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Bradley A. Plantz · Jackie Andersen
Leonard A. Smith · Michael M. Meagher
Vicki L. Schlegel

Detection of non-host viable contaminants in *Pichia pastoris* cultures and fermentation broths

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Abstract The ability to detect viable contaminants in cultures propagated from the original host-expression system ensures that the integrity and purity of seed banks, fermentation broths, and ultimately the final product are continually controlled and maintained. The method developed to detect such agents must be selective for a broad spectrum of microbes, which may be present at very low levels, while discriminating from the host organisms. Although *Pichia pastoris* strains are frequently used as cell lines for the expression of heterologous proteins, a method that is specific for monitoring culture purity has yet to be reported for this type of organism. An assay that is capable of recovering contaminating bacteria, fungi, and closely related yeast from cultures of *P. pastoris* at parts per million detection limits is described here.

Keywords *Pichia pastoris* · Non-host contaminants · Fermentation.

Introduction

Pichia pastoris expression systems have gained wide acceptance as industrial host strains for the production of heterologous proteins because of several factors. This yeast expresses proteins that are analogous to the primary, secondary, and tertiary structures of their natural counterparts [8]. An active protein is produced during the fermentation process that eliminates the need for any further downstream refolding processes. And, similar to other higher-level eukaryotic systems, *P. pastoris* strains efficiently carry out such post-translational modifications as glycosylation, proteolytic processing, and disulfide-bond formation [1,12,14,16]. *P. pastoris* organisms are also easy to cultivate on simple, inexpensive media and can be grown to high cell densities that are comparable to biomasses usually associated with bacterial host organisms, such as *E. coli* [6,7,10,13]. Due to these advantages, several proteins from *P. pastoris* recombinant cell lines are currently in various stages of clinical trials, with more products on the horizon.

Products derived from *P. pastoris* are manufactured from an indefinite number of raw materials, manipulations, and down-stream events. The purpose of these manipulations is to produce a sufficiently pure, biologically active material that complies with the standards required by governmental agencies, most notably the Food and Drug Administration (FDA). Entry and proliferation of a microorganism that does not originate from the host expression system can occur at any point in the manufacturing train. In regulatory parlance, this contaminating organism is referred to as either an adventitious agent or a non-host contaminant. Introduction of a non-host organism is particularly problematic when the product of interest is of biological origin because the host system, the matrix formulated for the drug, or even the desired final product can produce an ideal environment for supporting the growth of a viable contaminant. Even if killed or removed during other down-stream processes, non-host microbes can significantly compromise the integrity and purity of the final product. The International Committee on Harmonization (ICH) has thus issued specific guidance mandating the detection of possible non-host contaminants within process matrices [9].

B. A. Plantz · J. Andersen · M. M. Meagher
Department of Chemical Engineering,
University of Nebraska,
Lincoln, NE 68683 USA

L. A. Smith
United States Army Medical Research Institute
of Infectious Diseases, Fort Detrick,
Frederick, MD 21702–5011, USA

V. L. Schlegel (✉)
Department of Food Science and Technology,
University of Nebraska, Lincoln,
NE 68583–0919, USA
E-mail: vlschleg@unlnotes.unl.edu
Tel.: +1-402-4724695
Fax: +1-402-4721693

An effective bioburden risk minimization system is imperative for products that are destined for animal or human health care use [3]. Bioburden risk minimization consists of two complementary practices, the first being prevention. To prevent a contaminant from entering a process, environmental controls and process monitoring are required concomitant to the associated risk. The second component of a bioburden risk minimization is testing. For this approach, statistical plans must be developed because detection of contaminating organisms in the product requires the use of destructive procedures. The rigors and requirements of testing will vary dependent upon the demands of the process, but basically random samples are collected at critical steps and then analyzed for potential contaminants. The statistical plan must ensure that the seed inoculum, inoculum stages, and production fermentation propagate only pure cultures and that the final dosage form of the product is devoid of all microorganisms [3,4,9,17]. By combining both these practices and by establishing well-defined specifications, a risk minimization program designed specifically for a particular biopharmaceutical facility can be effectively initiated.

Despite the regulatory mandates, *P. pastoris* cultures are frequently monitored with tests designed for other types of recombinant host lines, which generate results that tend to be ambiguous and difficult to interpret. The need for a relatively simple non-host contamination assay relative to *P. pastoris* became apparent within our laboratory. During the development of a non-host detection method, specific media were formulated to inhibit growth of the host organism while enabling growth of contaminating microbes that may be present in a typical production environment.

Materials and methods

Microorganisms and stock cultures

P. pastoris (ATCC 28485) and *Williopsis saturnus* (ATCC 18119) were purchased from the American Type Culture Collection (Rockville, Md., USA). *P. pastoris* GS115, a common production strain, was a generous gift from the United States Army Medical Research Institute for Infectious Disease (Ft. Detrick, Frederick, Md., USA). *Saccharomyces cerevisiae* (ATCC 2601), *Aspergillus niger* (ATCC 16404), *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC, 8739), *and Bacillus subtilis* (ATCC 6633) were purchased from Microdiagnostics (St. Cloud, Minn., USA) as EZ-KITS. These organisms were handled according to the manufacturer's recommendation to provide 10–100 colony-forming units (CFUs), with viable counts being confirmed by spread plates. If higher densities of culture material were required, organisms were amplified from single-colony isolates.

Yeast cultures were routinely cultivated on yeast-peptone-dextrose broth (YPD) (Difco, Detroit, Mich., USA) at 30 ± 2 °C with continuous agitation at 150–200 rpm for 24–48 h. YPD agar plates were prepared by adding 1.5% agar to the broth just prior to autoclaving. Bacterial cultures were amplified in shake flasks to an optical density, measured at 600 nm (OD_{600}), of 2–4 by growth on Lauria-Bertani broth (LB; Difco) at 35 ± 2 °C and 150–200 rpm. Culture identity was verified by colony morphology, cell morphology and Gram stains.

Test media formulation

Media prepared in-house and/or purchased test kits were used to identify nutrients that supported heterotrophic organisms while inhibiting both *P. pastoris* wild-type and recombinant host strains. When solid media were necessary, microbiological grade agar was added to a broth, unless indicated otherwise, at a final concentration of 1.5% before the material was subjected to steam sterilization. All media were adjusted to pH 6.8 prior to sterilization.

1. *API test strips*: API-20 yeast identification kits were purchased from API (bioMerieux, Durham, NC, USA). Yeast and mold strains were inoculated into test wells according to the manufacturer's recommendation. The test strips were evaluated 48 and 72 h post-inoculation.
2. *Tryptic soy agar (TSA)*: TSA was supplied by Difco and prepared according to the manufacturer's instructions.
3. *Tryptic soy agar with cycloheximide (TSA-C)*: To ensure antibiotic activity, a cycloheximide stock solution was prepared fresh on the day of use, filter sterilized, and delivered to steam-sterilized TSA after it had cooled to 48–50 °C. Different final concentrations of cyclohixmide-based media were prepared, as listed in Tables 2 and 3. The media, in both broth and agar plates, were assigned a 2-week expiration date.
4. *Biotin-avidin complex media (BA)*: The basic composition of BA medium consisted of 2 g dextrose/l, 0.5 g ammonium sulfate/l, 1.0 g potassium phosphate di-basic/l, and 1.0 g acid hydrolyzed casamino acids/l. After the steam-sterilized medium cooled to approximately 48–50 °C, filter-sterilized yeast extract was added to a final concentration of 0.01 g/l. Avidin was then transferred from a filter-sterilized stock solution and the medium was dispensed. The medium was assigned a 4-week expiration date to ensure activity.
5. *Xylose/sucrose/maltose carbon source media (XCM/SCM/MCM)*: Yeast nitrogen base (YNB; Difco), 13.4 g/l and either xylose, sucrose, or maltose source at 10 g/l were combined as a 10× stock solution and filter sterilized. Working solutions of the final media formulation were prepared by diluting the 10× stock with steam-sterilized solutions of either water or agar.
6. *High osmolarity media (HOM)*: Increasing concentrations of either sucrose or potassium chloride were mixed into TSA medium and autoclaved.
7. *Nitrogen selective media (NSM)*: Nitrogen selective media was composed of 1 g glucose/l, 0.5 g di-basic potassium phosphate/l; and either ammonium sulfate, potassium nitrate, or potassium nitrite at a final concentration of 5 g/l. Filter-sterilized yeast extract was added to the medium as a source of vitamins.

Procedures

Cell densities were determined by measuring the OD_{600}. Viable counts were verified by preparing serial dilutions of the microbes in phosphate-buffered saline (PBS), pH 6, followed by spread-plating the dilutions onto appropriate agar plates. Quantitative data for CFUs and optical densities refer to the quantity of cells present in pure cultures. Pure cultures were diluted to 0.1–0.8 optical density units (ODU), within the linear range of the Beckman DU-70 spectrophotometer.

Initial screening conditions for the selection of bacteria, molds, and non-host yeast strains were determined by consulting bacteria and yeast manuals [2,18]. Carbon sources were further screened by using API-20 test strips. Candidate media formulations were first tested for selectivity by streaking pure cultures onto agar plates and by inspecting for growth. Judging growth from isolated colonies in the streak zone minimized the risk of carryover nutrients that would affect the growth results. Duplicate plates were prepared and

incubated at either 25 ± 2 °C or 37 ± 2 °C in humidity-controlled incubators. Temperature was monitored by disk chart recorders over the duration of incubation, which extended up to 7 days. Plates were inspected daily for both growth and excessive drying. A qualitative evaluation of growth was ascertained for all test media by comparing colony size on the test media to an identical culture but incubated on TSA plates as a positive control.

Preparation of frozen stocks for assaying

Detection limits for frozen stocks of *P. pastoris* GS115 were evaluated by first amplifying the cultures in YPD broth for 40–48 h with shaking. Pellets of the cultures were obtained by centrifugation. These pellets were then resuspended in fresh YPD without significant concentration or dilution. Steam sterilized glycerol was mixed into the cultures at a final concentration of 15% followed by chilling the suspension on ice. A sample of the culture was removed, diluted, and plated on YPD plates to determine viable cell counts.

Contaminants were prepared by one of two methods. If available in EZ-KITs, cultures of non-host contaminants were initially diluted 1:10 in sterilized water or transferred directly to an aliquot of the chilled *P. pastoris* GS115 sample, resulting in a predicted level of either 10 or 100 CFU/ml, respectively. If not available in EZ-KITs, bacterial agents were prepared by incubating the microbes overnight in LB broth; non-host yeast agents were grown for 2–3 days in YPD broth. Serial dilutions of the contaminants were plated on appropriate agar media to determine viable cell counts. Various serial dilutions of the contaminants were spiked into chilled *P. pastoris* GS115 cultures, mixed, and then either sampled directly or stored in the vapor phase of liquid nitrogen. Samples from the spiked *P. pastoris* GS115, cultures were prepared for cryopreservation by slowly freezing (∼Δ1 °C/min) to −80 °C followed by long-term storage in the vapor phase of liquid nitrogen. Over a 3-week period, the spiked cultures were removed from storage, thawed quickly, and 0.5-ml aliquots were transferred into the enrichment media, which had been dispensed into 17×150-mm test tubes and incubated 5 days to test for sterility before use.

Preparation of high-cell-density cultures for assaying

A description of the general fermentation methods is described elsewhere [15,19,20]. However, for these experiments, the basal salts medium was FM-22 [19], and the cultures were induced with methanol supplemented with 2 ml *Pichia* trace mineral salts 4 (PTM4)/l. Samples were then collected no earlier than 24 h post-induction. Model Bioflo 3000 fermentors (New Brunswick Scientific, Edison, NJ, USA) equipped with a 5-l working volume vessel were used for these studies. To minimize the risk of introducing environmental contaminants into a sample, the fermentor's sample port was drenched with 70% isopropyl alcohol before and after each sample was collected. The port was also flushed with 10–20 ml fermentation broth before a sample was removed and transferred into a sterile 50-ml conical tube. The OD_{600} of each sample was determined while the broth was maintained on ice.

After incubation, a given contaminant was centrifuged to concentrate the cells and a small volume of the concentrate was spiked into aliquots of fermentation broth at levels of 0.1, 0.01, 0.001, and 0.0001%, as determined by OD_{600} with respect to the fermentation broth. A 0.5-ml aliquot of the spiked culture was delivered to 5.0 ml enrichment broth. The enrichment tubes were placed on a rotary shaker set at either 25 ± 2 °C or 37 ± 2 °C and incubated for 24 ± 2 h. A 100-µl aliquot of enriched broth was spread uniformly onto agar plates of the same media components as the enrichment broth. The plates were incubated at 25 ± 2 °C or 37 ± 2 °C for up to 5 days. Tests were considered as "presumptive positive" when colonies grew on the plates. TSA streak plates were also prepared to confirm the identity of the cultures. Cells from individual colonies were either Gram-stained (bacteria) or simple-stained to assess cell morphology.

Results

Selectivity

A selective method was designed by initially categorizing possible non-host agents according to their expected difficulty in differentiating each type from *Pichia*. Heterotrophic bacteria were considered to be the most easily discernible microbes followed by fungi, heterotrophic yeast, and, lastly, closely related yeast. The heterotrophic bacterial strains *E. coli, S. aureus,* and *B. subtilis*, were chosen as potential contaminants based upon their routine use as test/host systems within a biopharmaceutical campaigning facility, their association with humans, or their persistent nature, respectively. *A. niger* was selected because the United States Pharmacopeia (USP) references this fungal strain as a standard test organism [17]. Representative heterotrophic yeast strains included *S. cerevisiae*, because it is used as a host cell line for expressing recombinant biologics, and *C. alibican,* because it is a human pathogen. The remaining yeast, *W. saturnus*, was selected because it is a methylotroph and, at one time, was considered to be closely related to *P. pastoris*.

Conditions that inhibited *P. pastoris* growth while supporting growth of the "chosen" non-host contaminants were identified by first comparing each of the organisms' phenotypic traits, which are shown in Table 1. For example, a carbon source was contemplated as a possible selective agent because *P. pastoris* growth is limited as compared to the phenotypic features of the other microbes. Because *P. pastoris* is a biotin auxotroph and biotin can be rendered biologically inactive by complexing with avidin, vitamin-free growth is presented as a phenotype. Table 1 also shows the potential of a phenotype-based assay to separate seven additional yeast and bacterial species that were not tested but pose risks based upon environmental persistence, pathogenicity, or relatedness to *P. pastoris*. Comparisons of the phenotypic traits were applied to fungi, with osmo-tolerance and optimum growth temperature being the additional specific tests.

API-20 test strips were used to ensure that the selected strains of yeasts and fungi matched their expected phenotypes as well as to broaden the carbon-utilization testing. As predicted, both *P. pastoris* wild-type and GS115 strains grew on only glucose and glycerol while the remaining three yeasts and *A. niger* grew on several other carbon sources. Sucrose, maltose, and xylose sustained all four contaminants. Raffinose sustained all non-host organisms except for *C. albicans*. The candidate carbon sources xylose, sucrose, and maltose were ultimately chosen for further development because detection of potential pathogenic organisms is imperative when producing biologically derived therapeutics, hormones, vaccines, etc.

The optimal growth temperature range for *P. pastoris* is 25–28 °C; thus, bacteria and human pathogens have a

Table 1 Phenotypic traits of selected organisms. + Growth, – no growth, *v* variable growth, *w* weak growth, *a* absent, *nr* not reported, *s* sensitive, *r* resistance, *a* acid formed, *n* no acid formed, *na* not applicable

	Strains of microorganism tested								Additional microbial strains for comparison													
									Yeast							Bacteria						
	Pichia pastoris[a]	*Williopsis saturnus*[a]	*Candida albicans*[a]	*Saccharomyces cerevisiae*[a]	*Aspergillus niger*[a]	*Escherichia coli*[b]	*Staphylococcus aureus*[b]	*Bacillus subtilis*[b]	*Pichia wickerhamii*[a]	*Pichia polymorpha*[a]	*Hansunela polymorpha*[a]	*Saccharomyces capensis*[a]	*Saccharomyces bailii*[a]	*Schizosaccharomyces pombe*[a]	*Torulopsis wickerhamii*[a]	*Klebsiella oxytoca*[b]	*Enterobacter cloacae*[b]	*Salmonella I*[b]	*Bacillus polymmyxa*[b]	*Bacillus schlegelii*[b]	*Staphylococcus epidermidis*[b]	*Staphylococcus hominis*[b]
Glucose	+	+	+	+	nr	a	a	a	+	+	+	+	+	+	+	+	+	+	a	n		
Lactose	–	–	–	–	nr	a	a	–	–	+	–	–	–	–	–	+	+	+		n	a	v
Sucrose	–	+	+	+	nr	v	a	–	+	+	+	+	v	+	–	+	+			n	a	a
Mannitol	+	+	+	v	nr	a	a	a	+	+	+	nr	+	–	+	+	+	+	a	n	n	n
Xylose	–	+	+	–	nr	a	n	a	+	+	–	–	–	–	+	+	+	+	a		n	n
Maltose	–	–	+	+	nr	a	a	nr	+	+	+	–	–	+	–	+	+	+			a	a
Glycerol	+	+	v	v	nr	nr	nr	nr	+	+	+	–	+	–	+	nr	nr	nr			nr	nr
Vitamin-free Growth	–	+	w	v	nr	na	na	na	–	+	–	v	a	–	p	na	na	na				
Nitrate assimilation	–	+	–	–	nr	nr	nr	na	–	–	+	–	–	–	nr	nr	nr	nr				
Cycloheximide	s	nr	nr	s	nr	res	res	res	nr	nr	nr	a	a	nr	nr	res	res	res			res	res

[a]From [11]
[b]From [5]

selective advantage when incubated at 37 °C and the data shown in Tables 2 and 3 support this hypothesis. In particular, when *P. pastoris* was cultured at its optimum temperature of 25 °C, cycloheximide did not completely limit colony formation. However, growth was completely suppressed at the higher, less permissive temperature of 37 °C; yet, the expected target contaminant groups flourished when incubated at this temperature. Temperature also enhanced the affects of cycloheximide. The growth of *C. albicans* on the avidin medium at 25 °C was variable between the experimental replicates but positive at 37 °C. Unexpectedly, *A. niger* grew at 37 °C in the presence of avidin.

The purpose of osmo-tolerance as a selection character was to enrich for fungi. TSA media containing different concentrations of sucrose or potassium chloride were prepared to enrich for fungi via osmo-tolerance. *A. niger* usually did not grow on any of the TSA/potassium chloride media when incubated at either temperature. When growth of the host organism occurred, *P. pastoris* isolates could not be differentiated from *A. niger* isolates until 3–4 days post-incubation. At this time, sufficient growth had occurred to visually inspect the colony morphologies. Greater discrimination between *P. pastoris* and *A. niger* was achieved on TSA supplemented with sucrose as the osmolite. *P. pastoris* GS115 did not grow in the presence of 2.5 M sucrose but *A. niger*, *C. albicans,* and *S. aureus* propagated with growth patterns that differed only slightly compared to those displayed on the control plates. *A. niger* was not predicted to grow at the lower osmotic pressure levels and at the less permissive temperature of 37 °C. Nonetheless, growth was robust suggesting that osmo-tolerance is a suitable criterion for the selection of a significant group of contaminants.

Although *Pichia* grew on ammonia, as expected, the host system also grew on nitrate, which was not expected. Still, nitrate/nitrite utilization may provide a suitable degree of selection because *P. pastoris* GS115 did not assimilate nitrite while all the other strains grew to varying degrees on this medium. A caveat may be associated with the nitrogen testing. The non-nitrogen medium components may contain a small but biologically relevant amount of organic and inorganic nitrogen as a contaminant. Nitrogen may also be present at adequate growth-sustaining levels from the yeast extract that was originally provided as a vitamin source.

To determine whether the contaminants could be recovered by using the tested media and growth conditions, aliquots of each contaminant were delivered to *P. pastoris* cultures. The suspensions spiked with *E. coli, S. aureus*, and *B. subtilis* were exposed to cycloheximide and avidin-based media and incubated at 37 °C. Sucrose and xylose media incubated at 25 °C were used for recovering *C. albicans, W. saturnus, S. cerevisiae* and *A. niger* from spiked cultures. In all cases, a lawn of growth occurred on the selected medium with no indication of more than one colony type. Follow-up identification of the lawns confirmed that growth was indeed

Table 2 Selective media screening at 25 °C. Each medium was repeated in duplicate for each organism as indicated by the two rows of results. All media were formulated as agar plates. The efficacy of all test conditions was determined by a qualitative comparison between the TSA control and the test condition. + + +, + +, and + reflect cell densities relative to a control. Blank cells indicate the organism was not tested at that condition

Organism		Control	Cycloheximide (mg/l)			Avidin (mg/l)			Osmo-tolerance							Carbon source			Nitrogen Source		
									Sucrose (molal)			Potassium chloride (molal)				Xylose	Sucrose	Maltose	NH_4	NO_2	NO_3
			75	125	150	0.25	0.5	1.0	2.0	2.5	3.0	1.0	1.5	2.5	3.0						
Yeast and fungi	*P. pastoris*	+++	+	+	+	–	–	–	+	–	–	+++	++	–	–	–	–	–	+	–	+
		+++	+	+	+	–	–	–	+	+	–	+++	++	+	–	–	–	+	+	–	+
	S. cerevisiae	+++	–	–	–	+	++	++	+	–	–	–	–	–	–	++	++	++	++	+	++
		+++	–	–	–	++	++	++	++	+	–	–	–	–	–	++	++	++	++	+	++
	C. albicans	+++	++	++	++	–	–	–	++	++	+	–	–	–	–	++	–	–	++	+	++
		+++	++	++	++	++	++	++	++	++	+	++	++	+	+	++	++	++	++	+	++
	W. saturnus	+++	–	–	–	++	++	++	–	–	–	+	–	–	–	++	++	++	++	+	++
		+++	–	–	–	++	++	++	–	–	–	+	–	–	–	++	++	++	++	+	++
	A. niger	+	–	–	–	+	+	+	++	++	+	+	–	–	–	–	+	+	++	+	++
		+++	–	–	–	++	–	+	++	++	++	+	–	–	–	–	++	++	++	+	++
Bacteria	*B. subtilis*	+++	++	++	++	–	–	–	+	–	–	++	+	–	–	–	–	–			
		+++	++	++	++	++	++	++	+	–	–	++	++	–	–	–	++	–			
	E. coli	+++	++	++	++	++	++	++	–	–	–	–	–	–	–	++	+	++			
		+++	++	++	++	++	++	++	–	–	–	–	–	–	–	++	+	+			
	S. aureus	+++				+	+	+	++	+	–	++	++	++	+	–	+	+			
		+++				++	++	++	++	+	–	++	++	++	+	–	+	+			

Table 3 Selective media screening at 37 °C. Each medium was repeated in duplicate for each organism as indicated by the two rows of results. All media were formulated as agar plates. The efficacy of all test conditions was determined by a qualitative comparison between the TSA control and test condition. + + +, + +, and + reflect cell density relative to a control. Blank cells indicate the organism was not tested at that condition

Organism		Control	Cycloheximide (mg/l)			Avidin (mg/l)			Osmo-tolerance							Carbon source			Nitrogen Source		
									Sucrose (molal)			Potassium chloride (molal)				Xylose	Sucrose	Maltose	NH_4	NO_2	NO_3
			75	125	150	0.25	0.5	1.0	2.0	2.5	3.0	1.0	1.5	2.5	3.0						
Yeast and Fungi	*P. pastoris*	++	–	–	–	+	–	–	+	–	–	+	+	+	–	–	–	–	+	–	–
		++	–	–	–	+	–	–	–	–	–	++	++	+	–	–	+	–	+	–	–
	S. cerevisiae	++	–	–	–	+	–	–	++	–	–	–	–	–	–	++	++	+	–	–	–
		++	–	++	++	++	++	++	++	–	–	–	–	–	–	++	++	+	–	–	–
	C. albicans	++	++	++	++	++	++	++	++	++	+	++	++	–	–	++	++	++	++	++	++
		++	++	++	++	++	++	++	++	++	+	++	++	+	–	++	++	++	++	++	++
	W. saturnus	++	–	–	–	++	++	++	–	–	–	–	–	–	–	++	++	+	–	+	–
		++	–	–	–	++	++	++	–	–	–	–	–	–	–	++	++	++	–	–	–
	A. niger	++				+	+	+	++	++	++	++	++	++	+	++	++	++	++	+	++
		++				++	++	++	++	++	++	++	++	++	+	++	++	++	++	+	++
Bacteria	*B. subtilis*	++	++	++	++	++	++	++	+	–	–	++	+	–	–	+	++	++			
		++	++	++	++	++	++	++	++	–	–	++	++	–	–	+	++	++			
	E. coli	++	++	++	++	+	++	+	–	–	–	++	–	–	–	++	+	++			
		++	++	++	++	++	++	++	–	–	–	++	–	–	–	++	++	++			
	S. aureus	++	++	++	++	++	++	++	++	++	–	++	++	++	++	–	++	+			
		++	++	++	++	++	++	++	++	++	–	++	++	++	++	–	++	+			

due to the test contaminant that had been mixed with the host cultures.

Detection limits

After a suitable medium and optimized growth conditions for recovering a given contaminant were determined, detection limits were established for that agent when mixed with *P. pastoris* cell lines. This was accomplished by adding fresh *P. pastoris* GS115 stock cultures with known, but varying concentrations (in CFUs) of a non-host agent. A sample of the spiked *P. pastoris* cells was transferred to the enrichment broth and grown under the conditions determined from the previous study and the corresponding plates were then inspected for growth. The lowest average concentration of a non-host agent that was initially transferred to the host system suspension and was positive for growth is listed in Table 4. Less than 15 CFU of the bacteria, molds, and the non-host yeast, *S. cerevisiae,* and less than 350 CFU of the remaining non-host yeasts were recovered from samples spiked into *P. pastoris* GS115 at a final CFU of $\sim 10^8$. When the spiked *P. pastoris* stock cultures were frozen, stored for 1–3 weeks in the vapor phase of liquid nitrogen and then re-tested, the detection limits of the assay remained relatively the same (data not shown).

Detection limits were determined for contaminants within high-cell-density fermentations. Because samples were randomly obtained from bench-top fermentations, which varied significantly in cell densities ranging between 180 and 430 OD_{600}, the data had to be normalized to percent detectable contaminant by optical density. The detection limit for each contaminant was subsequently determined on separate days from separate fermentation broths, and duplicate sets of data were obtained. The risk that recovery is dependent on the starting cell density of the fermentation broth was thereby minimized. As shown in Table 5, all of the non-host agents were recovered at less than 0.0001% contamination by optical density. Simple identity testing verified that the contaminant recovered was indeed the initial spiked adventitious agent. These results confirm that the test method is able to detect adventitious agents in at least the parts per million range with even lower limits possible with additional experimentation.

Simplicity/ruggedness

The test procedures were qualified for simplicity and ruggedness by transferring the assay to our departmental Quality Control (QC) laboratory. The QC personnel now routinely use the assay on all cell bank preparations and production fermentation broths. When indications

Table 4 Detection limits of contaminants within frozen stocks. The detection limit for the assay was determined from the average number of contaminant CFUs/ml spiked into the *P. pastoris* culture. Values recorded as "less than" indicate no further dilution was completed to determine an absolute minimum. Contaminant load was considered detectable after recovery and confirmation of the contaminant from the agar plate

	Detection limits/vial containing:			
Organism	Cycloheximide	Avidin	Sucrose	Xylose
E. coli	< 5.6 CFU	< 5.6 CFU		
S. aureus	< 4.6 CFU	< 4.6 CFU		
B subtilis	< 4.1 CFU	< 4.1 CFU		
C. albicans			< 320	< 320
W. saturnus			120	120
S. cerevisiae			13	13
A. niger			< 3.5	< 3.5
P. pastoris	No growth	No growth	No growth	No growth

Table 5 Detection limits of contaminants within fermentors. The detection limit for the assay is recorded as the percentage of optical density units of contaminant spiked into the fermentation broth sample compared to the sample. Contaminant load was considered detectable after recovery and confirmation of the contaminant from the agar plate

	Detection limits/*Pichia* cell density			
Organism	Cycloheximide	Avidin	Sucrose	Xylose
E. coli	< 0.0001%	< 0.0001%		
S. aureus	< 0.0001%	< 0.0001%		
B subtilis	< 0.0001%	< 0.0001%		
C. albicans			< 0.0001%	< 0.0001%
W. saturnus			< 0.0001%	< 0.0001%
S. cerevisiae			< 0.0001%	< 0.0001%
A. niger			< 0.0001%	< 0.0001%
P. pastoris	No growth	No growth	No growth	No growth

of contamination have been reported, the QC staff have responded appropriately without the aid of a trained microbiologist to interpret the results.

This, of course, is not necessarily an indicator that the test procedure is robust. However, the fermentation development laboratory suffered from an increasing number of failed fermentations. Some runs were obviously contaminated with bacteria that were morphologically similar to a Gram-positive bacillus. By using the non-host procedures as described, samples from fermentation broths and environmental swabs were tested. From these samples, seven unique species of microbes were identified by the University of Nebraska Veterinary Diagnostic Laboratory and included *Bacillus cereus, Bacillus licheniformis, Bacillus polymyxa, Xanthomonas maltophilia, Pseudomonas alcaligenes, Enterococcus casseliflavus*, and *Candida glabrata*. The primary source of contamination was traced to an autoclave that was not operating properly.

Discussion

A key component in demonstrating process control is to ensure the purity of a microbial culture through all stages of the development and production processes. Despite all the training and the controls implemented, the possibility of contaminating a culture always exists. Perhaps the most critical control point is maintaining microbial purity of the cultures used to initiate the manufacturing process, e.g., the master cell banks (MCBs) and the working cell banks (WCBs). Contract manufacturing facilities are particularly prone to contaminants introduced from research banks or MCBs/WCBs originating from outside sources. Screening incoming cultures can prevent facility-wide outbreaks of organisms, such as most species of *Bacillus*. If a seed culture or production fermentation suspension becomes contaminated, early detection will prevent wasted efforts of proceeding with a process that is out of control. Conversely, a sufficiently robust method for monitoring culture purity provides evidence that a process is in control and that a quality intermediate and ultimately a final product has been produced.

Prior to this study, assays dedicated specifically to the recovery of potential contaminating organisms in *P. pastoris* cell lines had yet to be reported. It was our goal to develop a test procedure for screening seed stock cultures and fermentation broths of *P. pastoris* cultures that would satisfy the needs of a typical biopharmaceutical facility. Three design criteria were thus specified to develop such a non-host detection method. First, the method had to detect a broad range of microorganisms. Second, the method had to recover low levels of contaminants within a high population of host cells. And lastly, the test procedures and data had to be explicit enough to be completed and interpreted by a technician not trained as a microbiologist. To meet these criteria, a phenotype-based enrichment and selection method was deemed most appropriate.

Additional guidelines were established to ensure that certain quality indicators were designed into a test method to minimize the possibility of an ambiguous result. The initial specification was that "the solid agar medium must inhibit the growth of *P. pastoris* colonies while supporting the growth of a given adventitious agent". If this objective could not be achieved, then "the colony morphology differences between *P. pastoris* and the contaminant had to be so distinctive that a clear differentiation could be made between both organisms". For example, *P. pastoris* colonies present as shiny smooth off-white colonies that are 2–3 mm in diameter on YPD plates when incubated for 48 h at a temperature of ~25 °C. Fungal mass, distinctive coloration, or wrinkled colonies would then be considered unambiguous colony morphologies. The test procedures also had to generate results that were so decisive that a sample could be deemed contaminated in practical "on-line" applications without immediate verification from confirmatory testing.

By using only four distinct media formulations, cycloheximide, avidin, sucrose, and xylose, incubated at either 25 or 37 °C, non-host agents can be recovered and, when necessary, distinguished from *P. pastoris* strains. Seed stocks or fermentation broths can further be examined for a greater range of contaminants by including osmo-tolerance, nitrogen sources, or additional carbon sources. Perhaps the most difficult group of organisms to discern from *P. pastoris* are other yeasts. The selectivity and power of this assay towards yeasts was demonstrated within our facility when the yeast, *C. glabrata*, was recovered from active fermentations. Another advantage of this test is a clear indication of positive (contamination) versus negative (no contamination) results is provided. We have repeatedly shown that *P. pastoris* cells do not proliferate extensively on the selective agar plates regardless of the sample media matrix. As a result, the presence of any colonies on the selective agar must be considered a suspect contaminant. An admonition to this observation is the importance of establishing and observing expiration dates for prepared media, as cycloheximide and avidin media retain their selective properties for no longer than 30 days post-preparation.

Furthermore, the method as developed is sensitive enough to detect 4–6 bacterial or mold CFUs from a frozen stock containing 10^8 viable *P. pastoris* cells. Initially, these results were viewed with skepticism, but mutually corroborating results were obtained from production fermentation data and frozen stock culture data. Even lower detection limits may be achieved, but the experiments have not been completed to date.

In conclusion, adaptation of DNA-based methods for the detection of adventitious agents is feasible and more than likely will prove to be a more powerful method with higher sample-throughput capabilities.

Still, highly technical and expensive instrumentation will be required. Supplies to carry out the phenotype-based method described here are usually readily available in most QC laboratories, particularly for those in small start-up biopharmaceutical companies.

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References

1. Bar KA, Hopkins SA, Sreekrshna K (1992) Protocol for efficient secretion of HAS developed from *P. pastoris*. Pharm Eng 12:48–51
2. Barnett JA, Payne PW, Yarrow D (1979) In: Barnett JA, Payne RW, Yorrow D (eds) A guide to identifying and classifying yeasts. Cambridge University Press, Cambridge
3. Center for Biologics Evaluation and Research (1993) Points to consider in the characterization of cell lines used to produce biologicals. Food and Drug Administration, Rockville, Maryland, USA
4. Code of Federal Regulations, General biological product standards: sterility, Title 21 Part 610.12. US Government Printing Office, Washington, DC,http://www.fda.gov/cdrh/devadvice/part610.html
5. Sneath PHA, Mair, NS, Sharpe ME (1984) In: Holt G (ed) Bergey's manual of systematic bacteriology, vol 2. Williams and Wilkins, Baltimore, pp 1016–1017, 1122–1123
6. Cregg JM, Tschopp JF, Stillman C, Siegel R, Akong M, Craig WS, Buckholz RG, Maden KR, Kellais PA, Davis GR, Smiley BL, Cruze J, Rorregrossa R, Velicelibi G, Thill GP (1987) High Expression and efficient assembly of hepatitis B surface antigen in methylotrophic yeast, *P. Pastoris*. Bio/Technol 5:479–485
7. Diggon ME, Tschoop JF, Grinna L, Lair SV, Craig WS, Velicelebi GR, Thill GP (1988) Secretion of heterologous proteins from methylotrophic yeast, *P. pastoris*. Dev Ind Microbiol 29:59–65
8. Higgins DR, Cregg JM (1998) Introduction to *Pichia Pastoris*. In: Higgins DR, Cregg JM (eds) *Pichia* protocols. Humana, Totowa, New Jersey, pp 1–15
9. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (1997) Q5D: Derivation and characterization of cell substrates used for production of biotechnological/biological broduct. Geneva, Switzerland
10. Larouch Y, Strme V, DeMuetter J, Messens J, Lauwerys M (1994) High level secretion and very efficient isotopic labeling of tich anticoagulent peptide (TAP) expressed in the methylotrophic yeast, *P. pastoris*. Bio/Technol 12:1119–1124
11. Lodder J, (ed) (1970) In: The yeasts. North Holland, Amsterdam
12. Peng YC, Acheson NH (1997) Production of active polyomavirus large T antigen in yeast, *P. pastoris*. Virus Res 49:41–47
13. Siegel RS, Brierley RA (1989) Methylotrophic yeast *P. pastoris* produced in high cell density fermentations with high cell yields as a vehicle for recombinant protein production. Biotechnol Bioeng 34:403–404
14. Smith PM, Suphioglu D, Griffith IJ, Theriault K, Know RB, Singh MB (1996) Cloning and expression in yeast, *P. pastoris*, of a biologically active form of Cynd1, the major allergen of Bermuda grass pollen. J Allergy Clin Immunol 93:331–343
15. Stratton J, Chinuvolu V, Meagher M (1998) High cell density fermentation. In: Higgins DR, Cregg JM (eds) *Pichia* protocols. Humana, Totowa, New Jersey, pp. 95–106
16. Tscopp JF, Sverlow G, Kosson, Craig RW, Ginna L (1989) High level secretion of glycosylated invertase in methylotrophic yeast, *P. pastoris*. Bio/Technol. 5:1305–1308
17. United States Pharmacopeia and National Formulary, (January 2000), Rockville, Maryland
18. van der Walt JP (1970) Criteria and methods used in classification. In: Lodder J (ed)The yeasts. North Holland, Amsterdam, pp 34–113
19. Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM (2000) Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnol Bioeng 70:1–8
20. Zhang W, Inan M, Meagher MM (2000) Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*. Biotechnol Bioprocess Eng 5:275–287