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Pichia anomala: cell physiology and biotechnology relative to other yeasts

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Abstract

Pichia anomala is a most interesting yeast species, from a number of environmental, industrial and medical aspects. This yeast has been isolated from very diverse natural habitats (eg. in foods, insects, wastewaters etc) and it also exhibits wide metabolic and physiological diversity. Some of the activities of *P. anomala*, particularly its antimicrobial action, make it a very attractive organism for biological control applications in the agri-food sectors of industry. Being a "robust" organism, it additionally has potential to be exploited in bioremediation of environmental pollutants. This paper provides an overview of cell physiological characteristics (growth, metabolism, stress responses) and biotechnological potential (eg. as a novel biocontrol agent) of *P. anomala* and compares such properties with other yeast species, notably *Saccharomyces cerevisiae*, which remains the most exploited industrial microorganism. We await further basic knowledge of *P. anomala* cell physiology and genetics prior to its fuller commercial exploitation, but the exciting biotechnological potential of this yeast is highlighted in this paper.

Keywords: *Pichia anomala*, physiology, biotechnology

Introduction

Pichia spp. represent very interesting yeasts from both fundamental and applied perspectives. For example, from a cell biology viewpoint, they have proved most valuable in studies of organelle biogenesis, structure and function; and from an applied viewpoint, they have widespread biotechnological significance ranging from human therapeutic protein production, food fermentations, biocontrol agents and biofuel production. One particular species, *Pichia anomala**, exhibits great diversity with regard to its natural habitat, growth morphology, metabolism, stress-tolerance, and antimicrobial properties. It has been isolated from the following sources: flowering plants, fruit skins, insect intestinal tracts, human tissue and faeces, dairy and baked food products, contaminated oil, wastewaters, tree exudates, salted foods, and from the marine environment. This represents a wider range of habitats in Nature compared with the well-known *Saccharomyces cerevisiae*

(brewer's or baker's yeast). *P. anomala* also differs from *S. cerevisiae* with regard to its mode of central carbon metabolism, in that it exhibits an insensitivity to glucose (i.e. is Crabtree negative). An interesting shared characteristic of both species relates to their killer yeast activity. However, do both yeasts kill other yeasts (or fungi) by the same mechanisms? The antifungal activity of *P. anomala* appears to be linked to cell wall hydrolysis (β glucanase-induced lysis) and/or to production of volatile metabolites (eg. ethyl acetate), whereas *S. cerevisae* produces a killer toxin (eg. K1 toxin peptide) that disrupts plasma membrane integrity. The antimycotic properties of *P. anomala* have led to this yeast being considered a valuable biocontrol agent against fungi of agronomical importance. Other potential biotechnological applications of *P. anomala* include environmental bioremediation, biopharmaceuticals and biofuels.

This paper reviews some of the unusual characteristics of *P. anomala* and will highlight these with reference to its potential biotechnological exploitation. Cell physiology of *P. anomala* will also be compared with better known yeast species, notably *S. cerevisiae*.

*** Footnote**: Throughout this paper the species will be referred to as *Picha anomala*, rather than *Wickerhamomyces anomalus* (see Kurtzman *et al*, 2008 and Kurtzman, 2010), mainly because it was the nomenclature used in the symposium from which this manuscript emanated (1st International *Pichia anomala* mini-Symposium).

P. anomala in foods and beverages

P. anomala possesses several attributes with regard to food and beverage production and food/feed preservation, and some of these are summarised in Table 1. Benefits of *P. anomala* relate to its positive (flavour enhancing) roles in food and beverage fermentations and in food preservation. One particularly beneficial characteristic of *P. anomala* with regard to food and feedstuffs lies in its ability to liberate soluble phosphate from insoluble plant-derived phytic acid, which represents a form of non-utilisable phosphorus for monogastric animals. Phosphate solubilisation is facilitated by the activity of a thermostable phytase enzyme in *P. anomala* (Olstorpe, Schnürer and Passoth 2009a; Satyanarayana, 2010).

There are some detrimental roles of *P. anomala* in relation to food production and storage (eg. Deak, 2008). As a food spoilage yeast, its contamination of yoghurts, bread, sugary cakes (Lanciotti *et al*, 1998), and wine (eg. Rojas *et al*, 2001) can lead to taints commonly referred to as "chemical adulteration". This may be due to the propensity of *P. anomala* to produce ethyl acetate (see below). In stored animal feeds such as silage, it can consume lactic acid (Jonsson and Pahlow, 1984) and this may elevate pH thus reducing periods of safe feed storage.

Although there are some reports of nosocomial infections caused by *P. anomala* (eg. Charkrabarti *et al*, 2001), with regard to food safety aspects *P. anomala* is classed at biosafety level 1 (De Hoog, 1996) and is considered safe for healthy individuals. In fact, *P. anomala* currently has QPS (qualified presumption of safety) status from EFSA (European Food Safety Authority) and this has benefits in terms of public perspectives of food biotechnology and acceptability of novel microorganisms in food (Sundh and Melin, 2010).

***P. anomala* in the environment**

With regard to the natural niche of *P. anomala*, this yeast has been isolated from very diverse sources. These include plants, soil, animals, insects, water, hospitals, and food (Table 2). In natural environments, *P. anomala* is believed to exist as a diploid yeast (Naumov, Naumova and Schnürer, 2000). In relation to known growth extremes of *P. anomala*, this yeast has been found by Fredlund *et al* (2002) to exhibit the following growth ranges: temperatures ranging from 3-37°C; pH values from 2-12; and osmotic conditions as low as a_w 0.92 (NaCl) and 0.85 (glycerol). *P. anomala* is therefore quite ubiquitous in Nature and is able to tolerate a relatively wide range of environmental growth conditions. This contrasts with *S. cerevisiae* which is actually quite a rare yeast in natural environments (eg. Martini, 1993) and possesses a narrower range of growth extremes compared with *P. anomala*.

P. anomala plays certain beneficial roles in the environment. For example, it exhibits (as do many other yeasts) saprophytic roles in the carbon cycle; it can help to alleviate pollution by bioremediation of recalcitrant chemicals/heavy metals in wastewaters; and it can act in the biological control of harmful microbes by combating biodeteriogenic fungi. Walker, MacLeod and Hodgson (1995) showed that *P. anomala* was able to inhibit certain wood decay basidiomycetous fungi and it also displayed fungistatic acitivity against plant pathogenic fungi, including the causative agent of Dutch Elm disease, *Ophiostoma novo ulmi*. El-Latif Hesham *et al* (2006) have shown that *P. anomala* can effectively degrade toxic chemicals such as the aromatic hydrocarbons naphthalene and benzopyrene, thus highlighting its potential role in environmental bioremediation processes (eg. oil-contaminated industrial, terrestrial and marine environments).

***P. anomala* in industry**

P. anomala has been shown to produce several metabolites that can be potentially exploited as biotechnological commodities (summarised in Table 3). These products range from bioremediation agents, biopharmaceuticals, biosurfactants, biofuels, biocides and biocontrol agents (BCAs). The latter are particularly attractive for large-

scale applications in the agri-food sectors of industry, for example, to prevent fungal spoilage in fruit and cereals in post-harvest storage conditions (Jijakli, 2010 and Olstorpe, 2010, respectively).

Antimicrobial activity of *P. anomala*

For a yeast, *P. anomala* exhibits very unusual broad spectrum antimicrobial properties. For example, it has been shown to suppress the growth of several fungi, yeast and bacterial species and viruses (Table 4).

P. anomala is a killer yeast and a variety of killer toxins are known in *P. anomala* strains. With regard to the genetic basis of the killer phenomenon in *P. anomala*, the killer factor proteins are thought to be chromosomally inherited, unlike *S. cerevisiae* killer toxins (such as K1) which are encoded on double-stranded RNA virus-like extra-chromosomal elements, or *Kluyveromyces lactis* toxins which are encoded on linear DNA plasmids. *P. anomala* killer toxins also differ from those of other killer yeasts in that they exhibit diversity in terms of broad spectrum antimicrobial activity, variable molecular mass (eg. from 3-300 kDa), and different pH and temperature optima (Passoth *et al*, 2006). Recently, De Ingeniis *et al* (2009) have shown that a *P. anomala* killer toxin (peptide of ~8kDa) possesses novel ubiquitin-like characteristics.

In addition to its activity as a classical killer yeast (i.e. displaying ability to kill other yeast species – Walker, 1998), *P. anomala* also exhibits antifungal effects. For example, Masih, Alie and Paul (2000) have shown that *Botrytis cinerea* (a major plant pathogen) displayed emptied hyphae in contact with *P. anomala* yeast cells. These workers showed that *P. anomala* protected vines (*Vinus vinifera*) against *Botrytis* infestation. Similar investigations by Mohamed and Saad (2009) have shown by scanning electron microscopy antagonistic effects of *P. anomala* cells interacting with the fungus *Botryodiplodia theobromae*, showing pitting and disruption in hyphal surfaces that were totally penetrated and killed by *P. anomala*. The antimycotic properties of *P. anomala* were originally described by Björnberg and Schnürer (1993) against grain-storage fungi, and whilst the mode of action is still unclear, it may be due to several factors acting singularly or in combination (Table 5). Jijakli and Lepoivre (1998) proposed that the suppression of *B. cinerea* by *P. anomala* is partly due to the activity of an exo-β-1,3-glucanase. Fredlund *et al* (2004b) have shown that secretion of a volatile ester, namely ethyl acetate, by *P. anomala* may be responsible (possibly together with other metabolities such as ethanol) for its mode of antifungal activity, particularly against grain-storage moulds such as *Penicillium* spp. (Druvefors *et al*, 2005). Ester biosynthesis in *P. anomala* appears to differ from that in other yeast species such as *S. cerevisiae* with ethyl acetate being produced via an inverse esterase from acetate, rather than from acetyl CoA via ethanol acetyltransferase (Fredlund, 2004). A recent study

(Kurita, 2008) has compared esterase activities in both *S. cerevisiae* and *P. anomala*. The secretion of ethyl acetate by *P. anomala* is an interesting (from an antifungal biocontrol perspective) and well-established characteristic, bearing in mind that the species was originally named *Saccharomyces acetaethylicus* by Beijerinick in 1892 (Lodder and Kreger-van Rij, 1952).

In addition to its action against biodeteriogenic fungi in the agri-food areas, *P. anomala* also has potential applications in medical mycology. For example, *P. anomala* has long been recognised as possessing anti-*Candida albicans* activity (eg. Hodgson, Button and Walker, 1995; Polonelli *et al*, 1983; Sawant, Abdelal and Ahearn, 1988; Buzzini and Martini, 2001). Polonelli *et al* (1990) were the first to show that *P. anomala* killer toxin was active *in vivo* in experimental mice. More recently, Izgü, Altinbay and Türeli (2006) have shown that the K5 killer toxin of *P. anomala* displays activity against selected dermatophytes (*Microsporum* spp. and *Trichophyton* spp). The K5 killer protein (named "panomycin") was previously shown by Izgü and Altinbay (2004) to exhibit exo-β-1,3-glucanase activity. Magliani *et al* (1997) and Polonelli, Magliani and Conti (2010) have discussed medical applications of *P. anomala* killer toxins, in particular the immunomodulatory activities of "antibiobodies".

Stress tolerance of *P. anomala*

Figure 1 summarises major physicochemical and biotic stresses to which yeasts, including *P. anomala*, are exposed to when exploited in industry, or when used in environmental biocontrol applications. *P. anomala* responds to such stressful conditions by: accumulating trehalose and secreting ethyl acetate under O_2 limitation; synthesising glycerol (at start) and arabitol (at end) during salt stress; inducing biosynthesis of heat/cold shock proteins and stress enzymes; and altering structure of cell membranes. These stress responses are also observed in other yeasts (Walker and Van Dijck, 2006), but arabitol accumulation in salt stressed cells of *P. anomala* (Bellinger and Larher, 1988) is not an observable phenomenon in *S. cerevisiae*. Regarding hypoxic stress, Fredlund (2004) has proposed that trehalose accumulation in *P. anomala* is involved as a specific response to oxygen-limitation. The ethyl acetate-secreting capabilities of *P. anomala* have been proposed by Fredlund (2004) to act as a stress protection measure, by preventing intracellular accumulation of toxic acetic acid (and at the same time suppressing the growth of competitor microbes). Although there are some conflicting reports of ethanol tolerance of *P. anomala* (eg. Kalathenos, Sutherland and Roberts, 1995; Stratford, 2006), this yeast is generally regarded as a resilient or "robust" yeast (Fredlund *et al* 2002; Passoth *et al* 2006; Lahlali *et al* 2008), and the stress adaptation mechanisms (both general and specific) it adopts must be very efficient. As testament to

the inherent stress tolerance of *P. anomala*, Melin *et al* (2005; 2007) and Mokiou and Magan (2008) have successfully preserved and stabilised this yeast at high viabilities in both liquid and desiccated formulations for environmental biocontrol applications.

Cell physiological aspects of *P. anomala*

The morphology of *P. anomala* exhibits diversity in terms of various cellular shapes with budding cells and branched pseudohyphae being evident in both liquid and solid culture media (Kurtzman, 1998). As with other yeasts, it is possible that Quorum-sensing mechanisms may be involved in governing morphological changes and cell density related growth inhibition in this yeast (Walker, 1998). Sexual reproduction in *P. anomala* is characterised by formation of hat-shaped spores (Kurtzman, 1998).

P.anomala exhibits wide diversity regarding the metabolism of carbon and nitrogen sources (Table 6) and Fredlund *et al* (2002) showed that this yeast can grown on selective media with solely starch and nitrate as C and N sources, respectively. It has also been reported to grow in vitamin-free media. Concerning oxygen requirements of *P. anomala*, this organism may be regarded as a facultative yeast, being able to grow in both oxygen-replete and oxygen-limited conditions. Respiratory growth of *P. anomala* is favoured under aerobic conditions and alcoholic fermentation is only induced by O_2-limitation. Fredlund *et al* (2004a) reported that *P. anomala* exhibited growth rates of 0.22 and 0.056 h^{-1} and biomass yields of 0.59 and 0.11 g/g glucose under aerobic and anaerobic conditions, respectively. When shifted to oxygen limitation, *P. anomala* rapidly induced key fermentative enzymes (pyruvate decarboxylase and alcohol dehydrogenase – Fredlund *et al*, 2006) and also lowered flux through the pentose phosphate pathway. *S. cerevisiae* is also regarded as a facultative yeast, but in this organism, glucose (rather than oxygen) governs central carbon metabolism. Fredlund *et al* (2004a) have shown that in aerobic conditions, pyruvate flux into *P. anomala* mitochondria is ~60% (c.f. only 7% under O_2-limitation) and that glucose consumption rate is faster under anaerobic conditions (4.6 c.f. 2.1 mmol/g biomass/h, respectively). All of this is demonstrative of the Pasteur Effect in *P. anomala* and an absence of the Crabtree Effect (Walker, 1998). This represents a major difference with *S. cerevisiae* regarding glucose catabolism under conditions of altered oxygen and glucose availability. For example, if glucose concentrations are high, *P. anomala* will respire under aerobic conditions, unlike *S. cerevisiae* which is a Crabtree-positive yeast that will predominantly ferment high glucose levels - even in the presence of oxygen (due to catabolite repression/inactivation of mitochondrial oxidative functions). Only when *P. anomala* is transferred to O_2-limited conditions will it concomitantly transfer to a fermentative mode of metabolism (Fredlund *et al*. 2004a).

The practical manifestation of these metabolic differences means that *P. anomala* can grow aerobically with high sugar concentrations at a relatively high growth rate and to higher cell densities than *S. cerevisiae*. The lack of a Crabtree Effect in *P. anomala* means that (unlike *S. cerevisiae*), there is no real necessity to keep sugar levels low and consequently no need to conduct *fed-batch* yeast propagation systems to control sugar feeding rates when attempting to maximise yeast biomass production.

Although these responses to oxygen and glucose represent major metabolic differences between *S. cerevisiae* and *P. anomala*, certainly similarities between the two yeasts do exist with regard to oxygen availability in that both species are unable to grow under strictly anaerobic conditions. This is because oxygen is required as an absolutely essential growth factor for sterol (ergosterol) and unsaturated fatty acid (oleic) synthesis (Walker, 1998) during plasma membrane biogenesis in *S. cerevisiae* and *P. anomala*. Nevertheless, *S. cerevisiae* grows at similar rates under aerobic and sterol/fatty acid-supplemented anaerobic conditions, whilst *P. anomala* grows slower under the latter conditions. It is apparent that both yeasts respond to available oxygen in different ways. Table 7 summaries the major differences and similarities between *P. anomala* and *S. cerevisiae* in terms of metabolism and cell physiology.

Conclusions and future perspectives

Pichia anomala exhibits interesting and potentially exploitable physiological and metabolic characteristics. These include: morphological diversity (budding, pseudomycelial); stress tolerance (to low pH, high osmotic pressure, low O_2, low a_w); enzyme secretion (invertase, lipase, peptidase, amylase, phytase); nutritional diversity (range of C, N, and P sources); biodegradation (of polyaromatic hydrocarbons, naphthalene, benzopyrene,); Crabtree negativity (glucose insensitivity); antimicrobial activity (yeasts, fungi, bacteria, viruses); and production of potential commercial metabolites.

Although *S. cerevisiae* remains the world's most exploited organism in industrial bioprocesses, other non-*Saccharomyces* yeasts like *Pichia* spp. have fantastic potential in biotechnology. Nevertheless, we still have much to learn about physiology and metabolism in non-*Saccharomyces* yeasts, including *P. anomala*, and enhancement of cell physiological knowledge in this yeast is a prerequisite for its fuller industrial exploitation. There are still several unresolved questions regarding carbon metabolism and its regulation in *P. anomala*. For example, it is conceivable that there is variability of the expression of the Crabtree effect in *P. anomala* strains, as previously demonstrated in *Kluyveromyces lactis* by Liti *et al* (2001), and the underlying mechanisms of such metabolic phenomena and their practical significance require further research. Other areas of *P. anomala*

research worthy of future investigation include: determination of modes of antimicrobial/antiviral action; and molecular understanding of the underlying reasons for stress tolerance. Stress tolerance and antimicrobial action are especially important *P. anomala* characteristics that can be exploited for future biotechnological applications.

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Table 1. Important roles of *Picha anomala* in foods, feeds and beverages

Food/beverage applications	**Examples of beneficial roles**	**References**
Flavour enhancement	Volatile (eg esters) and savoury (eg nucleotides) flavours	Eun-Kyoung et al (2003); Lee *et al* (2004)
Food/feed bio-preservation	Biological control of fungi in fruits and cereals	Petersson and Schnürer (1995); Jijakli (2010); Olstorpe *et al* (2009b)
Dairy fermentations	Probiotic effects	Mo *et al* (2004)
Baking	Sourdough fermentations (not necessarily beneficial)	Daniel *et al* (2010)
Winemaking	Volatile aromas, low alcohol wines, malic acid reduction	Ertin and Campbell (2001); Swangkeaw et al (2009)
Enzymatic food/feed processing	Phytase, amylase, peptidase	Ray and Nanda (1996); Satyanarayana (2010)
Brewing	Anti-gushing potential in malting barley	Olstorpe *et al* (2009a); Laitila *et al* (2007; 2010)

Table 2. Some diverse *Picha anomala* habitats

Isolation from:	**References**
Homo sapiens (human skin, faeces etc.)	Murphy *et al* (1986); Mo *et al* (2004)
Palm sugar	Nagatsuka *et al* (2005)
Bread/fermenting dough	Lanciotti *et al* (1998); Daniel *et al* (2010)
Insects (eg. *Drosophila* and malaria mosquito *Anopheles)*	Kurtzman (2001); Ricci *et al* (2010)
Sea urchins	Kajikazawa *et al* (2007)
Pharmaceutical wastewater	Recek, Cadez and Raspor (2002)
Cereal silos and silage	Olstorpe (2008)
Oil-contaminated soil	El-Latif *et al* (2006)
The sea	Wang *et al* (2007)
Hospitals	Thuler *et al* (1997); Chakrabarti *et al* (2001); Reyes (2004)

Table 3. *P. anomala* products of biotechnological potential

Product	Potential application	Reference
Sophorolipids	Biosurfactants	Thaniyavarn *et al* (2008)
γ-aminobutyric acid, GABA	Pharmaceuticals (GABA acts as a neurotransmitter, improves cerebral blood flow)	Kaku and Hagiwara (2008)
Volatile organic compounds	Fragrances	Buzzini *et al* (2003)
Isobutanol	Biofuels	US Patent (2009)
Beverage starter culture	Low-alcohol wines; aromas	Ertin and Campbell (2001)
Panomycocin	Novel zymocidial agents	Izgü, Altinbay and Türeli (2006)
Antiviral agent	Influenza virus therapy	Conti *et al* (2008)
Amoebicidal agent	Therapy of *Acanthamoeba* infections	Fiori *et al* (2006)
Anti-*Pneumocystis* agent	Therapy of *Pneumocystis carnii*	Seguy *et al* (1996)
Antibacterial agent	Therapy of Streptococcal infections	Conti *et al* (2002)
Biocontrol/biopreservative	Stored grain, vines, fruit	Jijakli (2010); Olstorpe (2010)
Enzymes	Phytase, esterase, peptidase, β-glucosidase, amylase	Ray and Nanda (1996); Satyanarayana, T (2010)
Bioethanol (indirectly)	Maintenance of airtight stored grain (biofuels)	Passoth *et al* (2009)

Table 4. Summary of antimicrobial properties of *P. anomala*

Antimicrobial characteristic	**Examples of microbes suppressed**	**References (examples)**
Antifungal	*Aspergillus*, *Botrytis*, *Penicillium*, *Fusarium*	Jijakli and Lepoivre (1998); Masih *et al* (2000); Jijakli (2010); Laitila *et al* (2007)
Antizymal	Various yeasts, incl. *C. albicans*	Sawant, Abdelal and Ahearn (1988)
Antibacterial	*Erwinia* spp.; *Enterobacteriaceae*; *Streptococci*	Polonelli and Morace (1986); Conti *et al* (2002)
Antiviral	Influenza virus	Conti *et al* (2008)

Table 5. Antimycotic activity of *P. anomala*: candidate antifungal agents

Antifungal agents or modes of antifungal action	Likely relative contribution (ranging from ***** predominant to * lesser importance)
Killer toxins	*****
Hydrolytic enzymes (eg. β-glucanase)	****
Volatile chemicals (eg. ethyl acetate)	***
Nutrient competition	**
Media acidification	*
Carbon dioxide	*
Predation/mycoparasitism	*
Other antifungal agents	Unknown

Table 6. Carbon and nitrogen growth source diversity in *P. anomala*

Carbon sources	Nitrogen sources
Saccharides: hexoses (glucose, galactose, fructose); pentoses (arabinose, xylose); disaccharides (sucrose, lactose), polysaccharides (starch; β-glucans) Alcohols: ethanol, glycerol Organic acids: acetate, citrate, lactate, malate, succinate Fatty acids: oleate, palmitate Aromatics: naphthalene, benzopyrene	Nitrate Nitrite Urea L-glutamine L-histidine

Table 7. Cell physiological and other characteristic differences between *P. anomala* and *S. cerevisiae*

P. anomala	*S. cerevisiae*
Budding/pseudomycelia	Mainly budding
Crabtree negative	Crabtree positive
Predominantly respiratory	Predominantly fermentative
Oxygen sensitive	Glucose sensitive
Glucose uptake by H^+ symport	Facilitated glucose diffusion
Malic acid utilisation	Malate only utilised with glucose
Several enzymes secreted	Few enzymes secreted
Antifungal action	Rarely antifungal (some strains)
High ethyl acetate	Low ethyl acetate
Widespread in Nature	Not widespread in Nature
Halotolerant	Not very halotolerant
Moderate ethanol tolerance	Ethanol tolerant
QPS (EFSA)	GRAS (FDA)
Opportunistic pathogen (some strains)	Doubtful opportunistic pathogenicity

Fig 1. Major environmental stress factors impacting on *P. anomala.*
Such stresses may be experienced by the yeast during growth in the natural environment or in industrial situations.