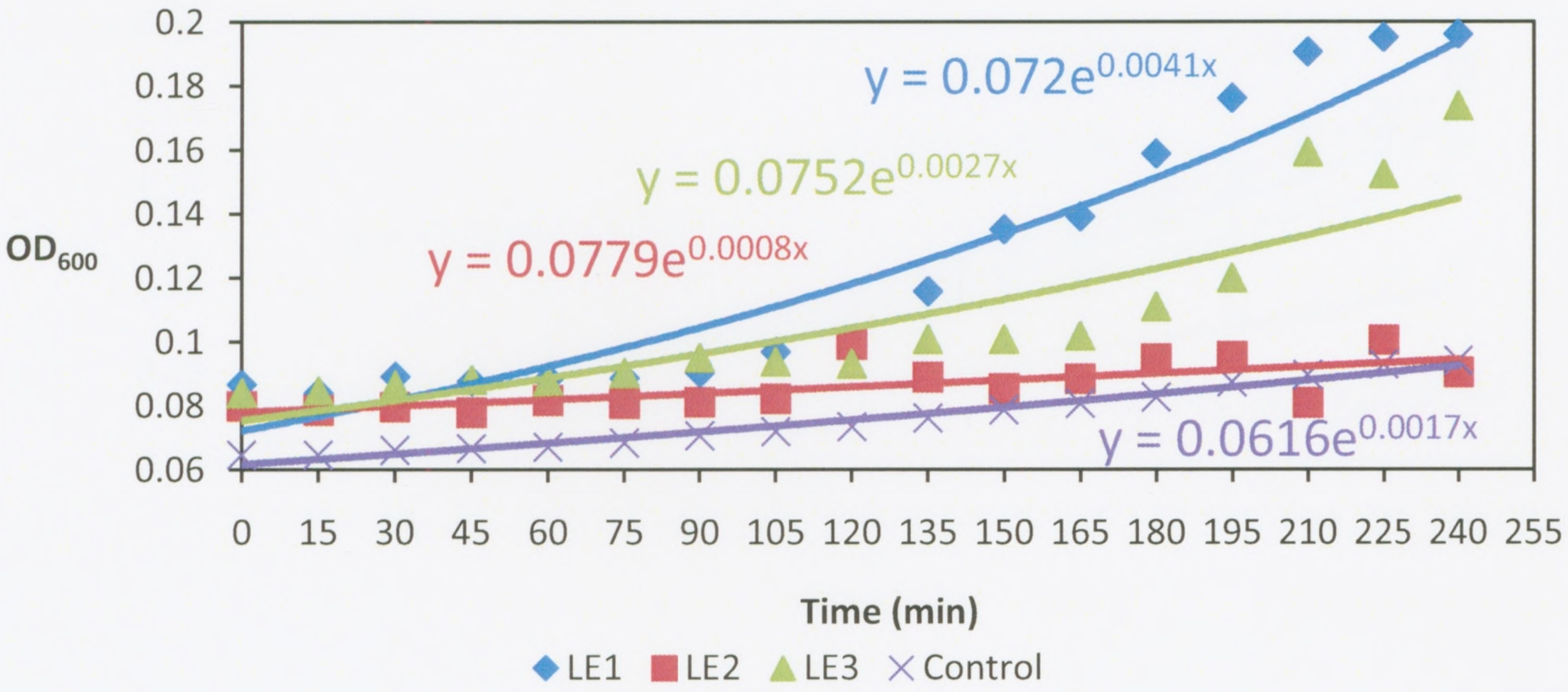


Figure 3.5 Comparison of growth curves of *Metschnikowia reukaufii* incubated with or without (A)1 µL, (B)5 µL, and (C) 10 µL larval extract over 4 hours.

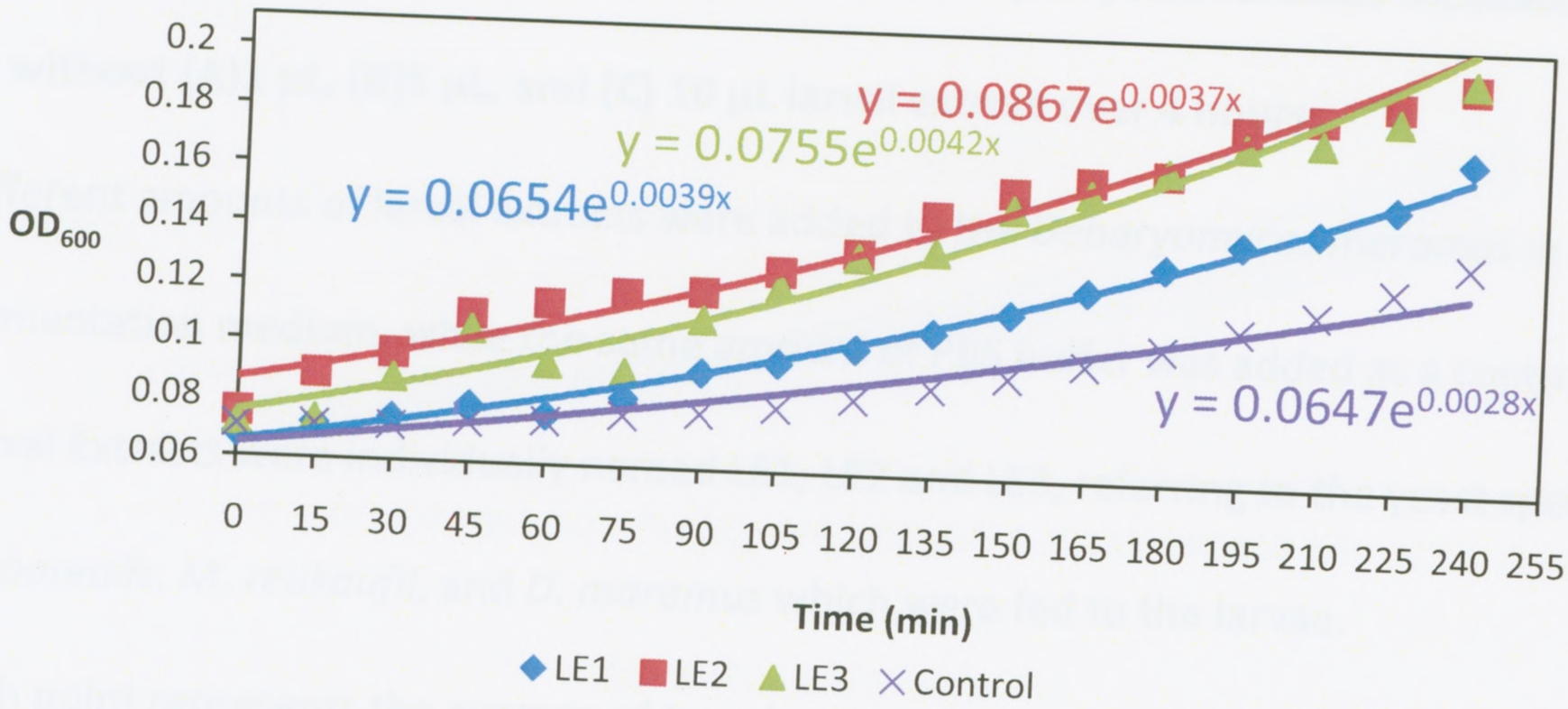
Different amounts of larval extracts were added to the *M. reukaufii* in liquid fermentation medium, while the same amount of PBS buffer was added as a control. Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae.

Each point represents the average of two determinations (N = 2). The rate of yeast growth was determined by the equation of each exponential curve.

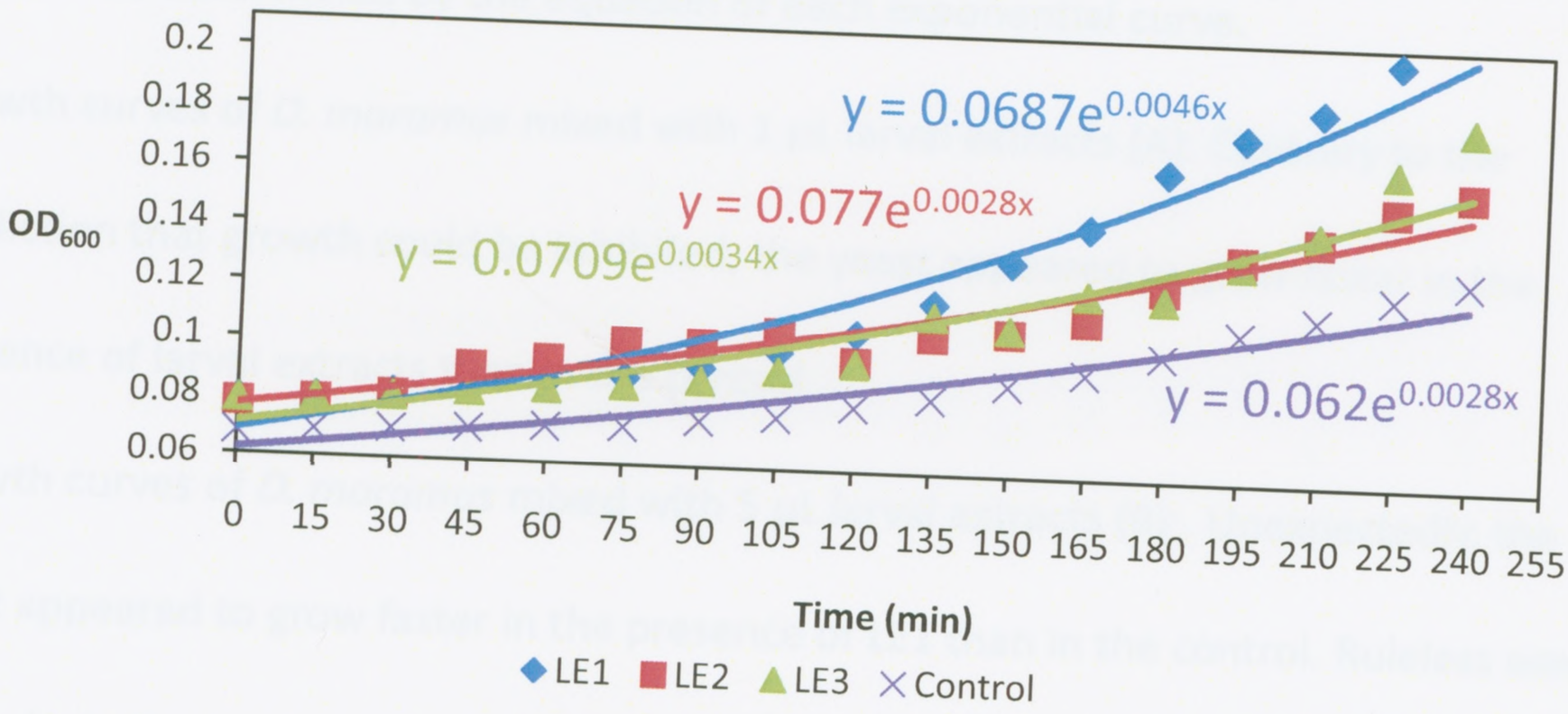
Growth curves of *M. reukaufii* mixed with 1 and 10 µL larval extracts (A and C): Contrary to the prediction that growth could be inhibited, the yeast appeared to grow faster in the presence of larval extracts than in the control.

Growth curves of *M. reukaufii* mixed with 5 µL larval extracts (B): Unexpectedly, the yeast appeared to grow faster in the presence of LE1 and LE3 than in the control.

(A) Growth curves of *Metschnikowia reukaufii* mixed with 1 µL larval extracts



(B) Growth curves of *Metschnikowia reukaufii* mixed with 5 µL larval extracts



(C) Growth curves of *Metschnikowia reukaufii* mixed with 10 µL larval extracts

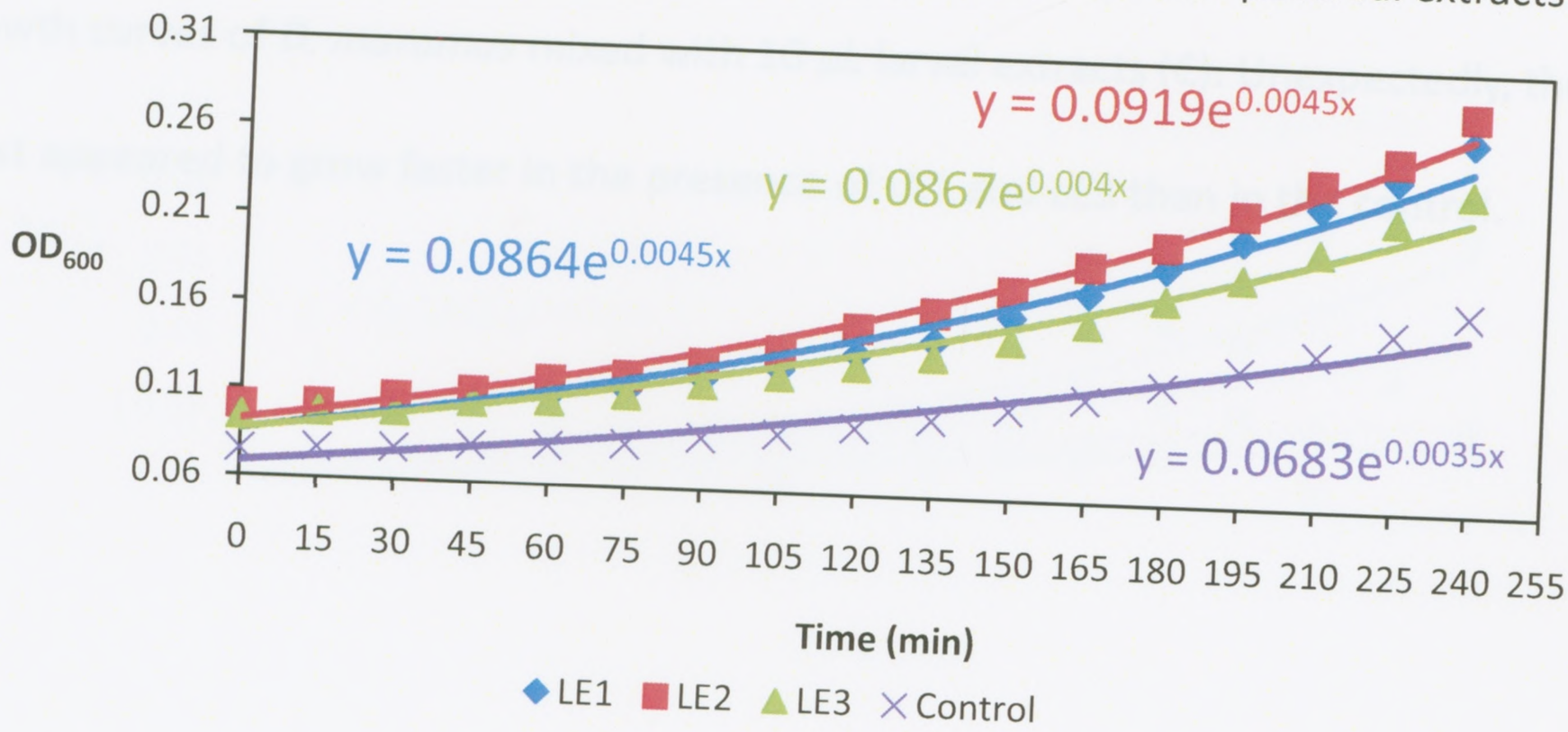


Figure 3.6 Comparison of growth curves of *Debaryomyces maramus* incubated with or without (A)1 µL, (B)5 µL, and (C) 10 µL larval extract over 4 hours.

Different amounts of larval extracts were added to the *Debaryomyces maramus* in liquid fermentation medium, while the same amount of PBS buffer was added as a control. Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae.

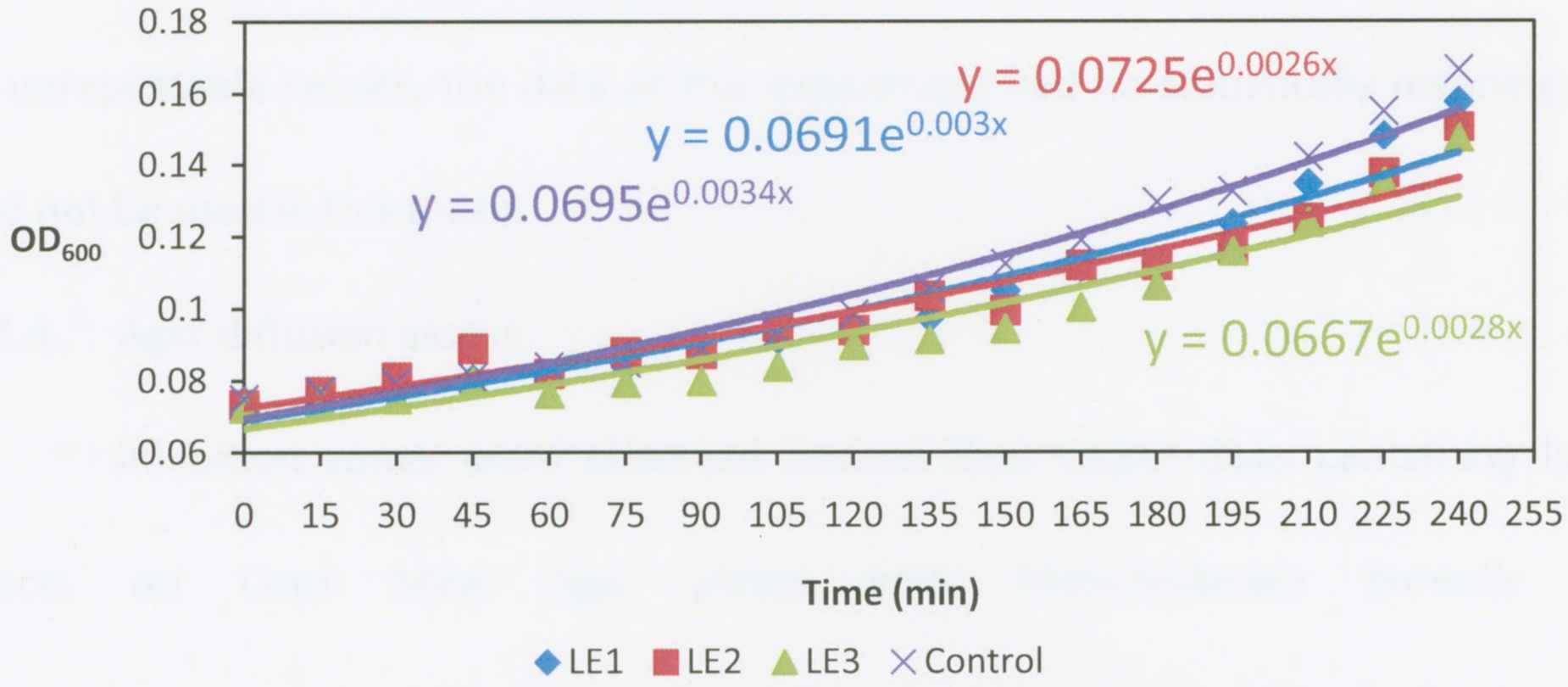
Each point represents the average of two determinations (N = 2). The rate of yeast growth was determined by the equation of each exponential curve.

Growth curves of *D. maramus* mixed with 1 µL larval extracts (A): Contrary to the prediction that growth could be inhibited, the yeast appeared to grow faster in the presence of larval extracts than in the control.

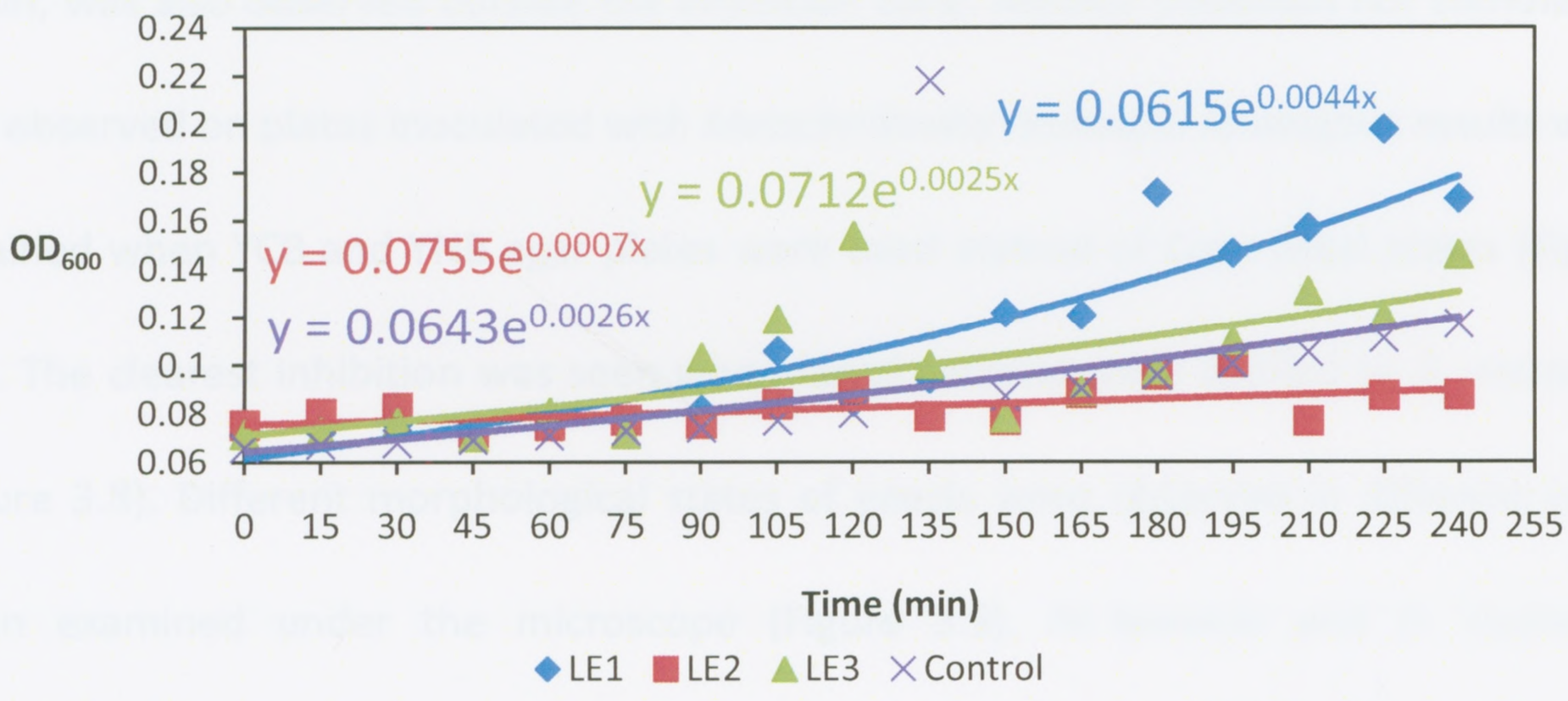
Growth curves of *D. maramus* mixed with 5 µL larval extracts (B):. Unexpectedly, the yeast appeared to grow faster in the presence of LE1 than in the control. Ruleless waves were shown in the growth curves.

Growth curves of *D. maramus* mixed with 10 µL larval extracts (C): Unexpectedly, the yeast appeared to grow faster in the presence of LE1 and LE3 than in the control.

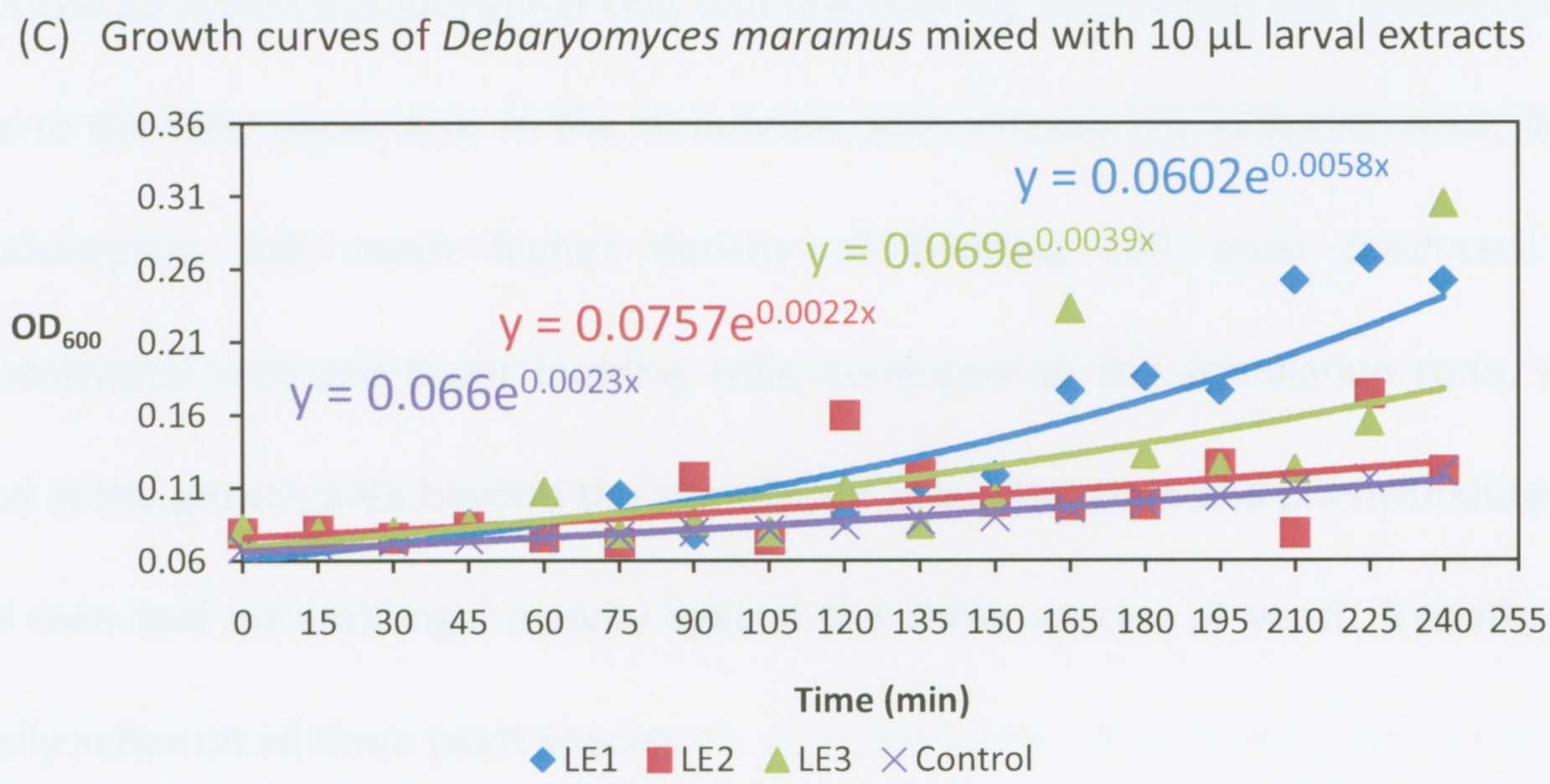
(A) Growth curves of *Debaryomyces maramus* mixed with 1 µL larval extracts



(B) Growth curves of *Debaryomyces maramus* mixed with 5 µL larval extracts



(C) Growth curves of *Debaryomyces maramus* mixed with 10 µL larval extracts



experiment was repeated three times on YM agar plates. However, due to contamination and unrepeatable results, the data of this experiment had no statistically meaning and could not be used in this thesis.

3.1.1.4. Agar diffusion assays

Inhibition zones were observed around filter paper disks containing larval extracts on Corn Meal agar plates with *Metschnikowia borealis* and *Debaryomyces maramus*. A growth stimulation area, where the growth density was higher, was also observed outside the inhibition zone. Neither inhibition nor stimulation was observed on plates inoculated with *Metschnikowia reukaufii*. Analogous results were obtained when YCB and YNB agar plates were used instead of Corn Meal plates (Figure 3.7). The clearest inhibition was seen when larval extracts were applied to *D. maramus* (Figure 3.8). Different morphological states of yeasts were observed in different areas when examined under the microscope (Figure 3.9). *M. borealis* and *D. maramus* produced abundant pseudohyphal cells and few budding cells within the inhibition zone close to the filter paper disk. In the stimulation zone outside the inhibition zone, fewer pseudohyphae but much higher density of budding cell were produced. No pseudohyphal cells and fewer budding cells, compared to the stimulation zone, were found in the growth area beyond the stimulation zone. Aprotinin and phenylthiourea on their own had no antifungal activity against the three species of yeast. Nystatin was equally active on all three yeast species.

3.1.2. Fractionation of antifungal peptides

3.1.2.1. Agar diffusion assays

Six fractions of each larval extract (< 3 kDa, 3 kDa-10 kDa, 10 kDa-30 kDa, 30 kDa-50 kDa, 50 kDa-100 kDa, and > 100 kDa fractions) were collected and tested with agar diffusion assays. Small inhibition zones and stimulation areas were observed on CM agar plates with *Metschnikowia borealis* and *Debaryomyces maramus* with the < 3 kDa fractions and > 100 kDa fractions for all three larval extracts (N = 3). In Figure 3.10, an inhibition zone is visible only for the > 100 kDa fraction of larval extract LE1. The < 3 kDa fractions and > 100 kDa fractions had no detectable activity against the four bacteria tested.

3.1.2.2. Isoelectric focusing of proteins of the > 100 kDa fractions

Proteins of the > 100 kDa fractions of three larval extracts were separated by isoelectric focusing. The protein concentration of the > 100 kDa fractions of three larval extracts was measured based on the equation from the standard curve (Figure 3.11). The average concentrations were 145, 137, and 182 µg/mL respectively for LE1, LE2 and LE3. Equal amounts of the proteins were applied to each lane and the gel is shown in Figure 3.12. The proteins in the > 100 kDa fractions of extracts LE1, LE2, and LE3 had the same distribution. The pI of the main proteins in the fractions ranged from 6.6 to 9.0.

3.1.2.3. Antifungal activity assays of individual proteins

No significant inhibition zone was observed using either the isoelectric focusing gel or protein blotting membrane. Budding cells and pseudohyphae were

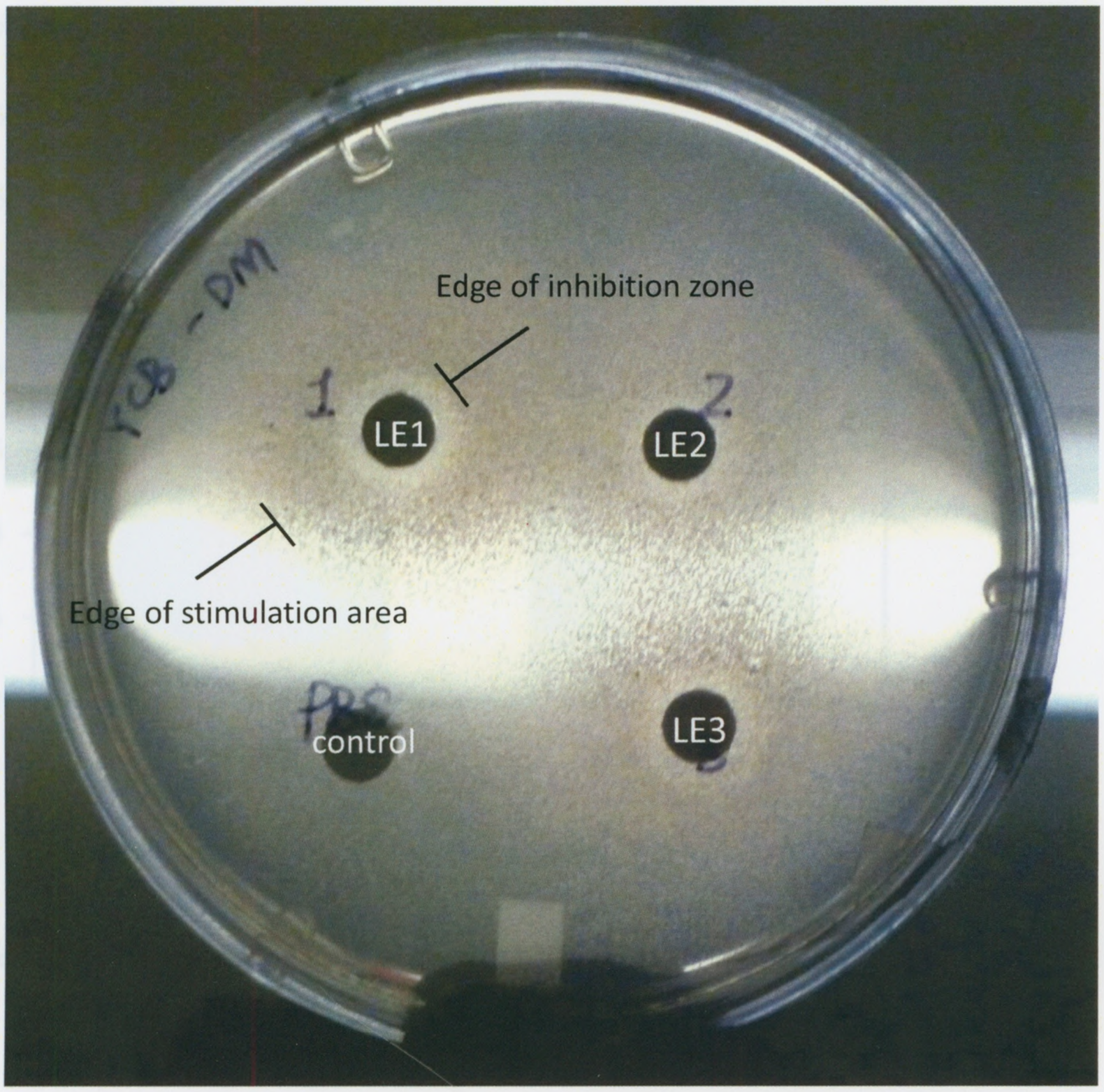


Figure 3.7 Inhibition and stimulation zones of larval extracts LE1, LE2, and LE3 on YCB agar plate with *Debaryomyces maramus*.

Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae.

Less yeast grew around the disk in the inhibition zone. Outside the inhibition zone, a stimulation area appeared where the concentration of yeast growth was higher than in other areas. All three larval extracts produced inhibition and stimulation zones with the yeast *D. maramus*.

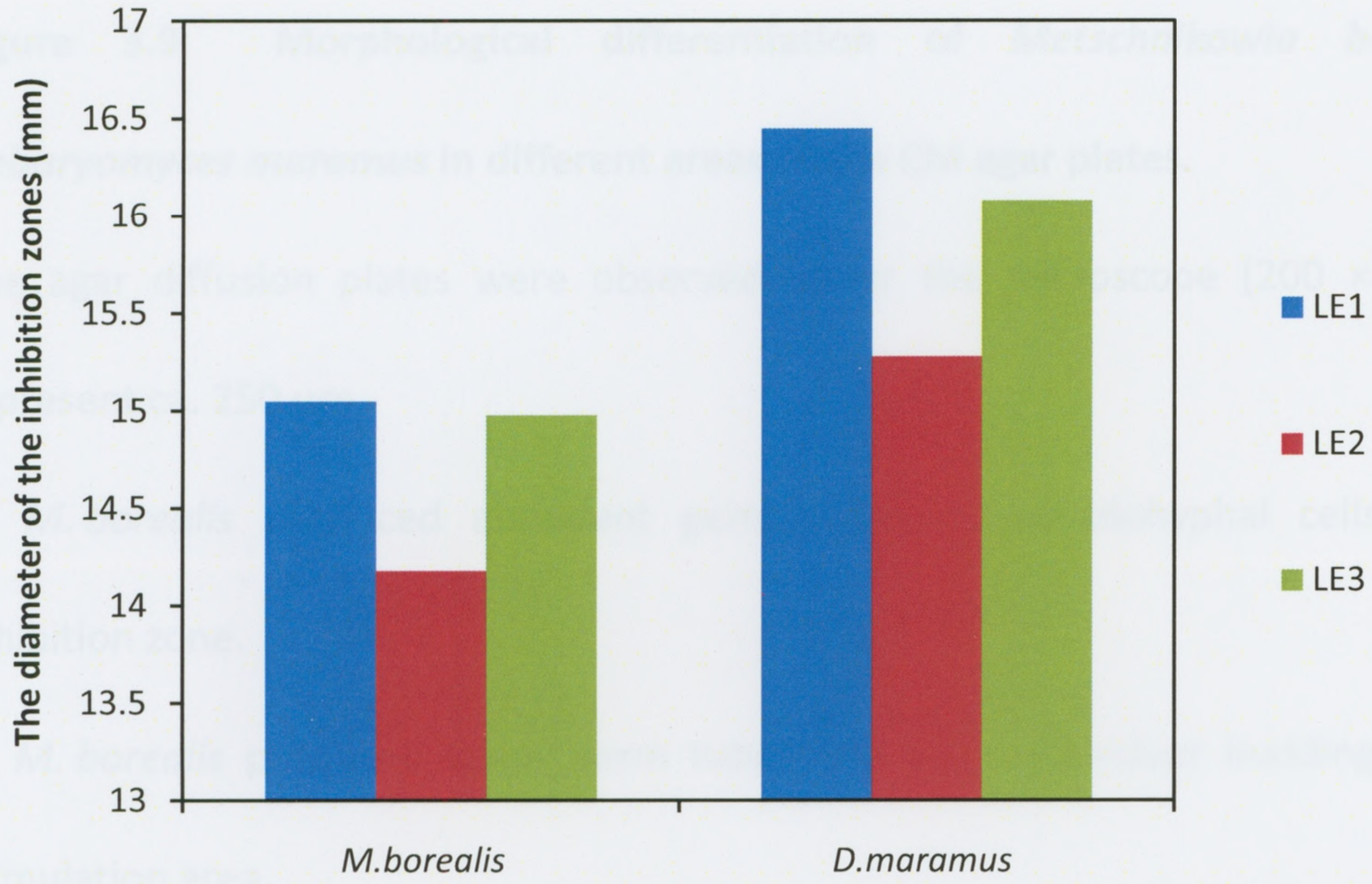


Figure 3.8 Comparison of the diameters of the inhibition zones of *Metschnikowia borealis* and *Debaryomyces maramus* incubated at 24 °C in 48 hours (N = 3).

Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae.

Inhibition zones were observed for all three extracts on plates with *M. borealis* and *D. maramus*. No inhibition was observed for *Metschnikowia reukaufii* or with the PBS buffer controls. The reported diameters included the diameter of the filter paper disk (= 1/4 in).

Figure 3.9 Morphological differentiation of *Metschnikowia borealis* and *Debaryomyces maramus* in different areas of the CM agar plates.

The agar diffusion plates were observed under the microscope (200 ×). Scale bars represent ca. 250 µm.

A: *M. borealis* produced abundant germ tubes or pseudohyphal cells within the inhibition zone.

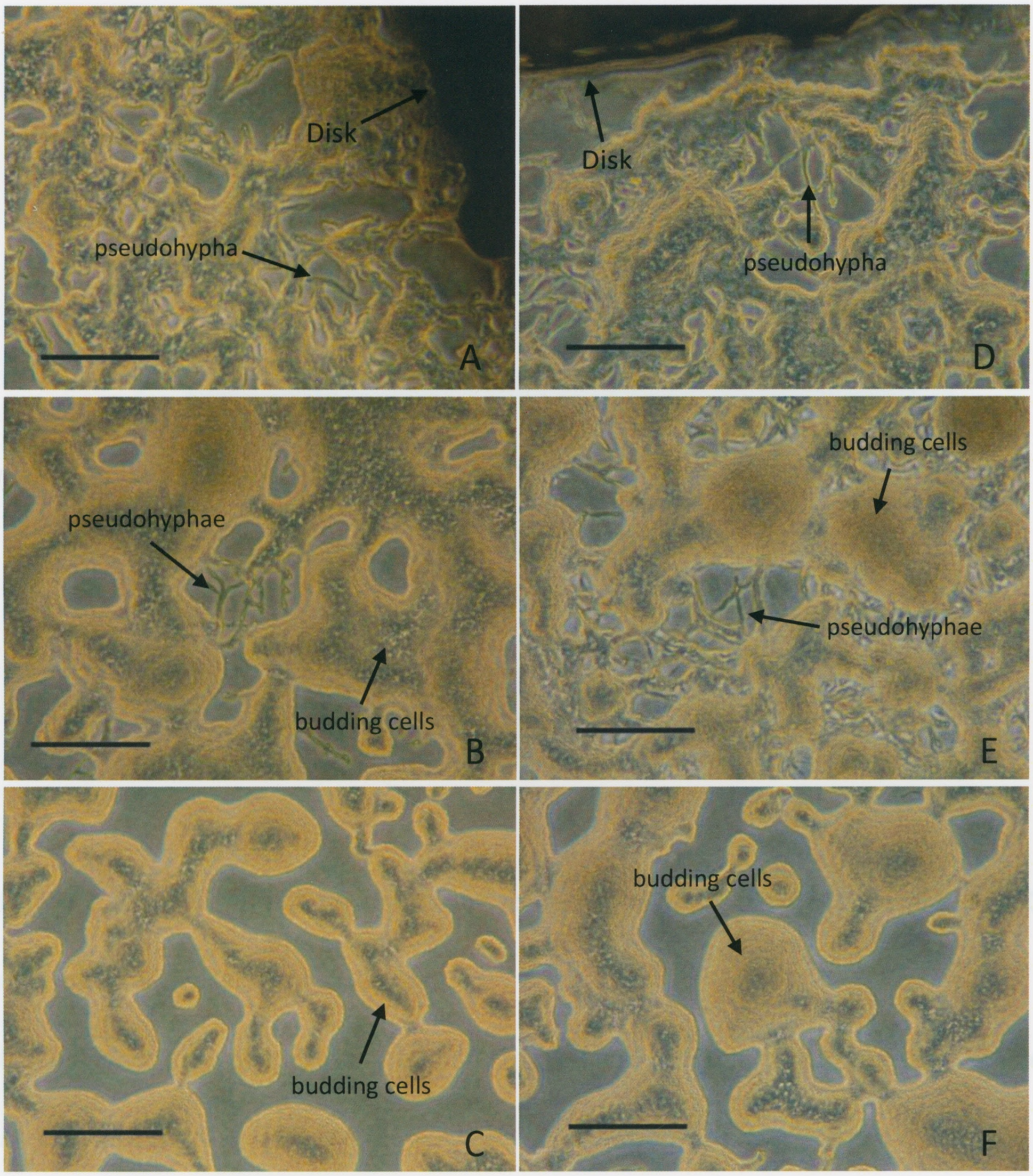
B: *M. borealis* produced fewer germ tubes and more abundant budding cells at the stimulation area.

C: *M. borealis* grew only as budding cells in the area outside of stimulation zone. The cells were less numerous and separated by larger gaps between colonies compared to the stimulation area.

D: *D. maramus* also produced abundant pseudohyphal cells in the inhibition zone.

E: Fewer pseudohyphal cells produced by *D. maramus* at the stimulation area. However, thicker layers of budding cells were shown at this area.

F: Similar to *M. borealis*, only budding cells of *D. maramus* appeared in the area outside of the stimulation area. The concentration of the cells in this area was lower than in the stimulation area.



Metschnikowia borealis *Debaryomyces maramus*

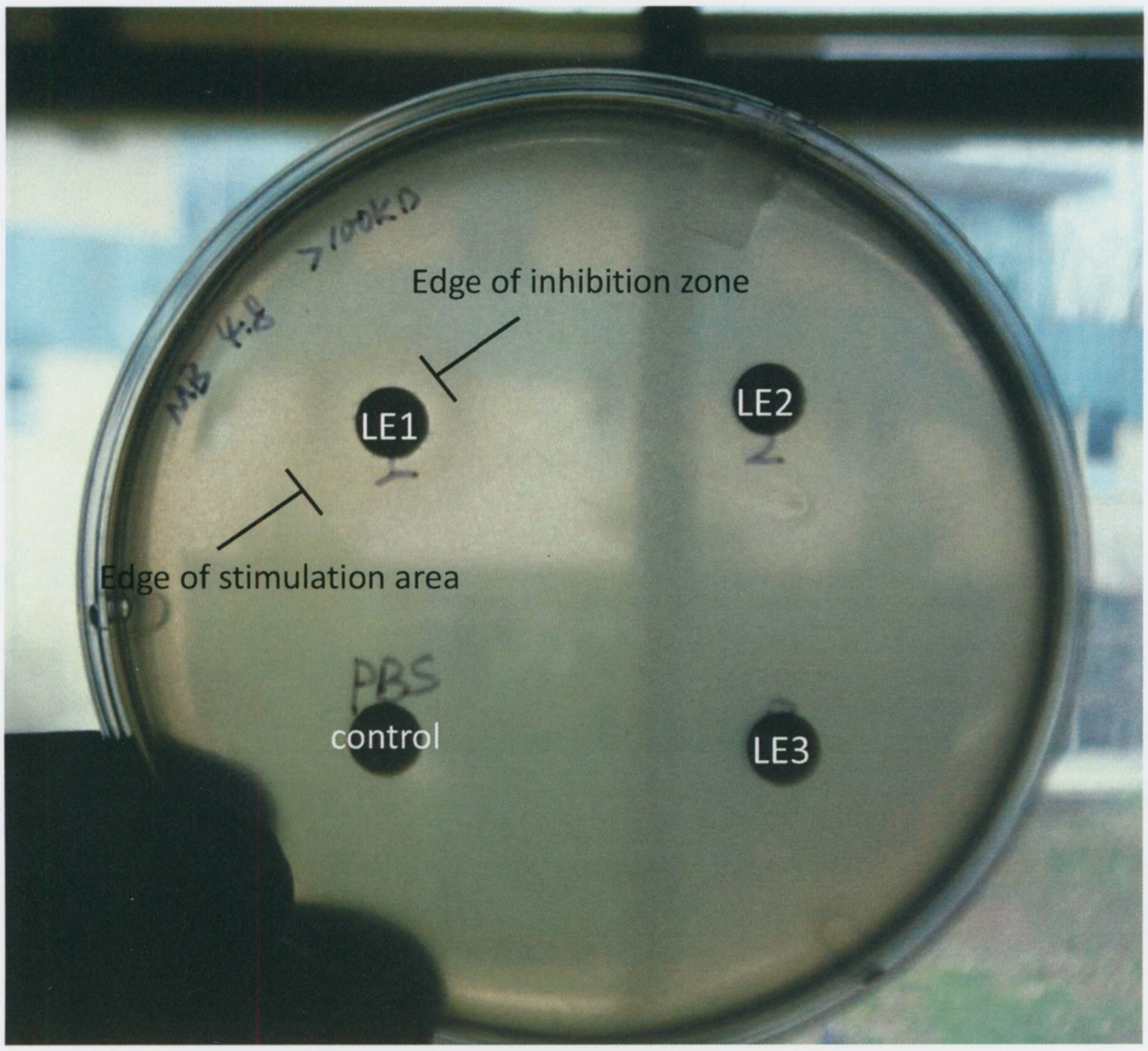


Figure 3.10 Inhibition and stimulation zones of the > 100 kDa fractions of LE1 on CM agar plate with *Metschnikowia borealis*.

Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae.

An inhibition zone is visible only for the > 100 kDa fraction of larval extract LE1 in this figure.

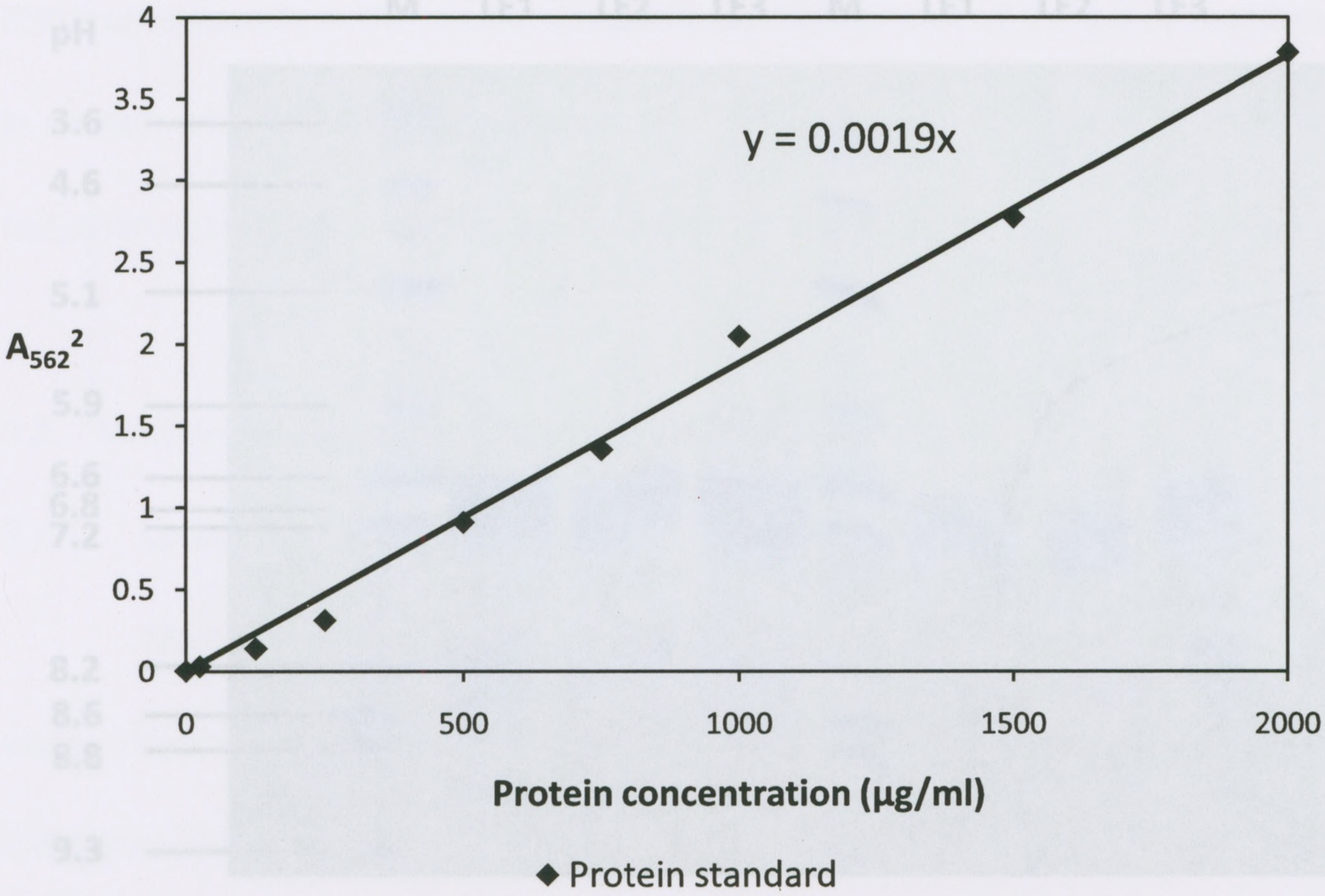


Figure 3.11 Standard curve of protein concentration assay of >100 kDa fraction of larval extracts made by nine different concentrations of protein standard.

The absorbance was measured at 562 nm with VERSAmax microtitre plate reader.

The standard curve was fit to a linear equation after squaring the absorbance values,

such that the concentration of protein could be estimated with the following equation:

$$\text{Concentration} = \frac{\text{Absorbance}^2}{0.0019}$$

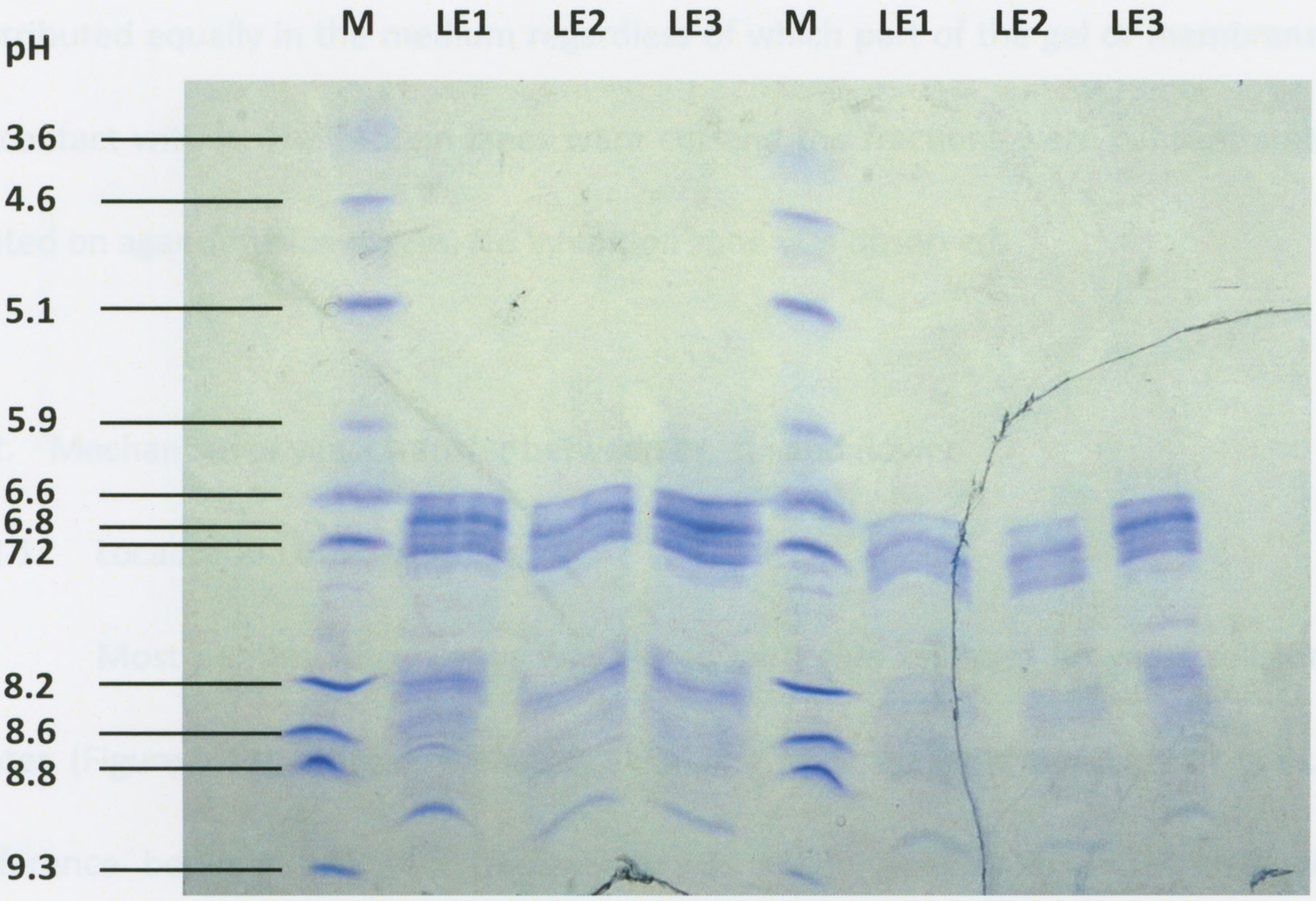


Figure 3.12 Proteins of > 100 kDa fractions of larval extracts LE1, LE2, and LE3 separated by isoelectric focusing.

Larval Extracts were individually named LE1, LE2 and LE3, referring to the yeast species *M. borealis*, *M. reukaufii*, and *D. maramus* which were fed to the larvae.

Lanes of fractions LE1, LE2 and LE3 on the left each contained ca. 35 µg proteins in 15 µL.

The lanes on the right each contained ca. 25 µg proteins in 10 µL. The proteins in the > 100 kDa fractions of extracts LE1, LE2, and LE3 had the same distribution. The pI of the main proteins in the fractions ranged from 6.6 to 9.0.

distributed equally in the medium regardless of which part of the gel or membrane was in contact with it. The protein lanes were cut and the fractions were concentrated and tested on agar diffusion assays. No inhibition zone was observed.

3.2. Mechanism of yeast transfer between beetle and flower

3.2.1. Localization of yeasts

Most beetles whose anus was closed with glue released no yeast cells on the plates (Figure 3.13). Unsealed beetles invariably released large numbers of cells. The difference between the two treatments was highly significant as assessed with a one-way analysis of variance ($F=41.8$, $P=1.7 \times 10^{-6}$). The beetles therefore appear to carry the yeasts in their digestive tract and release them during excretion.

3.2.2. Microorganisms in bindweed seeds

The presence of microorganisms in bindweed seeds was assessed in both liquid and solid media. However, no microorganisms were found in the seeds, indicating that the yeasts are not transmitted through the seeds.

3.2.3. Scanning electron microscopy of inner corolla of bindweeds

Less than ten yeast cells were found in each piece of inner corolla sample (1 cm x 0.8 cm) of young bindweed flowers when examined by scanning electron microscopy. A yeast cell with a bud scar was found on the flower tissue in Figure 3.14.

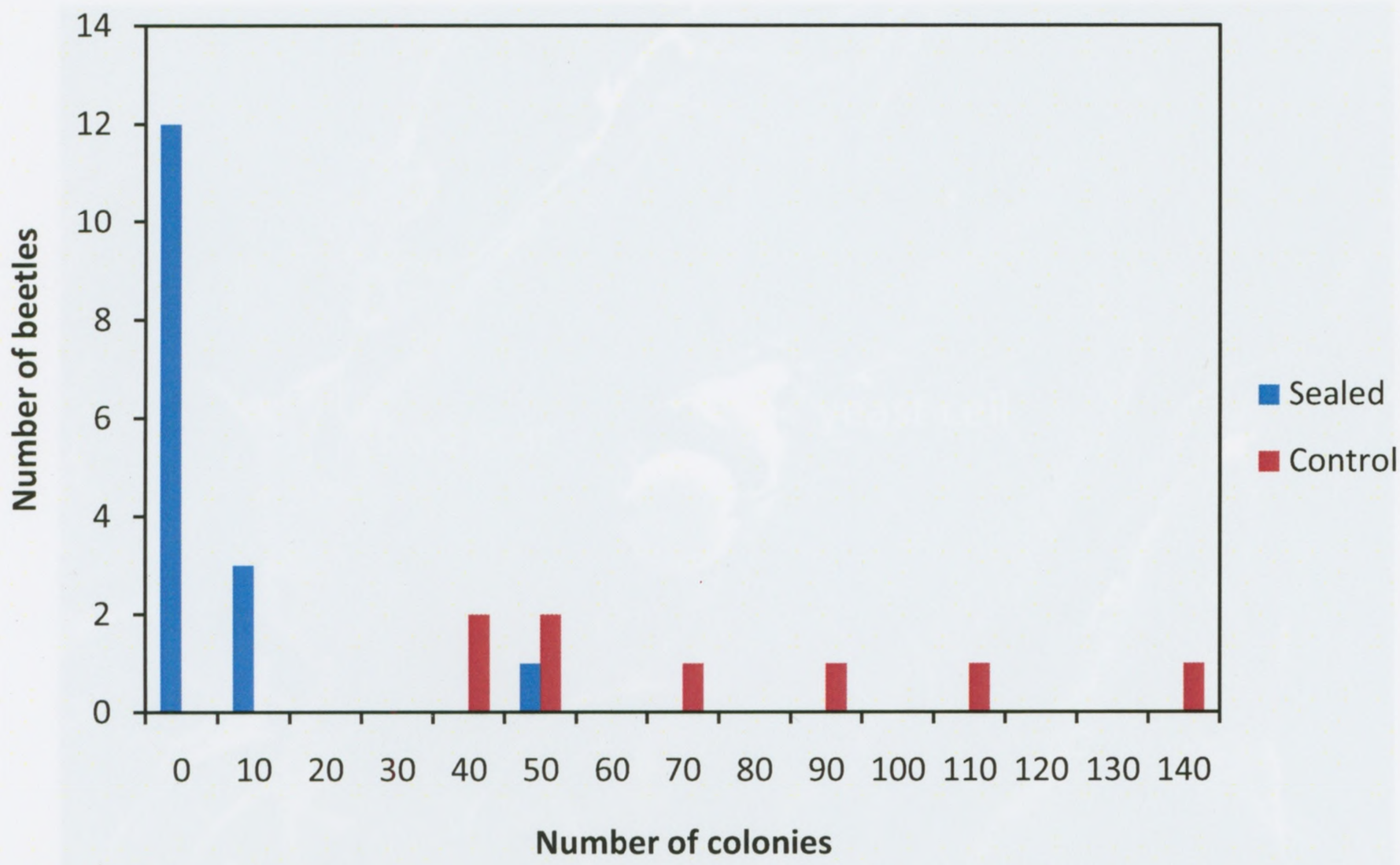


Figure 3.13 Effect of sealing the anus of *Conotelus* beetles on the release of *Metschnikowia borealis* cells on agar plates.

A one-way ANOVA showed that the difference is significant. ($F=41.8$, $P=1.7 \times 10^{-6}$).

A similar figure has been presented by Berkers (2010), as this work was performed jointly.

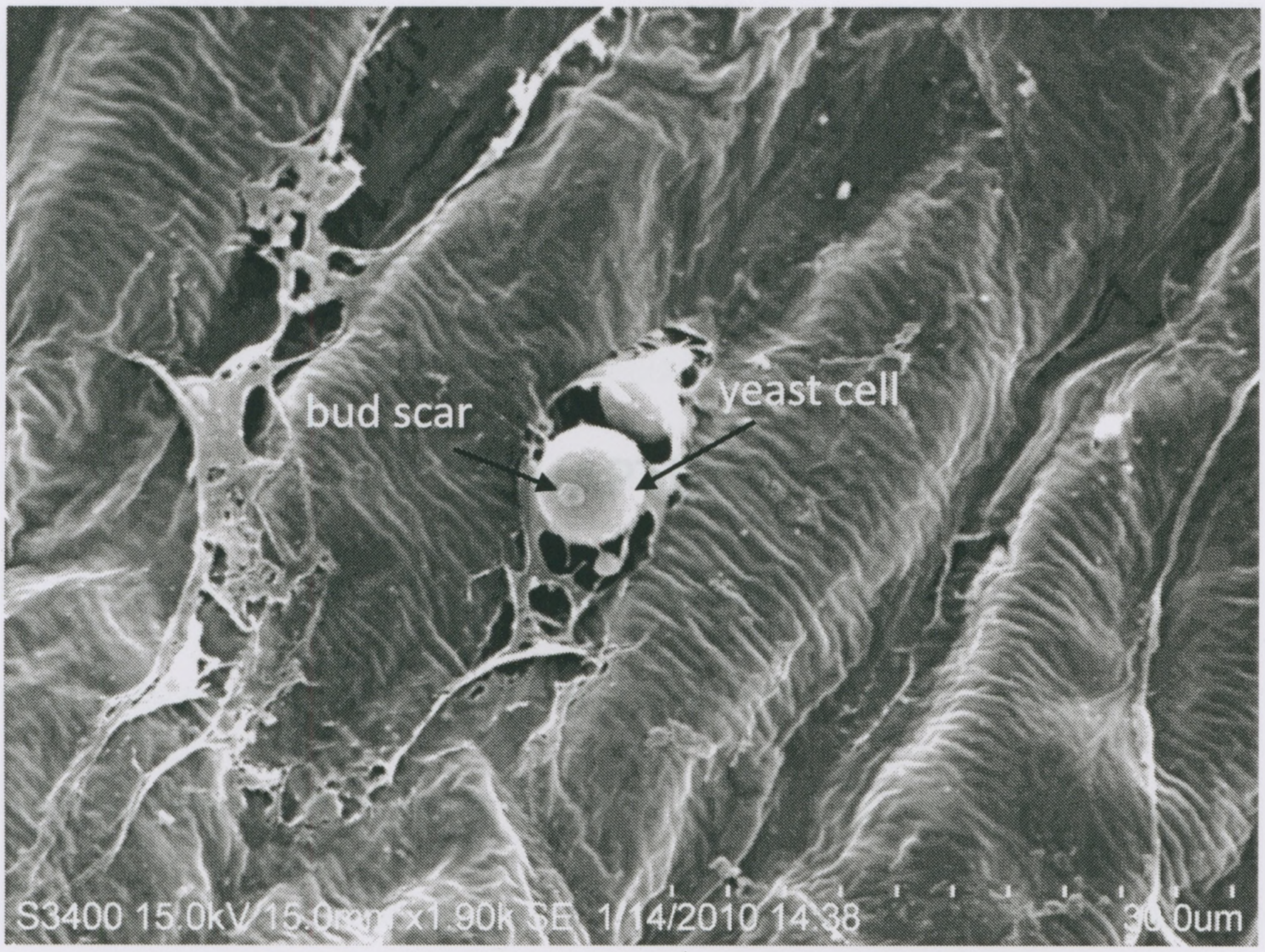


Figure 3.14 A yeast cell on the surface of inner corolla of bindweed flower examined by scanning electron microscope.

The sample was observed in a Hitachi 3400-N Variable Pressure Scanning Electron Microscope (VP-SEM) with an accelerating voltage of 15000 V.

CHAPTER 4

Discussion

4.1. Antimicrobial peptides of *Conotelus obscurus*

I hypothesized that the nitidulid beetle *Conotelus obscurus* produced specific antimicrobial peptides which select in favor of certain *Metschnikowia* species and that they could be induced by other yeast species. However, the results of the antifungal activity assays did not support these hypotheses. Instead, the fact that all of the larval extracts of *C. obscurus* produced both inhibition and stimulation zones when applied to the three yeasts tested suggests that the interactions between *Conotelus* beetles and their symbiotic yeasts are much more complex than anticipated.

4.1.1. Effects of larval extracts of *Conotelus obscurus* to different species of yeast

To test the antifungal activity of larval extracts of *C. obscurus*, densitometric assays, viable counts, and agar diffusion assays were conducted. No statistically significant difference in growth yields was observed (Figure 3.1, Figure 3.2, and Figure 3.3), which means the larval extracts might not affect the growth of yeasts under the conditions used in this assay.

Later, the densitometric assays were refined and the growth curves of yeasts with or without larval extract in four hours were determined. If the larval extracts had antifungal activity against the yeasts, the mixtures of yeasts with larval extracts should grow more slowly than the control, which was a mixture of yeasts with PBS buffer. Three different concentrations of three larval extracts (LE1, LE2, and LE3) were tested. The rates of yeast growth were determined as the slope of each exponential curve (Figure 3.4, Figure 3.5, and Figure 3.6). Unexpectedly, the extracts had no detectable inhibitory

effect on the yeasts and may even have stimulated their growth. It may be that the inhibitory effects of the extracts are overshadowed by their ability of serve as yeast nutrients.

The numbers of colonies formed by the yeasts and the number of cells were compared after treatment with larval extracts. The viable count was always lower than the total cell count irrespective of treatment, but a clear inhibition caused by larval extracts was not evident.

An inhibition zone around the filter paper disk containing larval extracts and a stimulation zone outside the inhibition zone were observed on the agar plates with *Metschnikowia borealis* and *Debaryomyces maramus* but not with *Metschnikowia reukaufii*. Both *M. borealis* and *D. maramus* produced abundant pseudohyphal cells within the inhibition zone. At the stimulation zone, there were fewer pseudohyphal cells but abundant budding cells. *M. borealis* and *D. maramus* are known to produce pseudohyphae under some conditions (Miller and Phaff, 1998; Nakase et al., 1998). Pseudohyphae are formed from budding yeast cells that remain attached to each other (Kron & Cow, 1995). Yeasts respond to stresses such as heat, osmotic pressure, salt, and starvation (Tanghe et al., 2006) by undergoing morphological changes, including pseudohyphal cell formation. For example, the transcription pathway of *Saccharomyces cerevisiae* can switch and produce pseudohyphae in response to nutrient limitation (Winderickx et al., 2003). The production of pseudohyphae by *M. borealis* and *D. maramus* suggested that the yeasts were experiencing stress at the inhibition zone. The

absence of pseudohyphae further away supported that conclusion. The results of the agar diffusion assays also indicated that some components stimulate the growth of *M. borealis* and *D. maramus.* I repeated these assays on YNB agar plates (lacking carbon source) and YCB agar plates (lacking nitrogen source) in an attempt to find out whether the larval extracts acted as carbon or nitrogen source. The stimulation areas appeared on both kinds of plates, which demonstrates that the larval extracts supplied both carbon and nitrogen nutrients. As all three larval extracts showed similar inhibition and stimulation activities, I must reject the hypothesis that the AMPs were induced by yeasts that are not *Conotelus obscurus* symbionts.

4.1.2. Fractionation assays of larval extracts of *Conotelus obscurus*

A larval extract is likely to contain many different components, for example, inorganic ions, free amino acids, organic acids, lipids, amino sugars, and carbohydrates (Wyatt, 1961). Usually, only proteins, lipids and some polysaccharides have very high molecular weights which are larger than 100 kDa. In the agar diffusion assays, the controls showed that aprotinin and phenylthiourea had no antifungal activity against the three species of yeast. Nystatin was equally active on all three species of yeast, including *M. reukaufii*, which ruled out the possibility that nystatin was carried over from the larval rearing plates and responsible for the inhibition. The lipids (chloroform extracts) of larval extracts showed no antifungal activity either, which means that the components responsible for yeast growth inhibition are likely to be peptides. In other words, the antifungal activity of the larval extracts to *M. borealis* and *D. maramus* may be due to

the presence of AMPs.

To find out about the nature of the inhibitory components of the larval extracts, six fractions containing different sizes of molecules were tested by agar diffusion. As both small inhibition zones and stimulation zones were found around the filter paper disks with the < 3 kDa fractions and > 100 kDa fractions, the components which have antifungal activity would seem to be peptides that are smaller than 3 kDa or large proteins that are larger than 100 kDa. The stimulatory components are probably small molecules such as carbohydrates or free amino acids and large molecules such as proteins or even lipids, as most yeasts found in *Conotelus* spp. are capable of assimilating lipids (Lachance et al., 2001a).

The proteins of all three fractions (LE1, LE2, and LE3) had similar bands in the isoelectric focusing gel, which suggested that the AMPs induced by three different yeasts may be the same overall. However, no inhibition or stimulation was observed in the antifungal activity assays of individual proteins. The absence of inhibition or stimulation activity of individual proteins could have been due to their low concentration. However, no inhibition or stimulation was observed in bands obtained from five-fold concentrated fractions. The absence of activity is therefore not likely to be due to a low concentration of peptides. It is possible that the AMPs do not work separately and that they have to combine with each other to achieve the antifungal activity observed with the whole > 100 kDa fractions. Also, the stimulation components might be large molecules other than proteins.

4.2. Interactions between *Conotelus obscurus* and *Metschnikowia borealis*

The larval extracts had inhibition activity against both *M. borealis* and *D. maramus*, but not *M. reukaufii*. The hypothesis that *Conotelus obscurus* produces specific antimicrobial peptides that select in favor of certain *Metschnikowia* species must, therefore, be rejected. However AMPs may still perform a very important role in the *Conotelus-Metschnikowia* selectivity and it is possible that the maintenance of specific *Metschnikowia* species is due to a mix of historical contingency and physiology (Lachance et al., 2003a), including an involvement by AMPs.

4.2.1. Mechanism of the association between *Conotelus obscurus* and its symbiotic yeasts

Larvae of *Conotelus obscurus* eat colonies of *Metschnikowia borealis* (and other species) as food (Lachance et al., 2003a). The larval extracts contain substances that can inhibit the growth of *M. borealis* and also contain some components that stimulate yeast growth. This suggests two possible relationships between *C. obscurus* and *M. borealis*: mutualism, where both parties benefit nutritionally, or parasitism, where the yeast gains nutrition at the detriment of the beetle.

Very little is known about the interactions between mutualistic microorganisms and the immune system of their hosts (Feldhaar and Gross, 2009). There are two possible mechanisms for this interaction: either the surviving microorganisms do not activate the immune responses of their host, or they are actively capable of evading the immune system (Feldhaar and Gross, 2008). *M. borealis*, *M. reukaufii*, and *D. maramus*

all appear to allow or induce the production of AMPs, suggesting that they were recognized as non-self. Alternatively, the AMPs may be constitutive. I must therefore reject the hypothesis that selectivity for *M. borealis* is due to lack of induction of AMPs by this yeast species.

There is also the possibility that *M. borealis* is a parasite of *C. obscurus*, or even a pathogen, which would explain why the beetle can produce AMPs against this yeast. However, evidence that *M. borealis* has detrimental effects on the growth of the beetle is lacking. For example, some pathogenic fungi are known to influence the behavior of their hosts in a way that favors their dispersal (Roy et al., 2006). The mycophagous behavior of *C. obscurus* may be stimulated by *M. borealis*, thus enhancing the yeast's dispersal.

4.2.2. Possibilities of further research

Although the initial hypothesis of this project was rejected, AMPs may remain very important in the interaction between beetle and yeast. The capability of *Metschnikowia* species to evade the immune response of the beetle, including inhibition by AMPs, may be one of the mechanisms of the beetle-yeast association. On the other hand, it is also possible that the beetle-yeast association is not due to AMPs and that other mechanisms are responsible. The major community of yeasts associated with the genus *Conotelus* is the large-spored *Metschnikowia* clade (Lachance et al., 2001a). Whether the large spores produced by these *Metschnikowia* species can affect the interaction between the beetle and the yeast is still unknown. Moreover, many symbiotic

microorganisms help their hosts in the battle against other parasites (Feldhaar and Gross, 2008). Can *Metschnikowia* species assist *Conotelus* in such a manner? Larval extracts contain substances that stimulate the growth of *M. borealis* but not *M. reukaufii*. Is the beetle-yeast association affected by the growth characteristics of the large-spored *Metschnikowia* species? Various new hypotheses can be proposed for further projects.

4.3. Yeast transfer between beetle and flower

There are two possible modes of transmission of yeasts between beetles: horizontal transmission where adult beetles transfer the yeast cells to each other during mating or feeding, and vertical transmission, where adult beetles transfer yeast cells to larvae of the next generation via the corolla where larvae feed. In either case, adult beetles may deposit yeast cells to the corolla of flowers. The extract manner in which the beetle carries the yeast cells is also unknown. To understand better the transfer mechanisms, three experiments were done.

The evidence obtained in this thesis suggests that the flowers do not participate on their own in the transmission of yeast and that adult *Conotelus* beetles harbour yeasts in their digestive tract and release them during excretion. Adult beetles are therefore able to transfer yeast cells to the corolla of flowers. The hypothesis that the transmission of the yeast cells occurs through the gut of beetles, the corolla of flowers, and ultimately to the larvae is therefore accepted.

CHAPTER 5

Summary

1. The larvae of the beetle *Conotelus obscurus* produced substances that inhibit the growth of the yeasts *Metschnikowia borealis* and *Debaryomyces maramus*.
2. All the larvae of *Conotelus obscurus* fed with *Metschnikowia borealis*, *Metschnikowia reukaufii*, and *Debaryomyces maramus* produced these substances.
3. The antifungal activity of these inhibitory components against *Debaryomyces maramus* is more pronounced than *Metschnikowia borealis*, and no antifungal activity against *Metschnikowia reukaufii* was shown.
4. The larval extracts of *Conotelus obscurus* served as both carbon and nitrogen sources for the growth of *Metschnikowia borealis* and *Debaryomyces maramus*. This may have counteracted the inhibitory effects of the extracts.
5. The inbibitory substances of *Conotelus obscurus* are smaller than 3 kDa or larger than 100 kDa, and they are probably antimicrobial peptides or proteins.
6. The selectivity of the beetle-yeast interaction may involve mechanisms other than immunity.
7. Adult *Conotelus* beetles carry the yeasts in their digestive tract and release them during excretion.
8. The bindweed flowers do not carry yeasts on their own.

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Appendix 1 Comparison of cell numbers (total count) and colony numbers (viable count) of *Metschnikowia borealis*, *Metschnikowia reukaufii*, and *Debaryomyces maramus* after incubation with larval extracts at room temperature.

The viable counts experiment was repeated three times on YM agar plates. However, due to contamination, only two replicates could be used.

		Yeast species					
		M.borealis		*M.reukaufii*		*D. maramus*	
Colonies	Cell number	84	80	128	86	189	86
LE1		47	26	58	8	72	32
LE2		31	44	47	10	123	24
LE3		37	25	68	8	84	35
Control		56	14	5	0	118	23