CHAPTER IV

RESULTS

4.1 Multilocus phylogenetic analyses and phenotypic characterization of tropical isolates of *Aureobasidium pullulans*

4.1.1 *Aureobasidium* isolates

The 53 fungal isolates used in this study (Table 4.1), 45 isolates (CU 1 through CU 47) were collected from 15 provinces in Thailand between August, 2005 and May, 2006 (CU 34 and CU 43 were excluded from this study). One isolate (DOUG) was collected from Washington Crossing, PA, USA, whereas 3 isolates (NRRL Y-12974, NRRL Y-6220, and NRRL Y-2311-1) were reference strains from the ARS Culture Collection, USDA, Peoria, IL, USA. The other 4 isolates (NRM2, BM1, HKW1, and PH1) were tropical comparative strains from the Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. All new isolates were deposited at the ARS Culture Collection, National Center for Agricultural Utilization Research, USDA, Peoria, IL, USA. The strain accession number was shown in Table 4.1. Moreover, they were deposited at Fungal Section, Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The strain accession number was shown in Appendix H.

Among the 45 new isolates from Thailand, 43 isolates were from leaf samples, one isolate was from a painted wall surface, and the other isolate was from a wood surface. Isolates were collected from Pathumthani (8 isolates), Ubonratchathani (7 isolates), Prachuapkhirikhan (6 isolates), Chanthaburi (5 isolates), Ratchaburi (4 isolates), Bangkok (3 isolates), Udonthani (2 isolates), Lopburi (2 isolates), Nongkhai (2 isolates), and Ayutthaya, Chiangmai, Nakhonratchasima, Rayong, Trang, and Trat (1 isolate each).

The 43 isolates from leaf samples were isolated from mango (8 isolates), banyan (6 isolates), needle pine (3 isolates), tamarind (3 isolates), Spanish cherry, East Indian

walnut, mangoesteen, palm, and rose apple (2 isolates each), and 13 other trees (1 isolate each) (Table 4.1).

The collection sites and period of collection and isolation were shown in Table 4.2. Even though, total rain, height above sea level and collection and isolation period were different, the isolates were not specific or limited at certain area. They could be dispersed in every clade as described in the next topic.

4.1.2 Phylogenetic analyses

DNA sequences determined in this study were deposited in GenBank under accession numbers EU719335 – EU719602 (Table 4.1). Data on the phylogenetic analyses of each of five loci and combined trees were summarized in Table 4.3. ITS and *EF-1α* sequences could not be used to differentiate individual isolates, while IGS1, *BT2*, and *RPB2* sequences had sufficient informative characters to classify all isolates into 12 main clades (Figure 4.1 – 4.7 and Table 4.4).

The IGS1 locus had the most informative characters, but the IGS1 locus of CU 26, CU 30, and NRRL Y-12974 could not be amplified under conditions used in this study, and therefore both four-locus combined and five-locus combined trees were made (Figure 4.6 - 4.7). The *RPB2* tree had a high number of phylogenetically uninformative variable characters as well as informative characters, and a low number of equally most parsimonious trees. The number of variable and informative characters in the *BT2* tree was not high enough to differentiate isolates in clade 1 from clade 2 (Figure 4.4).

Table 4.1 *Aureobasidium pullulans* isolates and other strains used in this study

Isolate[a]	Strain accession number	Sample	Host/Source	Collection site	GenBank accession number				
					ITS	IGS1	*EF-1α*	*BT*	*RPB2*
CU 1	NRRL 58514	leaf	*Mangifera indica* L. (Mango)	Ayutthaya, Thailand	EU719506	EU719454	EU719345	EU719400	EU719559
CU 2	NRRL 58515	leaf	*Lagerstroemia loudonii* Binn. (Laza wood)	Bangkok, Thailand	EU719517	EU719464	EU719356	EU719411	EU719570
CU 3	NRRL 58516	wall	Painted wall surface	Patumthani, Thailand	EU719527	EU719473	EU719366	EU719421	EU719580
CU 4	NRRL 58517	leaf	*Crescentia alata* HBK	Bangkok, Thailand	EU719536	EU719482	EU719375	EU719430	EU719589
CU 5	NRRL 58518	leaf	*Samanea saman* (Jacq.) (East Indian walnut)	Patumthani, Thailand	EU719537	EU719483	EU719376	EU719431	EU719590
CU 6	NRRL 58519	leaf	*Ficus annlata* (Banyan tree)	Patumthani, Thailand	EU719538	EU719484	EU719377	EU719432	EU719591
CU 7	NRRL 58520	leaf	*Desmos Chinensis*	Ratchaburi, Thailand	EU719539	EU719485	EU719378	EU719433	EU719592
CU 8	NRRL 58521	leaf	*Bambusa* sp. (Bamboo)	Ratchaburi, Thailand	EU719540	EU719486	EU719379	EU719434	EU719593
CU 9	NRRL 58522	leaf	*Eucalyptus citriodara* (Eucalyptus)	Ratchaburi, Thailand	EU719541	EU719487	EU719380	EU719435	EU719594
CU 10	NRRL 58523	leaf	*Ficus benjamina* L. (Banyan tree)	Ubonratchathani, Thailand	EU719496	EU719444	EU719335	EU719390	EU719549
CU 11	NRRL 58524	leaf	*Ficus benjamina* L. (Banyan tree)	Ubonratchathani, Thailand	EU719497	EU719445	EU719336	EU719391	EU719550
CU 12	NRRL 58525	leaf	*Tamarindus indica* L. (Tamarind)	Ubonratchathani, Thailand	EU719498	EU719446	EU719337	EU719392	EU719551
CU 13	NRRL 58526	leaf	*Tamarindus indica* L. (Tamarind)	Ubonratchathani, Thailand	EU719499	EU719447	EU719338	EU719393	EU719552
CU 14	NRRL 58527	leaf	*Ficus benjamina* L. (Banyan tree)	Ubonratchathani, Thailand	EU719500	EU719448	EU719339	EU719394	EU719553
CU 15	NRRL 58528	leaf	*Ficus benjamina* L. (Banyan tree)	Ubonratchathani, Thailand	EU719501	EU719449	EU719340	EU719395	EU719554
CU 16	NRRL 58529	leaf	*Ficus benjamina* L. (Banyan tree)	Ubonratchathani, Thailand	EU719502	EU719450	EU719341	EU719396	EU719555
CU 17	NRRL 58530	leaf	*Mimusops elengi* L. (Spanish cherry)	Patumthani, Thailand	EU719503	EU719451	EU719342	EU719397	EU719556
CU 18	NRRL 58531	leaf	*Mangifera indica* L. (Mango)	Lopburi, Thailand	EU719504	EU719452	EU719343	EU719398	EU719557
CU 19	NRRL 58532	leaf	*Chamaedorea erumpens* (Palm)	Lopburi, Thailand	EU719505	EU719453	EU719344	EU719399	EU719558
CU 20	NRRL 58533	leaf	*Mangifera indica* L. (Mango)	Patumthani, Thailand	EU719507	EU719455	EU719346	EU719401	EU719560
CU 21	NRRL 58534	leaf	*Garcinia Mangostana* L. (Mangosteen)	Chanthaburi, Thailand	EU719508	EU719456	EU719347	EU719402	EU719561

Table 4.1 (continued)

Isolate[a]	Strain accession number	Sample	Host/Source	Collection site	GenBank accession number				
					ITS	IGS1	*EF-1α*	*BT*	*RPB2*
CU 22	NRRL 58535	leaf	*Garcinia Mangostana* L. (Mangosteen)	Chanthaburi, Thailand	EU719509	EU719457	EU719348	EU719403	EU719562
CU 23	NRRL 58536	leaf	*Eugenia jumbos* (Rose apple)	Chanthaburi, Thailand	EU719510	EU719458	EU719349	EU719404	EU719563
CU 24	NRRL 58537	leaf	*Eugenia jumbos* (Rose apple)	Chanthaburi, Thailand	EU719511	EU719459	EU719350	EU719405	EU719564
CU 25	NRRL 58538	leaf	*Lansium domesticum* Corr.	Chanthaburi, Thailand	EU719512	EU719460	EU719351	EU719406	EU719565
CU 26	NRRL 58539	leaf	*Cerbera odollum* Gaertn.	Nakhonratchasima, Thailand	EU719513	_____[e]	EU719352	EU719407	EU719566
CU 27	NRRL 58540	leaf	*Kerriodoxa elegans* (Palm)	Prachuapkhirikhan, Thailand	EU719514	EU719461	EU719353	EU719408	EU719567
CU 28	NRRL 58541	leaf	*Pinus merkusii* Jungh. & de Vriese (Needle pine)	Prachuapkhirikhan, Thailand	EU719515	EU719462	EU719354	EU719409	EU719568
CU 29	NRRL 58542	leaf	*Pinus merkusii* Jungh. & de Vriese (Needle pine)	Prachuapkhirikhan, Thailand	EU719516	EU719463	EU719355	EU719410	EU719569
CU 30	NRRL 58543	wood	Wood surface	Prachuapkhirikhan, Thailand	EU719518	_____[e]	EU719357	EU719412	EU719571
CU 31	NRRL 58544	leaf	*Pinus merkusii* Jungh. & de Vriese (Needle pine)	Prachuapkhirikhan, Thailand	EU719519	EU719465	EU719358	EU719413	EU719572
CU 32	NRRL 58545	leaf	*Mangifera indica* L. (Mango)	Trat, Thailand	EU719520	EU719466	EU719359	EU719414	EU719573
CU 33	NRRL 58546	leaf	*Pandanus odoratissimus* (Screw pine)	Prachuapkhirikhan, Thailand	EU719521	EU719467	EU719360	EU719415	EU719574
CU 35	NRRL 58547	leaf	*Hopea ferrea* Laness.	Patumthani, Thailand	EU719522	EU719468	EU719361	EU719416	EU719575
CU 36	NRRL 58548	leaf	*Mangifera indica* L. (Mango)	Udonthani, Thailand	EU719523	EU719469	EU719362	EU719417	EU719576
CU 37	NRRL 58549	leaf	*Peltophorum pterocarpum* (Yellow Flamboyant)	Udonthani, Thailand	EU719524	EU719470	EU719363	EU719418	EU719577
CU 38	NRRL 58550	leaf	*Mangifera indica* L. (Mango)	Nongkhai, Thailand	EU719525	EU719471	EU719364	EU719419	EU719578
CU 39	NRRL 58551	leaf	*Mimusops elengi* L. (Spanish cherry)	Nongkhai, Thailand	EU719526	EU719472	EU719365	EU719420	EU719579
CU 40	NRRL 58552	leaf	*Sandoricum indicum* Cav. (Santol)	Chiangmai, Thailand	EU719528	EU719474	EU719367	EU719422	EU719581
CU 41	NRRL 58553	leaf	*Syzygium cumini* L. (Black plum)	Patumthani, Thailand	EU719529	EU719475	EU719368	EU719423	EU719582
CU 42	NRRL 58554	leaf	*Mangifera indica* L. (Mango)	Rayong, Thailand	EU719530	EU719476	EU719369	EU719424	EU719583
CU 44	NRRL 58555	leaf	*Saraca indica* (Canna or Indian shoot)	Ratchaburi, Thailand	EU719532	EU719478	EU719371	EU719426	EU719585

Table 4.1 (continued)

Isolate[a]	Strain accession number	Sample	Host/Source	Collection site	GenBank accession number				
					ITS	IGS1	*EF-1α*	*BT*	*RPB2*
CU 45	NRRL 58556	leaf	*Samanea saman* Jacq. (East Indian walnut)	Bangkok, Thailand	EU719533	EU719479	EU719372	EU719427	EU719586
CU 46	NRRL 58557	leaf	*Mangifera indica* L. (Mango)	Trang, Thailand	EU719534	EU719480	EU719373	EU719428	EU719587
CU 47	NRRL 58558	leaf	*Tamarindus indica* L. (Tamarind)	Patumthani, Thailand	EU719535	EU719481	EU719374	EU719429	EU719588
DOUG	NRRL 58559	wall	Painted wood surface	Washington Crossing, PA, USA	EU719543	EU719490	EU719383	EU719437	EU719596
NRM2[b]	NRRL 58560	leaf	Unknown	Nakhonratchasima, Thailand	AY225165	EU719492	EU719385	EU719439	EU719598
BM1[b]	NRRL 58561	grout	Bathroom	Bangkok, Thailand	EU719542	EU719489	EU719382	EU719436	EU719595
HKW1[b]	NRRL 58562	grout	Bathroom	Bangkok, Thailand	EU719544	EU719491	EU719384	EU719438	EU719597
PH1[b]	NRRL 58563	grout	Bathroom	Bangkok, Thailand	EU719545	EU719493	EU719386	EU719440	EU719599
	NRRL Y-2311-1[c]	stump	*Pinus elliottii* (Slash pine)	Fort Meyers, Florida, USA	EU719547	EU719495	EU719388	EU719442	EU719601
	NRRL Y-6220[c]	soil	Soil	Unknown	EU719548	EU719494	EU719389	EU719443	EU719602
	NRRL Y-12974[c]	leaf	Order: *Alismatales* (Seagrass)	Mangrove Cay, Florida, USA	EU719546	_____[e]	EU719387	EU719441	EU719600
	NRRL 187[d]	Unknown	Unknown	Unknown	EF652427	EU719488	EU719381	EF652251	EF652163

[a] CU 1-CU 47 and DOUG are new isolates collected for this study. CU abbreviates Chulalongkorn University. DOUG was named with kind thanks to Prof. Dr. Douglas E. Eveleigh, Department of Biochemistry and Microbiology, School of Environmental and Biological Sciences, Rutgers University, New Jersey, USA

[b] Comparative strains from Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University. NRM2 was isolated by Dr. Sehanat Prasongsuk (Prasongsuk *et al.*, 2005, 2007) and BM1, HKW1, and PH1 were isolated by Ms. Pacharawan Deenarn (Thesis for Degree of Master of Science in Biotechnology, Faculty of Science, Chulalongkorn University, 2006)

[c] Reference strains from the ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.

[d] Outgroup species (*Aspergillus nidulans*).

[e] Unsuccessful PCR amplification.

Table 4.2 Collection sites of *Aureobasidium pullulans* isolates and other strains used in this study

Isolate[a]	Sample	Host/Source	Collection site	Description of collection site during isolation period				Collection and isolation period
				Average temperature (°C)	Total rain (mm)	Height above sea level (m)	Area	
CU 1	leaf	*Mangifera indica* Linn. (mango)	Ayutthaya, Thailand	29.5	54.1	8	Lowland	August 2005
CU 2	leaf	*Lagerstroemia loudonii* Binn. (Laza wood)	Bangkok, Thailand	28.8	358.5	14	Lowland	September 2005
CU 3	wall	Painted wall surface	Patumthani, Thailand	28.0	117.7	4	Lowland	November 2005
CU 4	leaf	*Crescentia alata* HBK	Bangkok, Thailand	26.4	33.1	14	Lowland	December 2005
CU 5	leaf	*Samanea saman* (Jacq.) (East Indian walnut)	Patumthani, Thailand	25.9	5.5	4	Lowland	December 2005
CU 6	leaf	*Ficus annlata* (Banyan Tree)	Patumthani, Thailand	25.9	5.5	4	Lowland	December 2005
CU 7	leaf	*Desmos Chinensis*	Ratchaburi, Thailand	25.0	58.7	12	Lowland	December 2005
CU 8	leaf	*Bambusa* sp. (Bamboo)	Ratchaburi, Thailand	25.0	58.7	12	Lowland	December 2005
CU 9	leaf	*Eucalyptus citriodara* (Eucalyptus)	Ratchaburi, Thailand	25.0	58.7	12	Lowland	December 2005
CU 10	leaf	*Ficus benjamina* Linn. (Banyan tree)	Ubonratchathani, Thailand	23.4	0.9	125	Lowland	December 2005
CU 11	leaf	*Ficus benjamina* Linn. (Banyan tree)	Ubonratchathani, Thailand	23.4	0.9	125	Lowland	December 2005
CU 12	leaf	*Tamarindus indica* Linn. (Tamarind)	Ubonratchathani, Thailand	23.4	0.9	125	Lowland	December 2005
CU 13	leaf	*Tamarindus indica* Linn. (Tamarind)	Ubonratchathani, Thailand	23.4	0.9	125	Lowland	December 2005
CU 14	leaf	*Ficus benjamina* Linn. (Banyan tree)	Ubonratchathani, Thailand	23.4	0.9	125	Lowland	December 2005
CU 15	leaf	*Ficus benjamina* Linn. (Banyan tree)	Ubonratchathani, Thailand	23.4	0.9	125	Lowland	December 2005
CU 16	leaf	*Ficus benjamina* Linn. (Banyan tree)	Ubonratchathani, Thailand	23.4	0.9	125	Lowland	December 2005
CU 17	leaf	*Mimusops elengi* Linn. (Spanish cherry)	Patumthani, Thailand	25.9	5.5	4	Lowland	December 2005
CU 18	leaf	*Mangifera indica* Linn. (Mango)	Lopburi, Thailand	26.5	0	102	Lowland	January 2006
CU 19	leaf	*Chamaedorea erumpens* (Palm)	Lopburi, Thailand	26.5	0	102	Lowland	January 2006

Table 4.2 (continued)

Isolate[a]	Sample	Host/Source	Collection site	Description of collection site during isolation period				Collection and isolation period
				Average temperature (°C)	Total rain (mm)	Height above sea level (m)	Area	
CU 20	leaf	*Mangifera indica* Linn. (Mango)	Patumthani, Thailand	27.6	1.3	4	Lowland	January 2006
CU 21	leaf	*Garcinia Mangostana* Linn. (Mangosteen)	Chanthaburi, Thailand	26.8	0	9	Lowland	January 2006
CU 22	leaf	*Garcinia Mangostana* Linn. (Mangosteen)	Chanthaburi, Thailand	26.8	0	9	Lowland	January 2006
CU 23	leaf	*Eugenia jumbos* (Rose apple)	Chanthaburi, Thailand	26.8	0	9	Lowland	January 2006
CU 24	leaf	*Eugenia jumbos* (Rose apple)	Chanthaburi, Thailand	26.8	0	9	Lowland	January 2006
CU 25	leaf	*Lansium domesticum* Corr.	Chanthaburi, Thailand	26.8	0	9	Lowland	January 2006
CU 26	leaf	*Cerbera odollum* Gaertn.	Nakhonratchasima, Thailand	25.0	0	183	Lowland	January 2006
CU 27	leaf	*Kerriodoxa elegans* (Palm)	Prachuapkhirikhan, Thailand	27.5	10.7	1	Sea shore	February 2006
CU 28	leaf	*Pinus merkusii* Jungh. & de Vriese (Needle pine)	Prachuapkhirikhan, Thailand	27.5	10.7	1	Sea shore	February 2006
CU 29	leaf	*Pinus merkusii* Jungh. & de Vriese (Needle pine)	Prachuapkhirikhan, Thailand	27.5	10.7	1	Sea shore	February 2006
CU 30	wood	Wood surface	Prachuapkhirikhan, Thailand	27.5	10.7	1	Sea shore	February 2006
CU 31	leaf	*Pinus merkusii* Jungh. & de Vriese (Needle pine)	Prachuapkhirikhan, Thailand	27.5	10.7	1	Sea shore	February 2006
CU 32	leaf	*Mangifera indica* Linn. (Mango)	Trat, Thailand	28.1	129.8	4	Lowland	March 2006
CU 33	leaf	*Pandanus odoratissimus* (Screw Pine)	Prachuapkhirikhan, Thailand	28.3	91.3	1	Sea shore	March 2006
CU 35	leaf	*Hopea ferrea* Laness.	Patumthani, Thailand	30.1	134.8	11	Lowland	March 2006
CU 36	leaf	*Mangifera indica* Linn. (Mango)	Udonthani, Thailand	29.0	45.8	180	Lowland	April 2006
CU 37	leaf	*Peltophorum pterocarpum* (DC.) (Yellow Flamboyant)	Udonthani, Thailand	29.0	45.8	180	Lowland	April 2006
CU 38	leaf	*Mangifera indica* Linn. (Mango)	Nongkhai, Thailand	28.6	263.6	164	Lowland	April 2006
CU 39	leaf	*Mimusops elengi Linn.* (Spanish cherry)	Nongkhai, Thailand	28.6	263.6	164	Lowland	April 2006
CU 40	leaf	*Sandoricum indicum*, Cav. (Santol)	Chiangmai, Thailand	28.4	206.7	1,100	Highland	April 2006

Table 4.2 (continued)

Isolate[a]	Sample	Host/Source	Collection site	Description of collection site during isolation period				Collection and isolation period
				Average temperature (°C)	Total rain (mm)	Height above sea level (m)	Area	
CU 41	leaf	*Syzygium cumini* L. (Black plum)	Patumthani, Thailand	30.6	49.6	4	Lowland	April 2006
CU 42	leaf	*Mangifera indica* Linn. (Mango)	Rayong, Thailand	29.8	132.1	4	Lowland	April 2006
CU 44	leaf	*Saraca indica* (Canna or Indian shoot)	Ratchaburi, Thailand	30.5	61.6	12	Lowland	April 2006
CU 45	leaf	Samanea saman (Jacq.) (East Indian Walnut)	Bangkok, Thailand	30.0	250.1	14	Lowland	May 2006
CU 46	leaf	*Mangifera indica* Linn. (Mango)	Trang, Thailand	27.1	365.3	29	Lowland	May 2006
CU 47	leaf	*Tamarindus indica* Linn. (Tamarind)	Patumthani, Thailand	30.0	153.1	4	Lowland	May 2006
DOUG	wall	Painted wood surface	Washington Crossing, PA, USA	25.3	142.2	13	Lowland	July 2006
NRM2[b]	leaf	Unknown	Nakhonratchasima, Thailand	Unknown	Unknown	183	Lowland	Unknown
BM1[b]	grout	Bathroom	Bangkok, Thailand	Unknown	Unknown	14	Lowland	Unknown
HKW1[b]	grout	Bathroom	Bangkok, Thailand	Unknown	Unknown	14	Lowland	Unknown
PH1[b]	grout	Bathroom	Bangkok, Thailand	Unknown	Unknown	14	Lowland	Unknown
NRRL Y-2311-1[c]	stump	Slash pine	Fort Meyers, Florida, USA	Unknown	Unknown	3	Lowland	Unknown
NRRL Y-6220[c]	soil	Soil	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
NRRL Y-12974[c]	leaf	Seagrass	Mangrove Cay, Florida, USA	Unknown	Unknown	0	Cay	Unknown
NRRL 187[d]	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown

[a] CU 1-CU 47 and DOUG are new isolates collected for this study.

[b] Comparative strains from Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University.

[c] Reference strains from the ARS Culture Collection , National Center for Agricultural Utilization Research, Peoria, IL, USA.

[d] Outgroup species (*Aspergillus nidulans*).

Isolate CU 9 was located in clade 1 in the IGS1 tree, but found in clade 2 in the *RPB2* tree (Figure 4.2). The reference strain NRRL Y-12974 was separated from other clades by *BT2* and *RPB2* sequences. The sequences of 2 isolates, CU 26 and CU 30, in clade 12 were distinct from others and classified as not *A. pullulans*. In addition, ITS sequences of CU 26 (EU719513) and CU 30 (EU719518) contained ca. 500 bp of presumptive intron. All other terminal groups of isolates were concordant with strong statistical support in both bootstrap and Bayesian analyses, supporting the terminal groupings.

The DNA sequences of *EF-1α* and *RPB2* of reference strain CBS 584.75 (ex-neotype) were previously reported in the database in GenBank. They were distinctly different from all *A. pullulans* isolates in this study, but were part of the ingroup clade (Figure 4.3 and 4.5).

The eleven ingroup clades were strongly supported by both the bootstrap and Bayesian posterior probability statistics. Trees generated from the individual loci either were not informative (ITS, *EF-1α*) or produced trees with the same terminal groups. A branch was considered strongly supported if the bootstrap proportion was 90 – 100% and if the posterior probability was 0.9 – 1.0. Statistical support for some of the deeper branches was lacking at some loci.

Table 4.3 Summary of tree statistics from parsimony analyses of 5 loci in *Aureobasidium pullulans*

Parameter	ITS locus	IGS1 locus	*EF-1α* locus	*BT2* locus	*RPB2* locus	Combined 4-locus tree[a]	Combined 5-locus tree
Number of taxa	58	51	56	54	56	54	51
Total characters	511	520	610	378	757	2284	2804
Variable characters	110	119	67	48	108	341	524
Informative characters	29	298	51	97	213	370	530
Number of equally parsimonious trees	>100	26	>100	4	10	>100	4
Consistency index	0.9627	0.7596	0.7360	0.7403	0.6728	0.8097	0.7532
Retention index	0.9310	0.9351	0.8368	0.9040	0.8849	0.8978	0.9105

[a]4 loci; ITS, *EF-1α*, *BT2*, and *RPB2*.

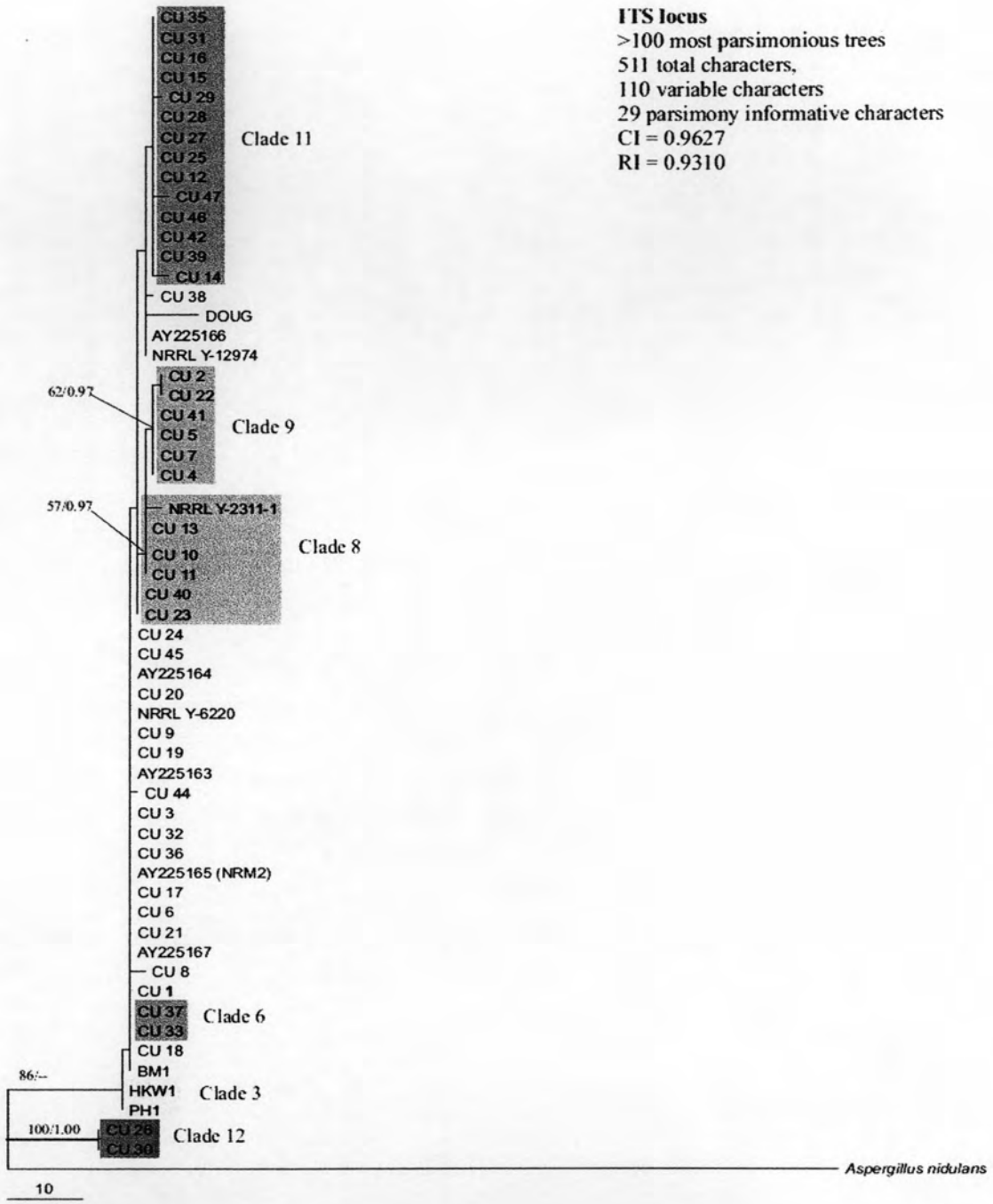

Figure 4.1 Maximum parsimony tree of the ITS region DNA from *Aureobasidium pullulans* isolates. Only the branch leading to CU 26 and CU 30 is strongly supported by the bootstrap and Bayesian posterior probabilities. Numbers above branches are the bootstrap/Bayesian posterior probabilities.

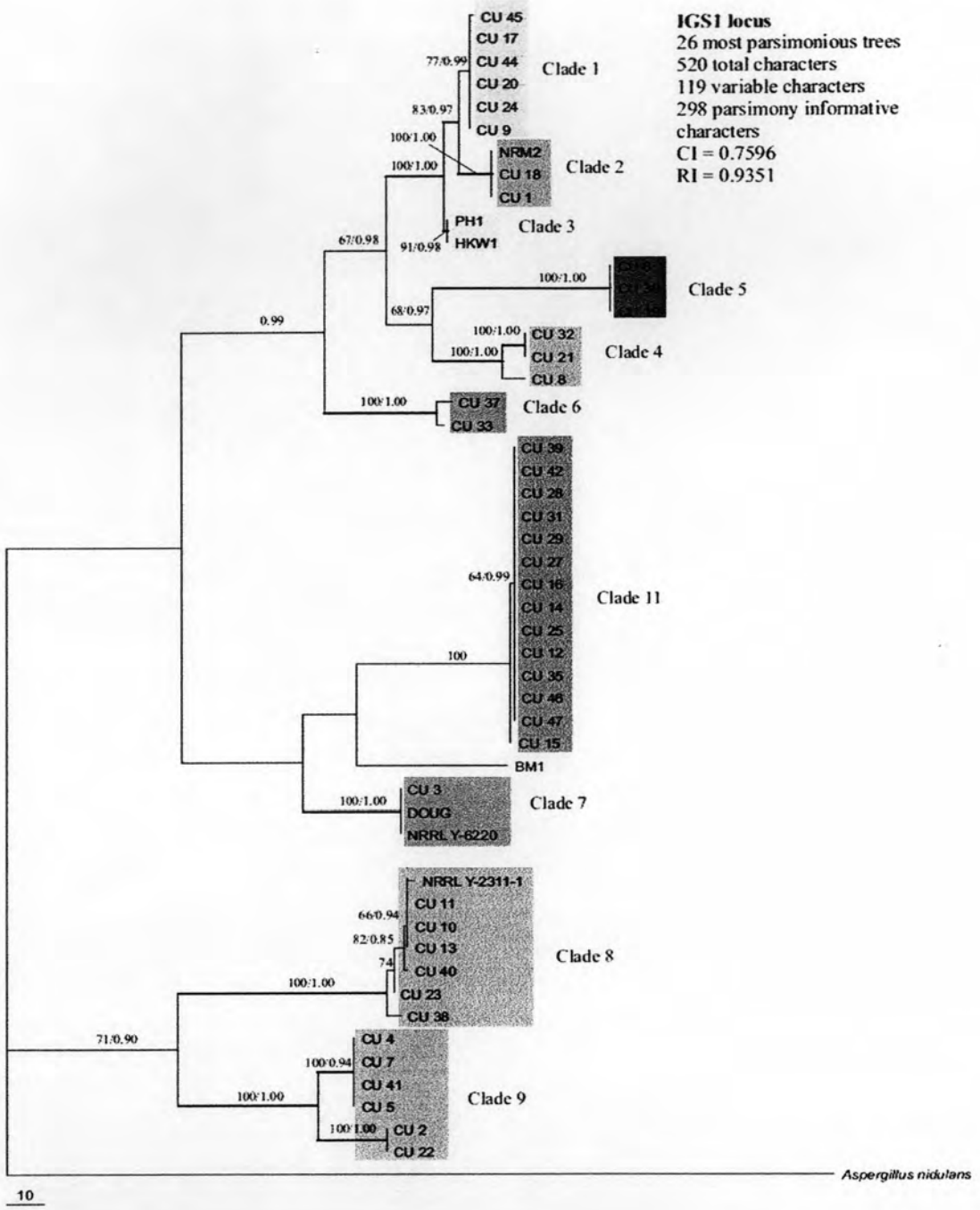

Figure 4.2 Maximum parsimony tree of the IGS1 region DNA from *Aureobasidium pullulans* isolates. IGS1 from NRRL Y-12974, CU 26 and CU 30 could not be amplified under our conditions. While most of the terminal groups are strongly supported in both statistics, deeper branches in the tree are mostly not statistically significant. Numbers above branches are the bootstrap/Bayesian posterior probabilities.

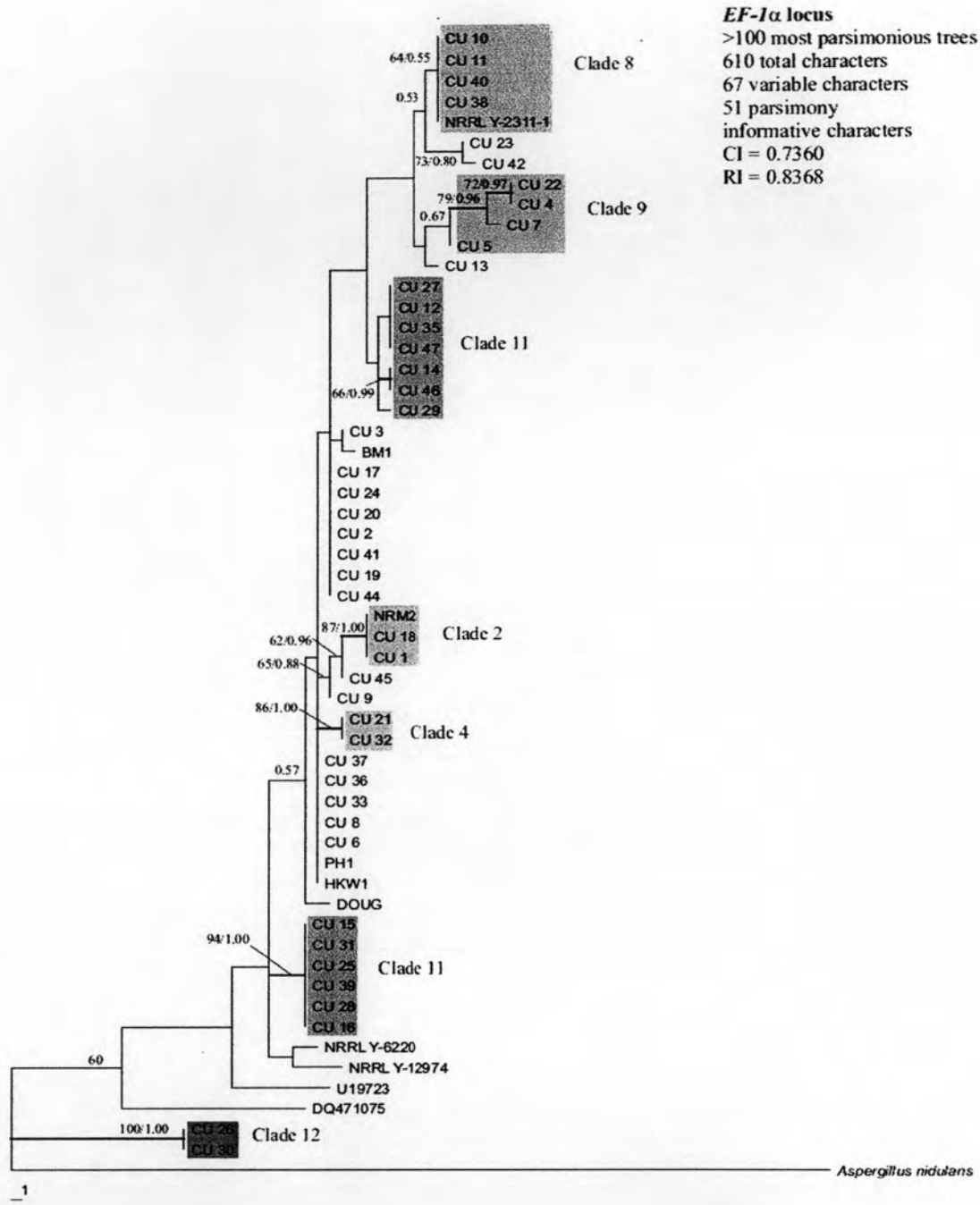

Figure 4.3 Maximum parsimony tree of the EF-1α region DNA from *Aureobasidium pullulans* isolates. CU 26 and CU 30 form a strongly supported clade outside of the ingroup. Sequence DQ471075 was obtained from GenBank and is from the ex-neotype isolate of *A. pullulans* (CBS 584.75). Only a few of the terminal groups are strongly supported in both statistics, deeper branches in the tree are mostly not statistically significant. Numbers above branches are the bootstrap/Bayesian posterior probabilities.

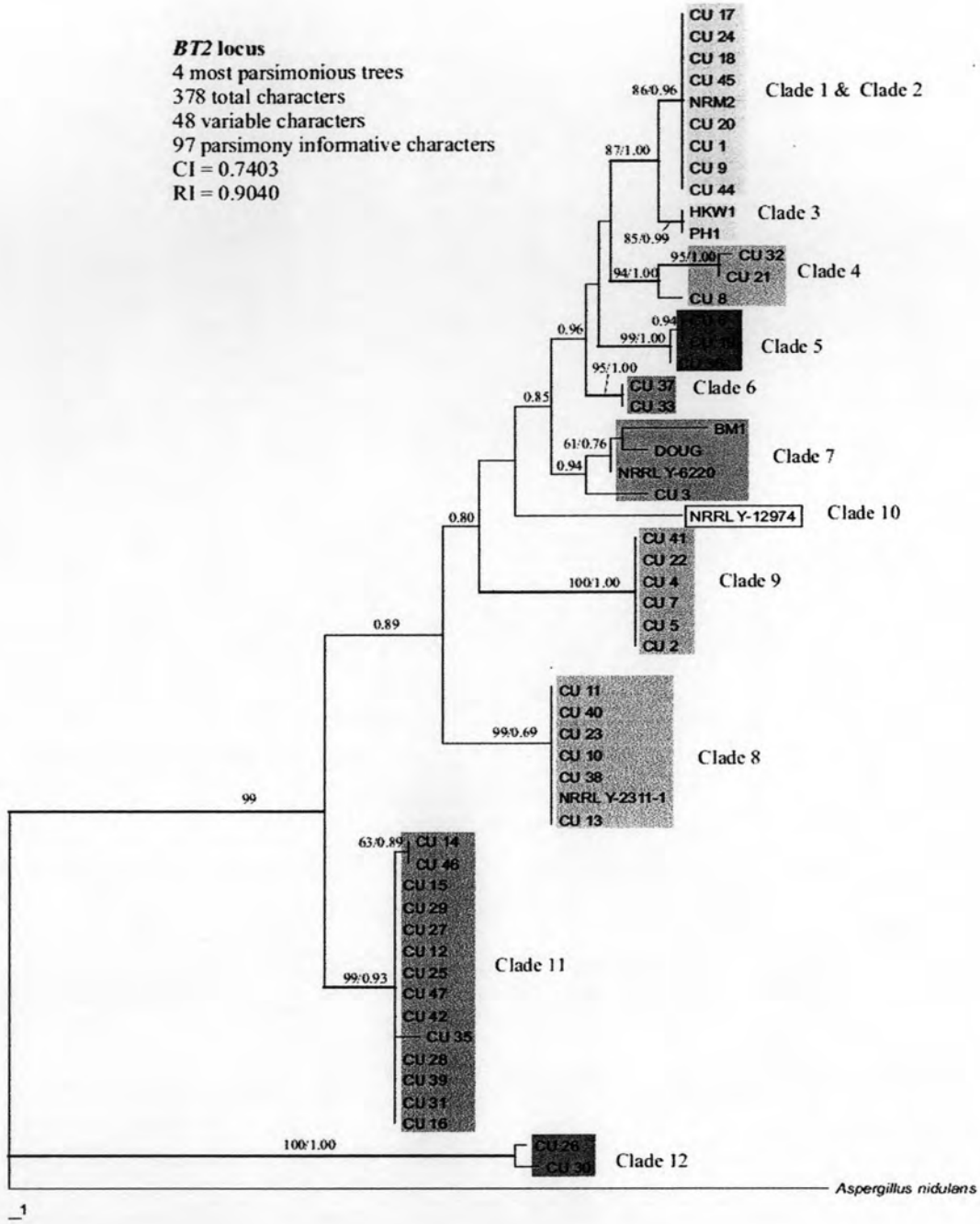

Figure 4.4 Maximum parsimony tree of the BT2 region DNA from *Aureobasidium pullulans* isolates. CU 26 and CU 30 form a strongly supported clade outside of the ingroup. Most of the terminal groups are strongly supported in both statistics, deeper branches in the tree are mostly not statistically significant. Isolates of clades I and II are identical at this locus. Numbers above branches are the bootstrap/Bayesian posterior probabilities.

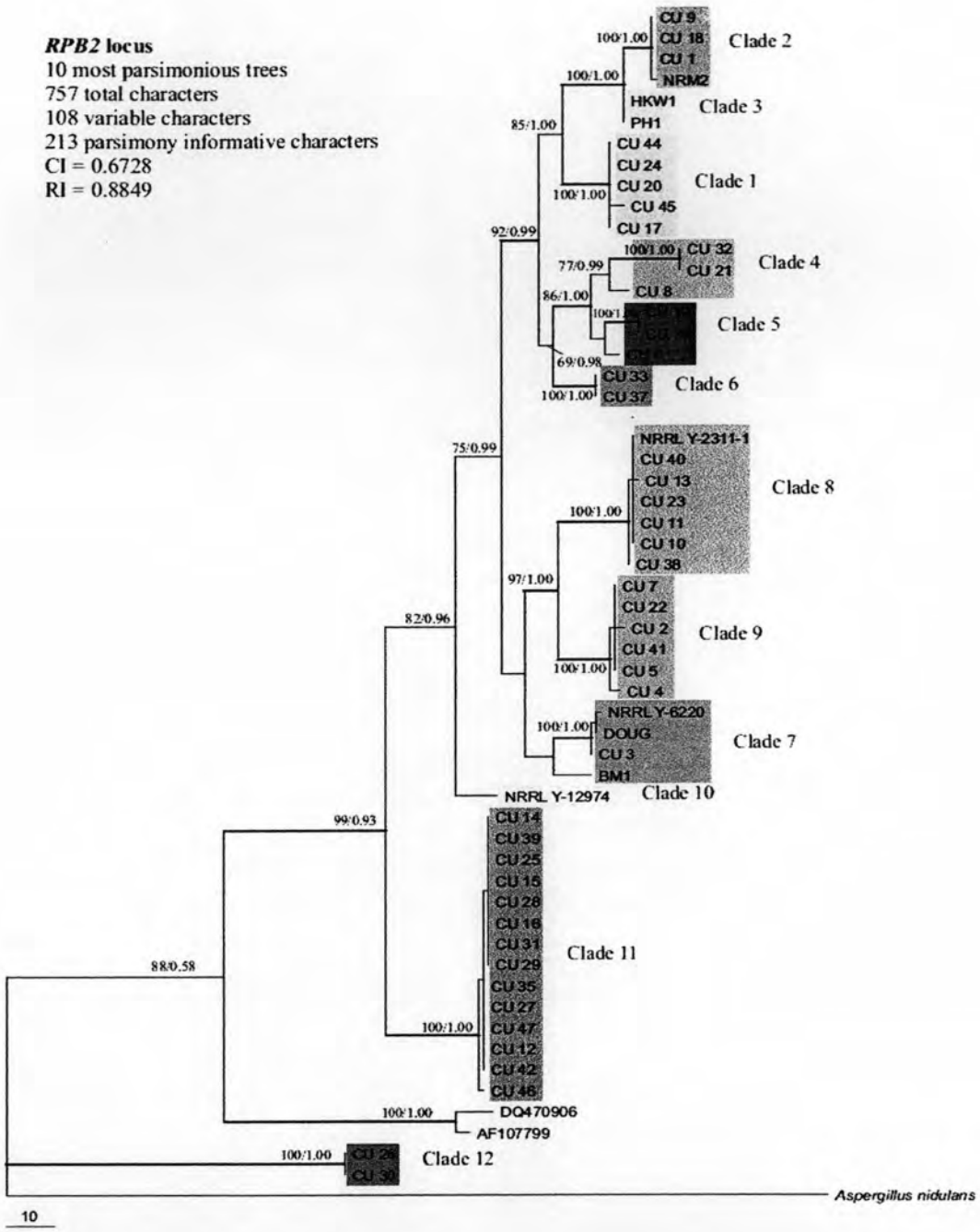

Figure 4.5 Maximum parsimony tree of the RPB2 region DNA from *Aureobasidium pullulans* isolates. CU 26 and CU 30 form a strongly supported clade outside of the ingroup. Sequence DQ470906 was obtained from GenBank and is from the ex-neotype isolate of *A. pullulans* (CBS 584.75). Most of the terminal groups are strongly supported by both statistics, not all deeper branches in the tree are statistically significant. Numbers above branches are the bootstrap/Bayesian posterior probabilities.

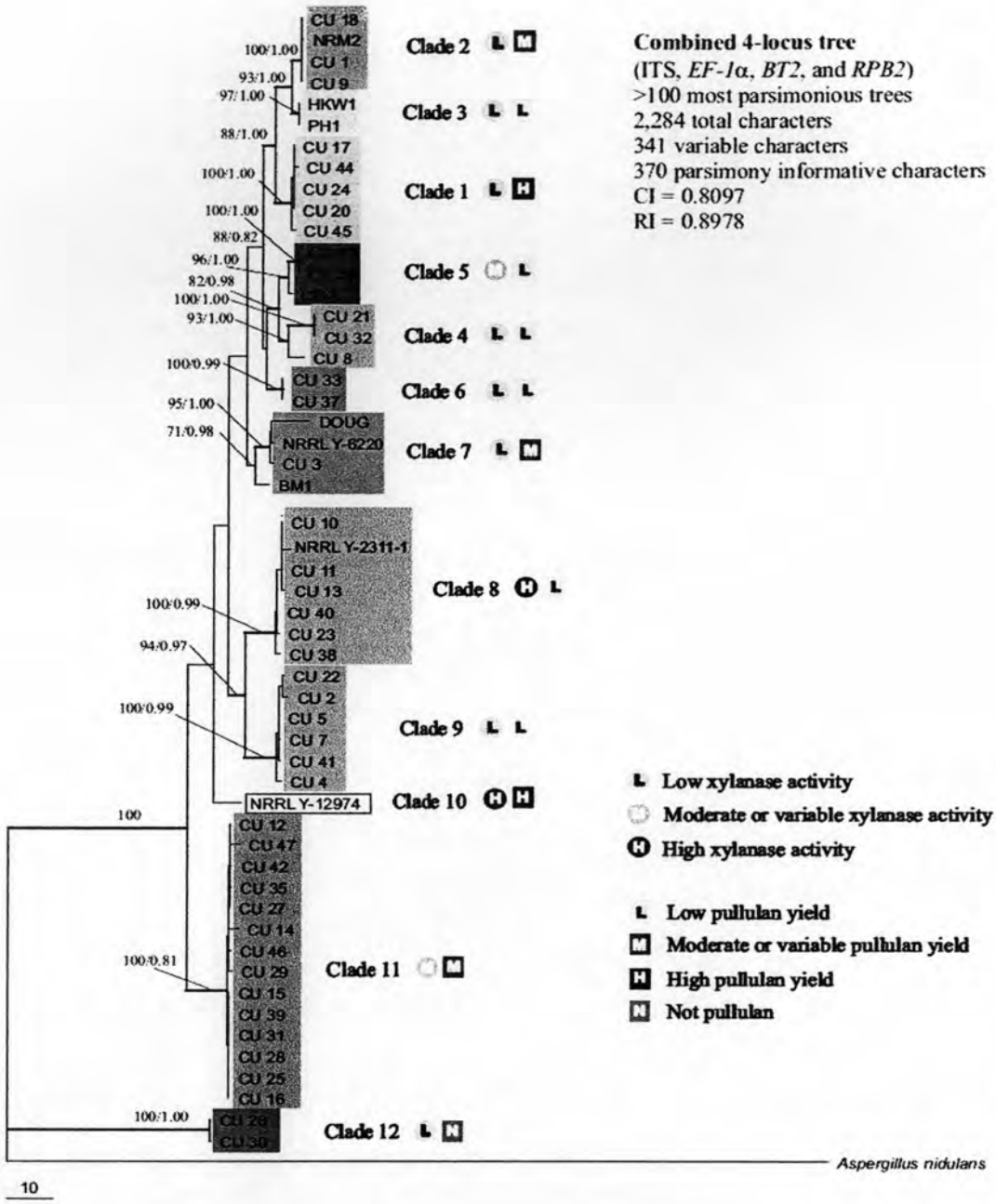

Figure 4.6 Maximum parsimony tree of the combined data from BT2, RPB2, EF-1α and ITS region DNA from *Aureobasidium pullulans* isolates. CU 26 and CU 30 form a strongly supported clade outside of the ingroup. Most of the terminal groups are strongly supported in both statistics, deeper branches in the tree are mostly not statistically significant. Numbers above branches are the bootstrap/Bayesian posterior probabilities.

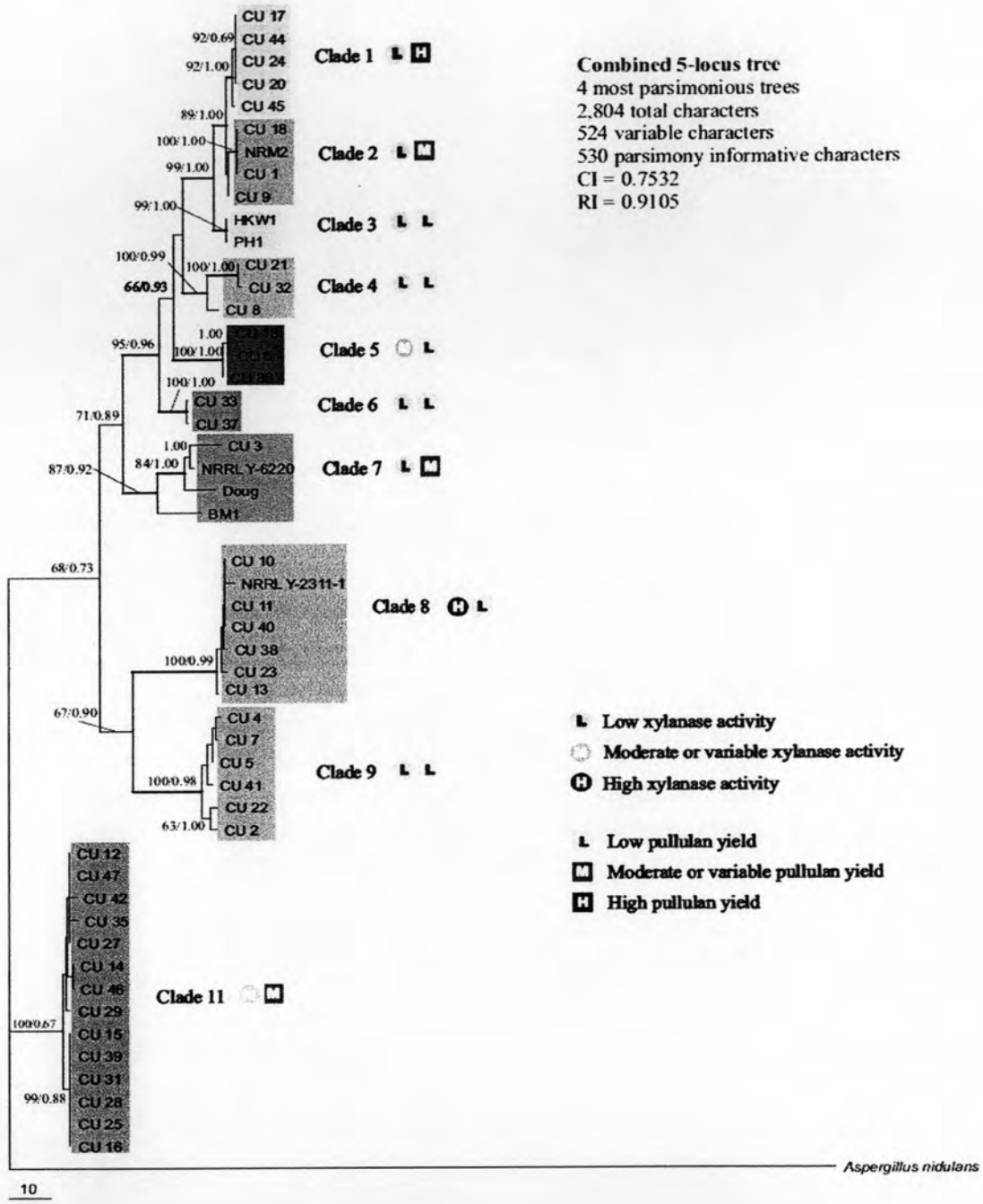

Figure 4.7 Maximum parsimony tree of the combined data from IGS1, BT2, RPB2, EF-1α and ITS region DNA from *Aureobasidium pullulans* isolates. Most of the terminal groups are strongly supported in both statistics, deeper branches in the tree are often not statistically significant. Numbers above branches are the bootstrap/Bayesian posterior probabilities.

Table 4.4 Clades of *Aureobasidium pullulans* and a closely related species with their phenotypes according to multilocus phylogenetic trees

Clade	Characteristics	Isolate	Color of colonies on		Pigmentation of pullulan production culture	Oil production	EPS production		Xylanase activity (U/ml)
			MEA at day 7	YMA at day 7 (and 2 months)			EPS (g/L)	g EPS/g cell	
1	High pullulan production White pullulan	CU 17	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to dark olivaceous)	Cream	-	29.0 ± 1.9	5.7	21.9 ± 0.2
		CU 20	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to black)	Cream	-	27.9 ± 1.0	4.5	22.4 ± 1.0
		CU 24	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to olivaceous)	Cream	-	21.9 ± 2.2	4.3	16.3 ± 0.1
		CU 44	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to olivaceous)	Cream	+	25.1 ± 2.7	5.4	18.4 ± 1.0
		CU 45	Dark olivaceous hyphae at center and white hyphae	Pink, (cream)	Cream	+	22.2 ± 1.2	4.3	19.6 ± 0.2
2	Closely related to clade 1 but lower pullulan yield	CU 1	Dark olivaceous hyphae at center and white hyphae	Pink, (dark olivaceous to black)	Light olivaceous	-	18.5 ± 1.3	2.6	20.4 ± 0.7
		CU 18	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to black)	Cream	-	11.3 ± 0.9	1.6	14.8 ± 0.4
		NRM2	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to dark olivaceous)	Cream	+	12.6 ± 0.6	1.9	13.4 ± 0.5
		CU 9	Brown (yeast like) at center, white hyphae, and orange red pigment diffused in agar	Pink and dark olivaceous at edge, (pink to black)	Orange pink	-	20.8 ± 0.4	3.5	26.8 ± 0.2
3	Low yield and dark pullulan Never turns black on YMA	HKW1	Dark olivaceous hyphae at center and white hyphae	Pink, (pink)	Olivaceous	-	7.0 ± 0.6	0.9	13.4 ± 0.3
		PH1	Dark olivaceous hyphae at center and white hyphae	Pink, (pink)	Olivaceous	-	5.6 ± 0.4	0.6	7.7 ± 0.5
4	Very low pullulan yield	CU 21	Dark olivaceous hyphae at center and white hyphae	Pink and dark olivaceous at edge, (cream, black at edge)	Cream	-	1.7 ± 0.2	0.2	21.5 ± 0.6
		CU 32	Dark olivaceous hyphae at center and white hyphae	Pink, (cream, black at edge)	Pale pink	-	1.9 ± 0.3	0.3	9.2 ± 1.1
		CU 8	Black hyphae at center and white hyphae	Pink, (black)	Dark olivaceous	+	2.9 ± 0.2	0.4	16.8 ± 0.9
5	Purple-red in PM Highly viscous EPS	CU 6	White hyphae with dark vinaceous pigment	Dark vinaceous, (brown to black)	Dark vinaceous	-	3.5 ± 0.3	0.3	41.1 ± 1.3
		CU 19	White hyphae with dark vinaceous pigment	Dark vinaceous, (brown, black at edge)	Dark vinaceous	-	6.8 ± 1.3	0.7	24.2 ± 0.7
		CU 36	White hyphae with dark vinaceous pigment	Dark vinaceous, (brown to dark olivaceous)	Dark vinaceous	-	10.2 ± 0.8	1.1	8.3 ± 0.6
6	Low yield and dark pullulan Turns black on YMA	CU 33	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to dark olivaceous)	Olivaceous	+	3.9 ± 0.1	0.6	17.0 ± 0.4
		CU 37	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to dark olivaceous)	Olivaceous	-	4.9 ± 0.5	0.7	25.2 ± 0.4

Table 4.4 (continued)

Clade	Characteristics	Isolate	Color of colonies on		Pigmentation of pullulan production culture	Oil production	EPS production		Xylanase activity (U/ml)
			MEA at day 7	YMA at day 7 (and 2 months)			EPS (g/L)	g EPS/g cell	
7		CU 3	Dark olivaceous hyphae at center and white hyphae	Pink, (pink to black)	Olivaceous	-	21.5 ± 1.5	2.9	28.9 ± 0.2
		DOUG	Pink, yeastlike at center and white hyphae	Pink, (pink to olivaceous)	Cream	-	13.8 ± 0.3	2.3	25.5 ± 0.6
		NRRL Y-6220	Dark olivaceous hyphae at center and white hyphae	Pink, (cream to olivaceous)	Cream	-	20.6 ± 1.8	3.2	17.2 ± 0.9
		BM1	Dark olivaceous hyphae at center and white hyphae	Pink, (green to dark olivaceous)	Dark olivaceous	-	2.3 ± 0.1	0.2	13.7 ± 0.3
8	Most exhibit color rings on YMA High xylanase activity Low pullulan production	CU 10	White hyphae with yellow pigment	Color ring[a], (cream and orange)	Pale pink	-	5.2 ± 0.5	0.8	94.3 ± 1.8
		CU 11	White hyphae with yellow pigment	Color ring[a], (cream and orange)	Pale pink	-	4.3 ± 0.4	0.7	91.4 ± 1.6
		CU 13	White hyphae with yellow pigment	Color ring[a], (cream, pink and orange)	Cream	-	6.9 ± 0.2	0.9	93.9 ± 1.2
		CU 23	White hyphae with yellow pigment	Color ring[a], (cream and pink)	Light olivaceous	-	2.7 ± 0.3	0.4	77.5 ± 0.5
		CU 38	White hyphae with light yellow pigment only at center	Pink, (cream and pink)	Pale pink	-	8.5 ± 0.4	0.9	53.8 ± 1.1
		CU 40	White hyphae with yellow pigment	Color ring[a], (cream and pink)	Light olivaceous	+	6.9 ± 0.5	1.0	78.3 ± 1.9
		NRRL Y-2311-1	White hyphae with yellow pigment	Color ring[a], (cream, pink and orange)	Cream	-	4.7 ± 0.1	0.9	152.4 ± 6.5
9	Initially black in PM Low pullulan production	CU 2	Dark olivaceous hyphae at center and white hyphae	Pink, (cream to black)	Olivaceous	+	1.3 ± 0.1	0.1	10.4 ± 0.4
		CU 4	Dark olivaceous hyphae at center and white hyphae	Pink, (dark olivaceous to black)	Dark olivaceous	-	3.9 ± 0.1	0.3	8.4 ± 0.1
		CU 5	Dark olivaceous hyphae at center and white hyphae	Pink, (cream to black)	Light olivaceous	+	2.0 ± 0.5	0.3	7.6 ± 0.2
		CU 7	Dark olivaceous hyphae at center and white hyphae	Pink, (cream to black)	Light olivaceous	+	1.9 ± 0.5	0.2	8.6 ± 0.0
		CU 22	Dark olivaceous hyphae at center and white hyphae	Pink, (cream to black)	Light olivaceous	+	4.7 ± 0.2	0.6	10.8 ± 0.1
		CU 41	Dark olivaceous hyphae at center and white hyphae	Pink, (cream to dark olivaceous)	Light olivaceous	+	1.5 ± 0.1	0.2	9.2 ± 0.3
10		NRRL Y-12974	Cream (yeast like)	Pink, (cream, black at edge)	Cream	-	23.4 ± 1.8	4.3	45.4 ± 0.4
11	Never turns black on YMA (after 2 months)	CU 12	Pink, yeastlike at center and white hyphae	Pink, (cream)	Cream	+	16.9 ± 0.8	2.2	39.9 ± 0.4
		CU 14	Pink, yeastlike at center and white hyphae	Pink, (cream and pink)	Cream	-	13.0 ± 0.6	2.4	50.9 ± 1.8
		CU 15	Pink (yeast like) and dark olivaceous sector	Pink, (cream)	Cream	+	6.3 ± 0.2	1.0	79.7 ± 3.8
		CU 16	Olivaceous hyphae at center and white hyphae	Pink, (cream)	Olivaceous	+	9.0 ± 1.1	1.5	49.9 ± 0.6
		CU 25	Pink, yeastlike at center and white hyphae	Pink, (cream)	Olivaceous	-	11.5 ± 0.7	1.2	53.5 ± 0.8
		CU 27	Pink, yeastlike at center and white hyphae	Pink, (cream)	Pale pink	-	17.2 ± 0.6	1.4	32.3 ± 1.7
		CU 28	Pink, yeastlike at center and white hyphae	Pink, (cream)	Olivaceous	-	12.9 ± 0.6	1.6	47.2 ± 2.2

Table 4.4 (continued)

Clade	Characteristics	Isolate	Color of colonies on		Pigmentation of pullulan production culture	Oil production	EPS production		Xylanase activity (U/ml)
			MEA at day 7	YMA at day 7 (and 2 months)			EPS (g/L)	g EPS/g cell	
11		CU 29	Pink, yeastlike at center and white hyphae	Pink, (cream)	Olivaceous	-	8.1 ± 0.4	0.8	49.6 ± 2.1
		CU 31	Pink, yeastlike at center and white hyphae	Pink, (cream and pink)	Olivaceous	+	10.2 ± 0.2	1.4	37.3 ± 1.5
		CU 35	Pink, yeastlike at center and white hyphae	Pink, (cream)	Pale pink	+	15.0 ± 1.9	2.1	38.1 ± 0.5
		CU 39	Pink, yeastlike at center and white hyphae	Pink, (cream)	Cream	+	15.0 ± 1.3	1.6	64.7 ± 1.1
		CU 42	Pink, yeastlike at center and white hyphae	Pink, (cream)	Cream	+	13.3 ± 0.7	2.2	41.1 ± 0.3
		CU 46	Pink, yeastlike at center and white hyphae	Pink, (cream)	Pale pink	-	13.3 ± 1.1	2.2	38.3 ± 0.6
		CU 47	Pink, yeastlike at center and white hyphae	Pink, (cream)	Pale pink	+	17.2 ± 0.1	2.1	31.1 ± 0.7
12	Not *A. pullulans* Non pullulan producer	CU 26	Black at center and white hyphae	Small pink colonies, (cream to black)	Cream	-	1.9 ± 0.3	0.3	7.1 ± 0.1
		CU 30	Black at center and white hyphae	Small pink colonies, (black)	Cream	-	2.0 ± 0.5	0.2	10.5 ± 0.3

[a] Apparent only with diurnal light cycles.

4.1.3 Phenotypic analyses

Colonial morphologies varied among isolates grown on YMA and MEA for 7 days (Figure 4.8 – 4.9, Table 4.4, and Appendix G). Most colonies were smooth, moist, yeast-like, pale pink, and primarily composed of blastoconidia on YMA, while dark septate hyphae were mostly observed on MEA. After day 7, mature colonies on YMA developed a velvety texture and dark brown or black color with a grayish fringe. Microscopically, early cultures showed polymorphic forms of *A. pullulans* with blastospores, swollen cells, and pseudohyphae, with later cultures showing hyphae and chlamydospores (Figure 4.10 and Appendix G).

The order of clades was derived from the 5-locus combined tree, with clade 10 and clade 12 derived from the 4-locus combined tree. Table 4.4 lists the specific characteristics of each isolate, including morphology, EPS or pullulan yield, and xylanase activity. Based on previous studies (Leathers, 1988), high pullulan yields are generally >20 g/L (>40% conversion), moderate yields are 10-20 g/L (20-40% conversion), and low yields are <10 g/L (<20% conversion).

Clade 1 (CU 17, CU 20, CU 24, CU 44, and CU 45) was represented by high pullulan producers with white pullulan, low xylanase activity, white hyphae with dark olivaceous centers on MEA (Figure 4.9A), pale pink color on YMA (Figure 4.8A), and cream-colored cultures in PM. Isolates in this clade thus produced high levels of pullulan with low melanin contamination, which could be beneficial in commercial pullulan production.

Clade 2 (CU 1, CU 18, NRM2, and subclade CU 9) was very close to clade 1, and the two could not be separated by sequence analysis of *BT2*. These isolates were moderate producers of white pullulan, with low xylanase activity, white hyphae with dark olivaceous centers on MEA (Figure 4.9A), pale pink color on YMA (Figure 4.8A), and cream color in PM. Isolate CU 9 was differentiated by *RPB2* as a subclade in this group. It produced rather high pullulan yields but low xylanase activity. The color of CU 9 on YMA was pink with a grayish fringe and its color on MEA was brown at the center of colonies, with white hyphae, and an extracellular red pigment (Figure 4.9E). The color of CU 9 in liquid PM was orange-pink and when polysaccharides were precipitated with

ethyl alcohol the supernatants exhibited a distinctive orange-red color. Furthermore, total DNA isolation of CU 9 showed a band of about 4 kb that possibly was extrachromosomal DNA or a plasmid (Figure 4.11).

Clade 3 isolates (HKW1 and PH1) exhibited white hyphae with dark olivaceous centers on MEA (Figure 4.9A) but did not become black on YMA (Appendix G), even after 2 months. Cultures in liquid PM were olivaceous green and consequently made dark green pullulan. These isolates produced very low yields of pullulan and had low xylanase activity.

Clade 4 isolates (CU 21, CU 32, and subclade CU 8) had the lowest yields of pullulan and exhibited low xylanase activity. Colonies were white hyphae with dark olivaceous centers on MEA (Figure 4.9A) and mostly pink on YMA (Figure 4.8A). Subclade CU 8 was slightly different in the color of colonies on MEA. It showed black hyphae at the center not dark olivaceous (Figure 4.9F and Table 4.4), and was dark olivaceous green in liquid PM.

Clade 5 (CU 6, CU 19, and CU 36) was distinctive from the others because these strains produced a purple-red (vinaceous) pigment on YMA (Figure 4.8B), MEA (Figure 4.9B) and in PM. Pullulan was white with low yields. However, relatively high viscosity EPS was obvious when culture supernatants were precipitated with ethanol. Moreover, supernatants exhibited an orange-red color when polysaccharides were precipitated with ethyl alcohol. The activity of xylanase was variable, from moderate to high.

Clade 6 (CU 33 and CU 37) was similar to clade 3 in both morphological and physiological characters, except the color of colonies turned dark green (olivaceous) after 2 months on YMA (Appendix G).

Clade 7 (CU 3, DOUG, NRRL Y-6220, and subclade BM1) included typically pigmented reference strain NRRL Y-6220, and colonies were pink to olivaceous on MEA (Figure 4.9D and Table 4.4). Isolate CU 3 was a high pullulan producer with green pigment. In contrast, subclade BM1 was very low in pullulan production. The activity of xylanase was low.

Clade 8 (CU 10, CU 11, CU 13, CU 23, CU 38, CU 40, and NRRL Y-2311-1) was a quite distinctive group, including the color variant reference strain NRRL Y-2311-1

described by Wickerham and Kurtzman (1975). With the exception of CU 38, all of these isolates showed color rings of red, orange, and pink when grown on YMA for 7 days (Figure 4.8C and Figure 4.12). These color rings depended on regular (diurnal) cycles of daylight. Colonies showed only a pink color when grown under constant light, and incubation in constant darkness produced a darker orange color. Most isolates grown on MEA exhibited whilte hyphae with yellow pigment (Figure 4.9C) except CU 38 did not produce yellow pigment (Figure 4.9G). Isolates in this clade produced the highest xylanase activities, although they had low pullulan yields.

When initially isolated, clade 9 strains (CU 2, CU 4, CU 5, CU 7, CU 22, and CU 41) produced black pigment in liquid PM cultures. However, this trait appears to be somewhat unstable, as most isolates in this clade, except CU 4, produced less melanin as cultures were maintained over time. Nevertheless, the pullulan yields and xylanase activites of this clade were very low. Strain CU 22 was unusual in that ethyl alcohol supernatants exhibited an orange-red pigment similar to that produced by strain CU 9 and strains of clade 5. Colonies were white hyphae with dark olivaceous centers on MEA (similar to Figure 4.9A) and pink on YMA (similar to Figure 4.8A).

Clade 10 (NRRL Y-12974) consisted of only one isolate which produced a high pullulan yield and rather high xylanase activity. This isolate, which was previously described as a color variant strain (Leathers, 1986), exhibited cream colored yeast-like colonies on MEA (Figure 4.9H) and pink colonies on YMA (Figure 4.8A).

Clade 11 (CU 12, CU 14, CU 15, CU 16, CU 25, CU 27, CU 28, CU 29, CU 31, CU 35, CU 39, CU 42, CU 46, and CU 47) was the largest group, located in a different lineage from the others. Like clade 3, this clade was interesting in that colonies did not turn black on YMA, even after 2 months. Colonies were mostly white hyphae with pink, yeast-like centers on MEA (similar to Figure 4.9D) except CU 15, had dark olivaceous sector (Figure 4.9I), and CU 16, olivaceous center (Figure 4.9J), and pink on YMA (Figure similar to 4.8A). Pullulan color and yields were variable, while the activity of xylanase was moderate to high.

Clade 12 (CU 26 and CU 30) apparently was not *A. pullulans*, but in fact a closely related species. Data from four loci (ITS, *BT2*, *EF-1α*, *RPB2*) showed that these 2 isolates were distinguished from others by being located in different lineage.

Furthermore, these strains did not produce authentic pullulan. The ^{1}H-NMR spectra of individual EPS samples from isolates in clades 1-11 showed the two signals of α-1,4 and one signal of α-1,6 glycosidic bonds characteristics of pullulan (See Appendix F). However, EPS samples from isolates CU 26 and CU 30 exhibited distinctively different NMR spectra not consistent with pullulan (Figure 4.13). Colonies were smaller than other *A. pullulans* isolates on both YMA and MEA (Figure 4.8E and Figure 4.9K).

There was no apparent relationship between EPS yield and xylanase activity in each isolate. Although isolates produced a range of xylanase activities, all strains (including those of clade 12) exhibited proteins of about 20-21 kDa characteristic of xylanase from *A. pullulans* (Figure 4.14). (It may be noted however that isolates in clade 4 produced a slightly smaller xylanase). A number of strains in several clades were observed to secrete a heavy oil in liquid PM cultures (Table 4.4).

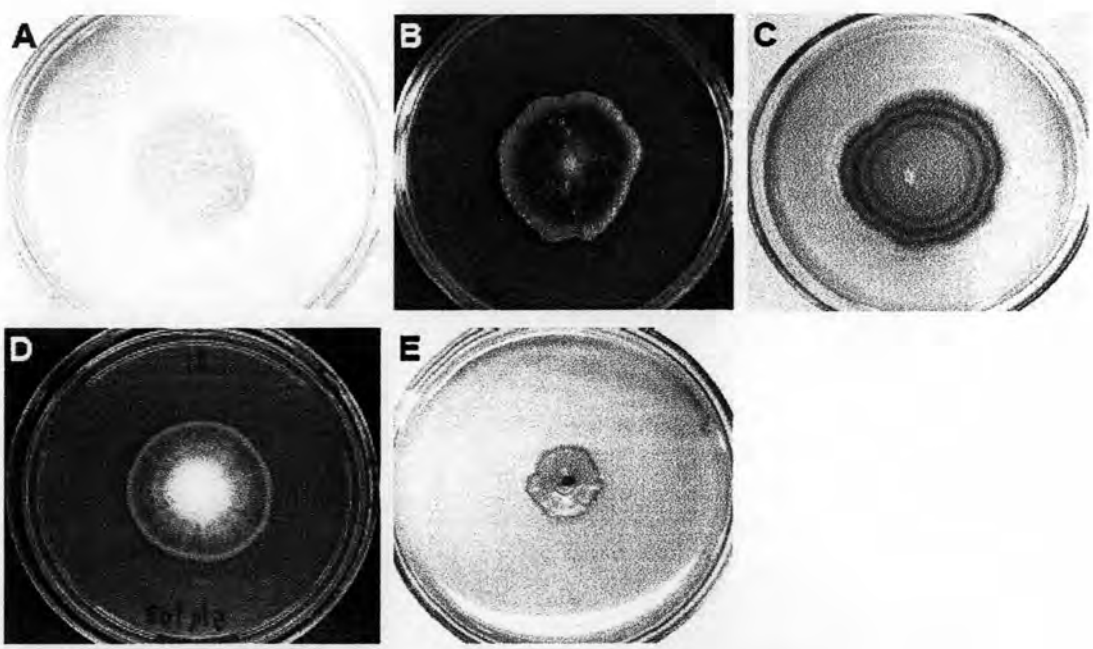

Figure 4.8 Representatives of colonial morphology of *Aureobasidium pullulans* and its allies on YMA at day 7. (A) Pink, isolate CU 42. (B) Dark vinaceous, isolate CU 6. (C) Color rings, isolate CU 10. (D) Pink and dark olivaceous at edge, isolate CU 21. (E) Small pink colony, isolate CU 26 (not *A. pullulans*).

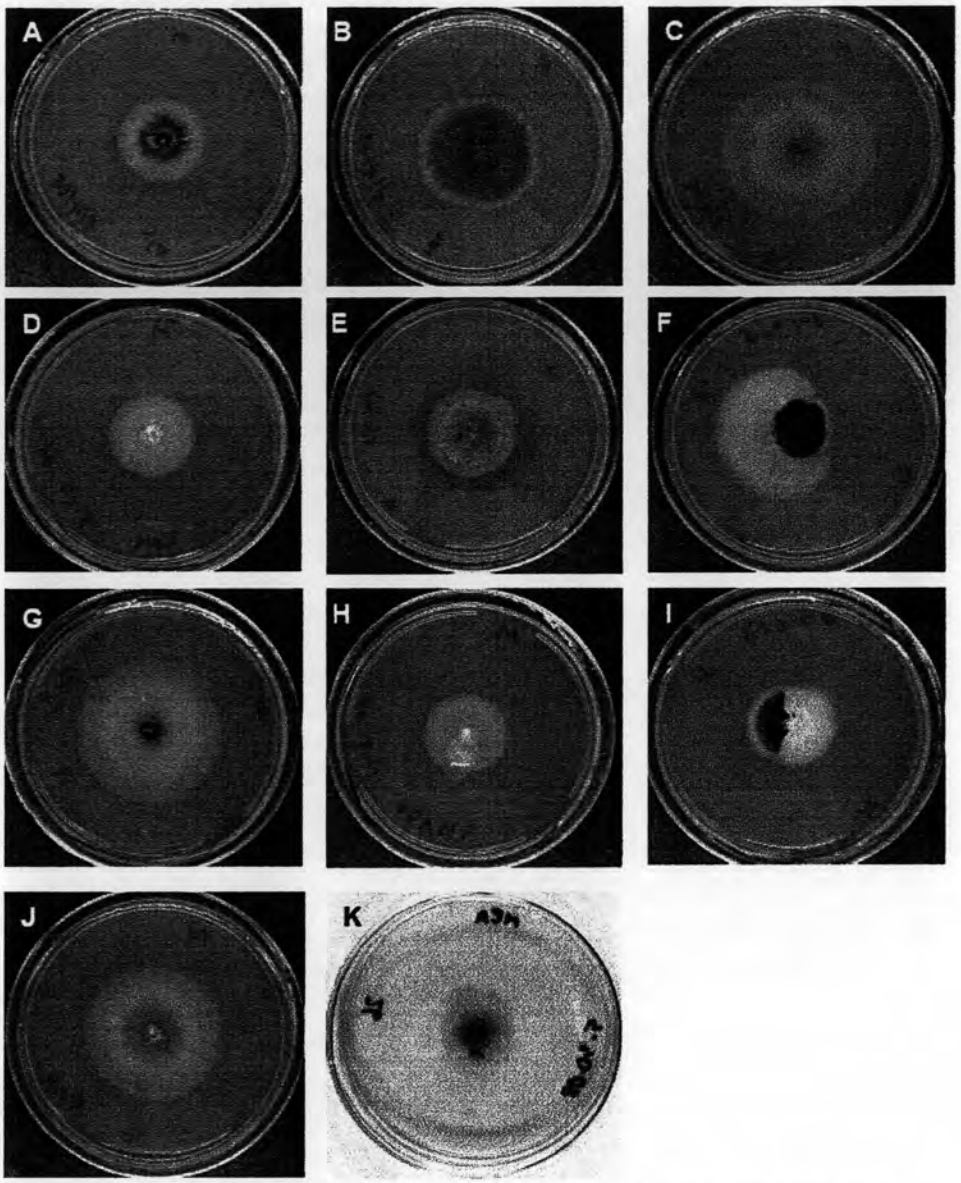

Figure 4.9 Representatives of colonial morphology of *Aureobasidium pullulans* and its allies on MEA at day 7. (A) Dark olivaceous hyphae at center and white hyphae, isolate CU 45. (B) White hyphae with dark vinaceous pigment, isolate CU 6. (C) White hyphae with yellow pigment, isolate CU 13. (D) Pink, yeastlike at center and white hyphae, isolate DOUG. (E) Brown (yeast like) at center, white hyphae, and orange red pigment diffused in agar, isolate CU 9. (F) Black hyphae at center and white hyphae, isolate CU 8. (G) White hyphae with light yellow pigment only at center, isolate CU 38. (H) Cream (yeast like), strain NRRL Y-12974. (I) Pink (yeast like) and dark olivaceous sector, isolate CU 15. (J) Olivaceous hyphae at center and white hyphae, isolates CU 16. (K) Black at center and white hyphae, isolate CU 26 (not *A. pullulans*).

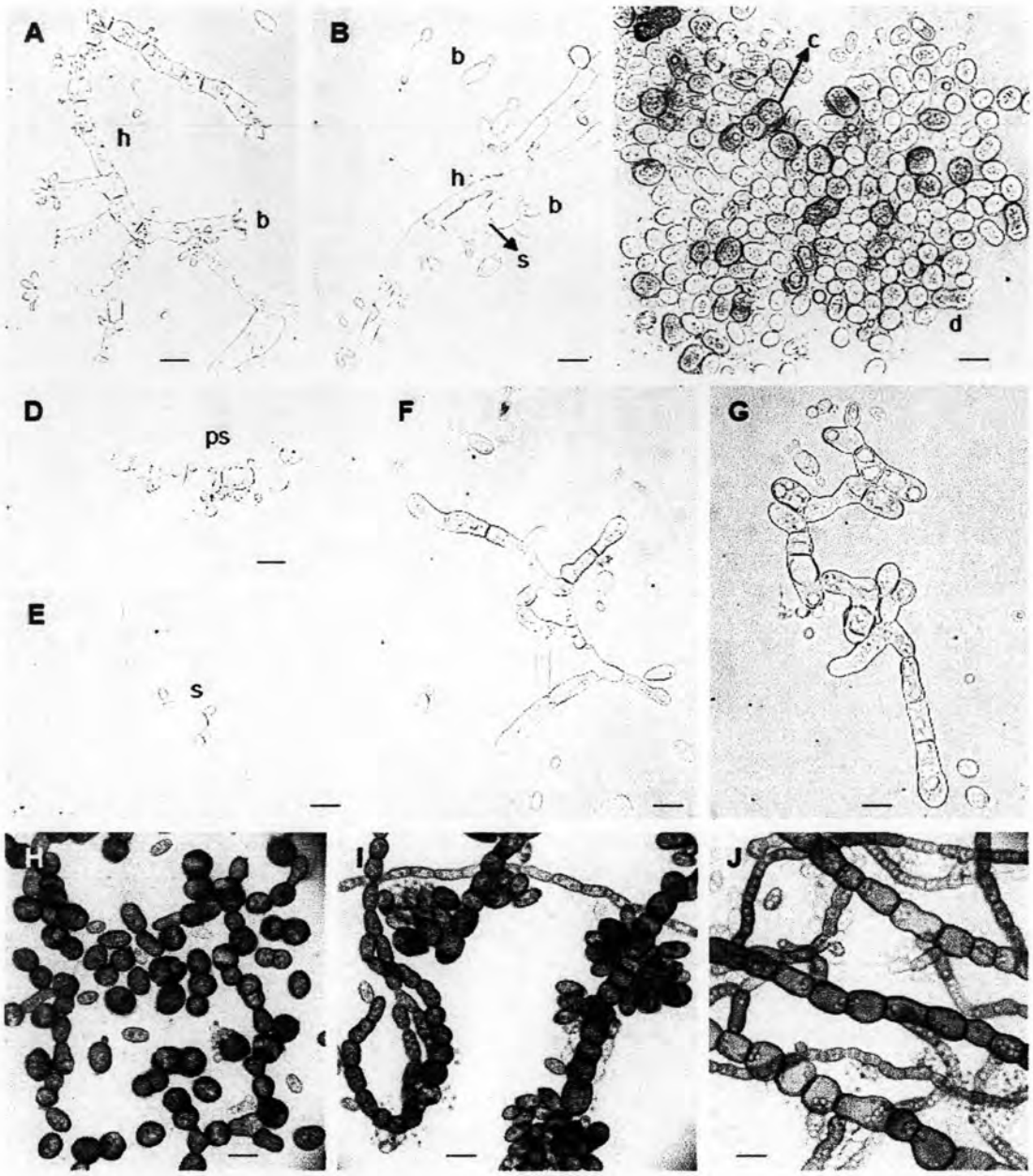

Figure 4.10 Representative microscopic morphology. (A)-(E) Polymorphic forms of *Aureobasidium pullulans* cultured in YM for 1–2 days. (A) CU 23 after 2 days. (B) CU 1 after 1 day. (C) CU 20 after 2 days. (D) CU 2 after 1 day. (E) CU 6 after 2 days. (F)–(G) Cellular morphology of *A. pullulans*–like isolates CU 26 and CU 30, respectively, after 2 days in YM. (H)–(J) Dark pigmented cells and hyphae of *A. pullulans* cultured on MEA for 7 days. (H)-(I) PH1. (J) CU 21. Bars = 10 µm for all figures. b = blastospore, c = chlamydospore, d = cell with dark pigment, h = hyphae, ps = pseudohyphae, s = swollen cell.

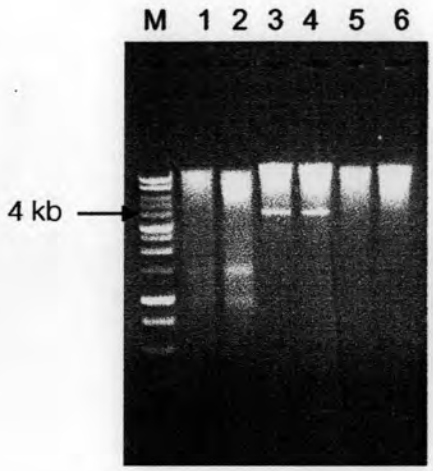

Figure 4.11 Possible natural plasmid found in total DNA isolation from *A. pullulans* isolate CU 9 (lanes 3 and 4). Lanes 1 and 2 were isolated total DNA from isolate CU 4, lanes 5 and 6 were from isolate CU 12. Lane M was 1 Kb DNA ladder (Promega, Madison, WI, USA). DNAs were separated on 1.0% agarose gel.

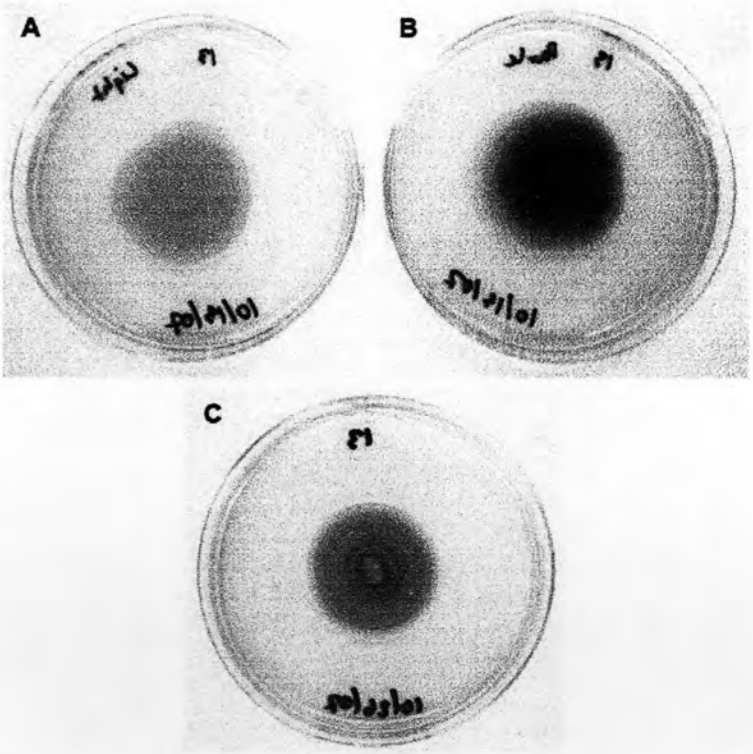

Figure 4.12 Color rings of colonies of *Aureobasidium pullulans* isolate CU 13 in clade 8 after it was cultured on YMA for 7 days under (A) light, (B) dark, and (C) continually alternating light and dark.

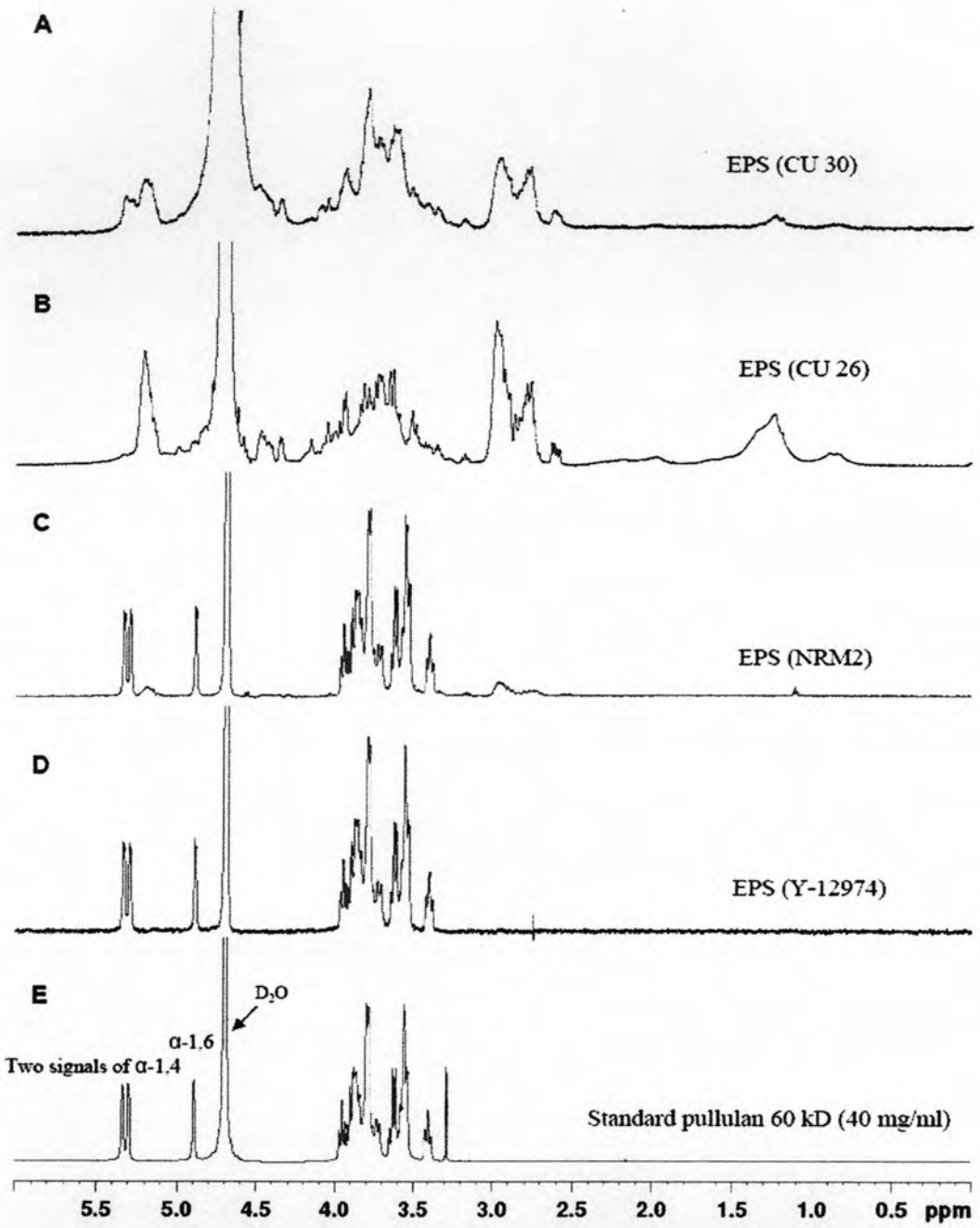

Figure 4.13 ^{1}H-NMR spectra of exopolysaccharides (EPS) of *Aureobasidium pullulans*. (A) Isolate CU 30. (B) Isolate CU 26. (C) Reference strain NRM2. (D) Comparative strain NRRL Y-12974. (E) Standard pullulan 60 kD (40 mg/ml; Sigma, St Louis, MO).

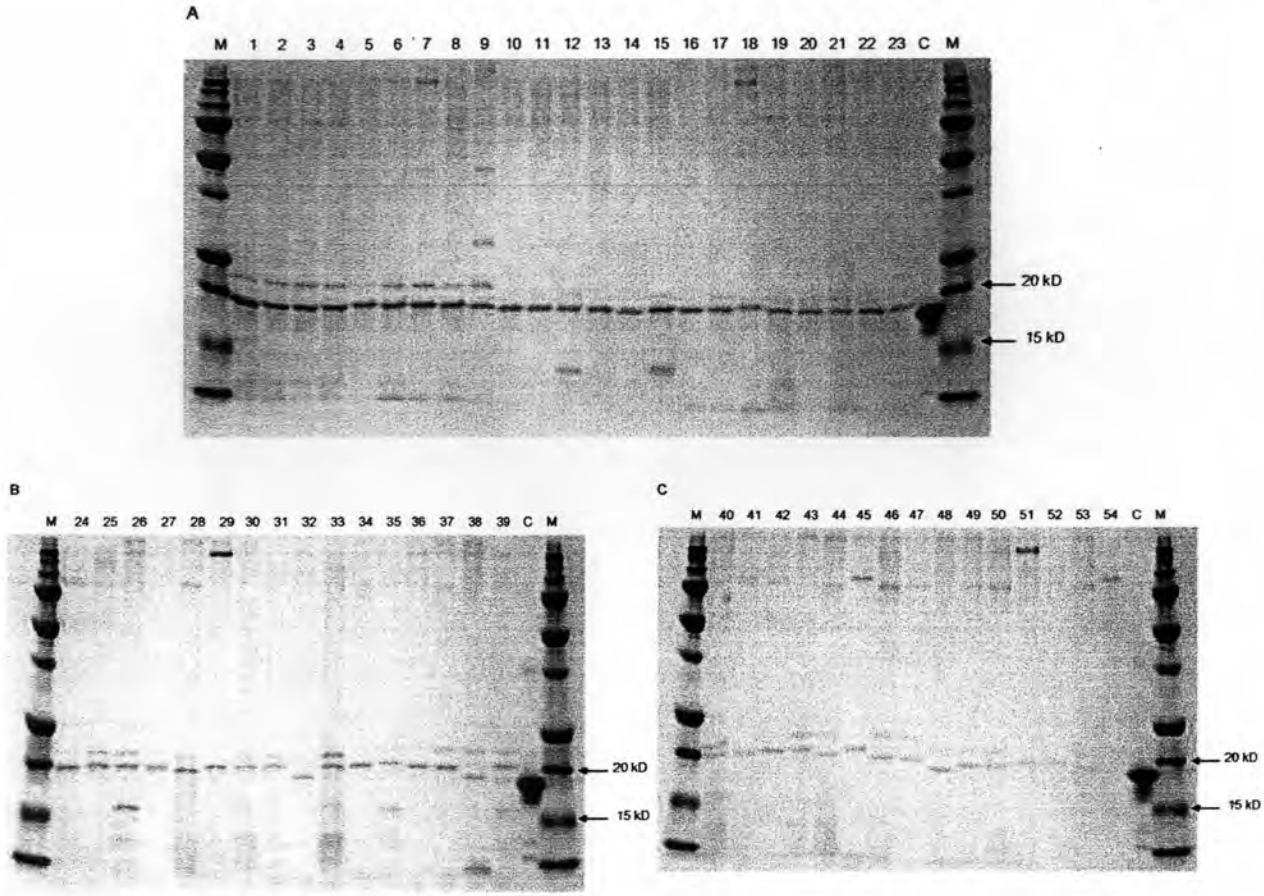

Figure 4.14 Analysis of xylanase activity on SDS-PAGE loaded on precast 8 – 16% gels (Criterion, Bio-Rad Laboratories, Inc., Hercules, CA) in order from the highest activity to the lowest activity. (A) Lanes 1 – 23 were NRRL Y-2311-1, CU 10, CU 13, CU 11, CU 15, CU 40, CU 23, CU 39, CU 38, CU 25, CU 14, CU 16, CU 29, CU 43, CU 28, NRRL Y-12974, CU 42, CU 6, CU 12, CU 46, CU 35, CU 31, CU 27, respectively. (B) Lanes 24 – 39 were CU 47, CU 3, CU 9, DOUG, CU 37, CU 19, CU 20, CU 17, CU 21, CU 1, CU 45, CU 44, NRRL Y-6220, CU 33, CU 8, CU 24, respectively. (C) Lanes 40 – 55 were CU 18, BM1, HKW1, NRM2, CU 22, CU 20, CU 2, CU 41, CU 32, CU 7, CU 4, CU 36, PH1, CU 5, CU 26, respectively. C = control xylanase (1% (w/v) in 43% glycerol,0.18 M Na/K phosphate , pH 7.0, diluted 1:100 in MilliQ water) (Hampton Research, Aliso Viejo, CA, USA). M = Precision Plus Protien Standard (Bio-Rad, Laboratories, Inc., Hercules, CA), 23.4 µg.

4.2 Relationship between α-amylase activity and pullulan profiles

4.2.1 EPS analyses and α-amylase activity

To investigate any involvement between α-amylase and the pullulan yield, five *A. pullulans* isolates were selected including CU 3, CU 20, CU 36, NRM2, and NRRL Y-12974. Isolate CU 3 was representative of clade 7. It produced olivaceous (dark green) pigment in PM and caused pigment contamination in pullulan production. Strain CU 20 was representative of *A. pullulans* in clade 1, which was one of the best pullulan producer without pigmentation. Isolate CU 36 was in clade 5, and produces a vinaceous (purple) pigment in PM, although the pigment did not contaminate pullulan precipitated from cultures. NRM2 was a reference strain from tropical isolates (Prasongsuk *et al.*, 2005, 2007). It was classified in clade 2 with moderate pullulan yield. Reference strain NRRL Y-12974 (Leathers *et al.*, 1988) was the only strain in clade 10 which produced a high yield of pullulan.

Representative isolates were cultured in standard PM (containing 5.0% sucrose and 0.1% N-sources) and cultures were sampled at day 2, 4, 6, and 8. Samples were evaluated for OD_{600} (cell growth), pH of culture, EPS or pullulan yield (g/L and g EPS dry weight /g cell dry weight), molecular weight and viscosity of EPS, and also α-amylase and pullulanase activities. Furthermore, strain NRRL Y-12974 was grown in both standard and modified PM media to compare the results with different carbon sources and concentration of nitrogen sources (Figure 4.15 – 4.22 and Appendix D).

For all isolates except NRRL Y-12974, cell growth gradually increased over 8 days in standard PM containing 5.0% sucrose and 0.1% N-sources (Figure 4.15). NRRL Y-12974 showed a drop in OD_{600} after day 4. However, cell growth of NRRL Y-12974 in modified PM gradually increased (Figure 4.16). Moreover, growth curves were also plotted in semi-log graphs. They seemed to be hyperbola curves (see Appendix D). In contrast, the pH in each culture decreased during cultivation (Figure 4.17). The pH of cultures in standard PM dropped from an initial pH of 6.5 to a final pH of 3.5, while the pH of NRRL Y-12974 cultured in modified PM dropped to about pH 5.5 (Figure 4.18).

The EPS yields (in g/L) of the five representative isolates increased during cultivation (Figure 4.19). In standard PM, the highest EPS yields were from strain NRRL

Y-12974, followed in order by isolates CU 20, CU 3, NRM2, and CU 36. For NRRL Y-12974 (Figure 4.20), the highest EPS yields were from cultures grown in standard PM (5.0% sucrose and 0.1% N-sources), followed in order by cultures grown in modified PM with 5.0% starch, 0.1% N-sources; modified PM with 5.0% starch, 0.3% N-sources; and modified PM with 5.0% sucrose, 0.3% N-sources. Thus, the EPS yields from NRRL Y-12974 grown in media containing 0.3% N-sources were lower than those grown in media containing 0.1% N-sources (Figure 4.20). The EPS yields (in g EPS dry weight / g cell dry weight) were slightly different. Yields from isolates CU 3 and CU 36 gradually decreased (Figure 4.21). The EPS yields of NRRL Y-12974 in starch PM were very high at day 2 and dramatically dropped in each day (Figure 4.22)

The molecular weight of EPS from each isolate gradually decreased after day 2 (Figure 4.23). However, the molecular weight of EPS from NRRL Y-12974 cultured in PM containing 5.0% starch was extremely low at all times (Figure 4.24). Generally, the viscosity of EPS decreased as a function of molecular weight. Interestingly, the viscosities of EPS from CU 36 and CU 3 were exceptional as they were not decreased. The viscosity of EPS from strain CU 3 gradually rose over 8 days, while the viscosity of EPS from strain CU 36 increased dramatically (Figure 4.25). Furthermore, viscosity was higher when grew NRRL Y-12974 in higher nitrogen concentration, while viscosity of EPS from starch PM was extremely low (Figure 4.26). In addition, EPS yield from PM with 0.3% N-sources was higher than that from PM with 0.1% N-sources, however, its molecular weight and viscosity were lower.

Even though the molecular weight of EPS from sucrose-grown cultures decreased over time, α-amylase activities were very low, under the detectable limit (Table 4.5). From the standard curve of α-amylase activity and pullulanase activity (See Appendix C), the calculated lower limits of detection for these assays were 0.02 and 0.005 U/ml, respectively. For all strains grown in sucrose media, α-amylase activities were below the limit of detection. Strain NRRL Y-12974 showed only low levels of α-amylase in modified PM containing 5.0% starch (Figure 4.27 and Table 4.6). Activities were slightly higher in cultures containing 5.0% starch and 0.3% N-sources than in cultures containing 5.0% starch and 0.1% N-sources. None of the cultures produced detectable pullulanase activity.

Combine data among (1) cell growth, pH changes, and EPS yield and (2) EPS yield, α-amylase activity, molecular weight, and viscosity measurements of each strain were shown in Appendix E.

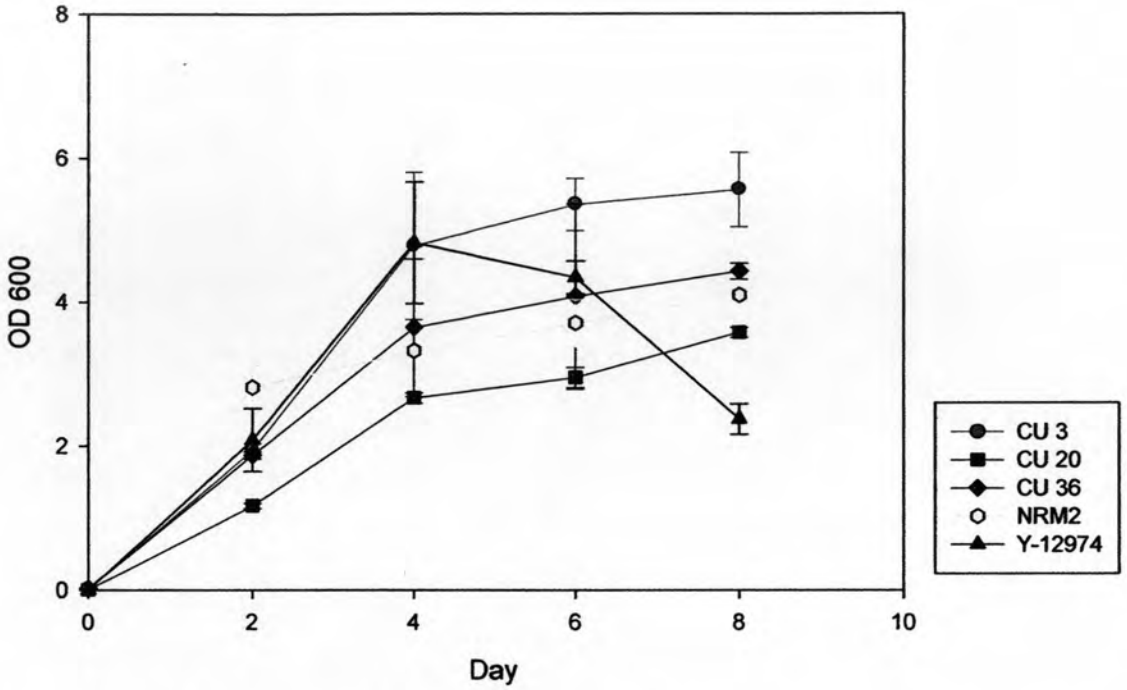

Figure 4.15 Growth curve of *A. pullulans* cultured in standard PM (5.0% sucrose and 0.1% N-sources) at day 0, 2, 4, 6, and 8. Bars represent standard error.

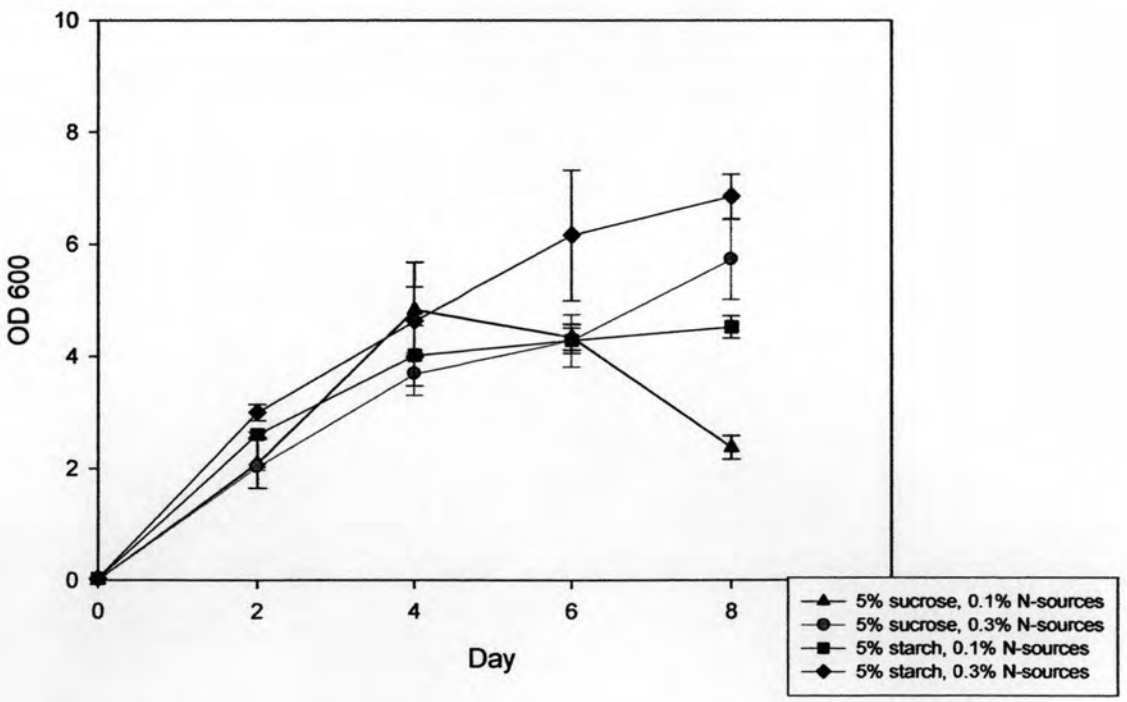

Figure 4.16 Growth curve of *A. pullulans* NRRL Y-12974 cultured in modified PM at day 0, 2, 4, 6, and 8. Bars represent standard error.

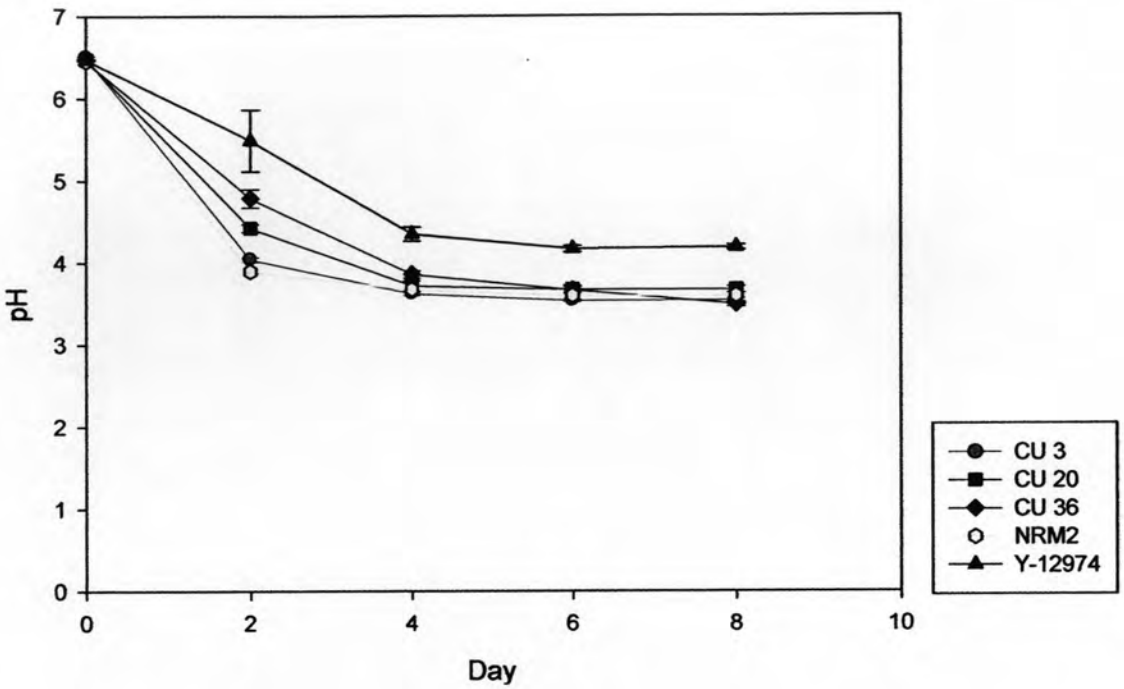

Figure 4.17 pH profiles of culture supernatants of *A. pullulans* grown in standard PM (5.0% sucrose and 0.1% N-sources) at day 0, 2, 4, 6, and 8. Bars represent standard error.

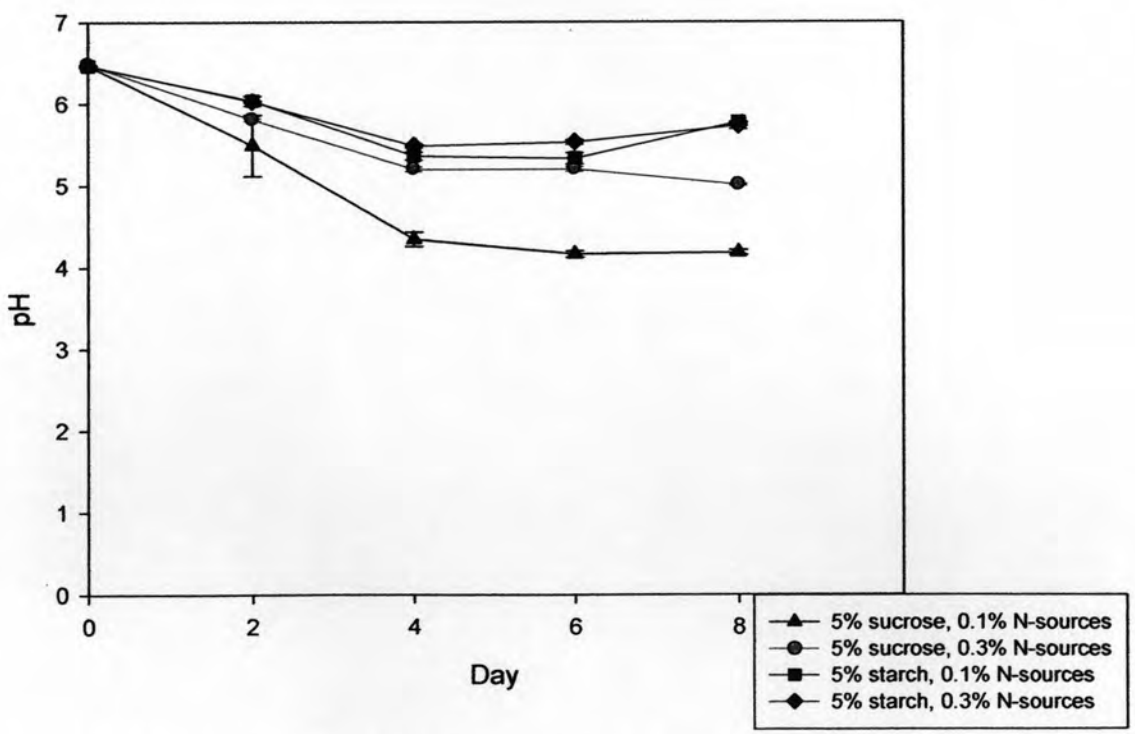

Figure 4.18 pH profiles of culture supernatants of *A. pullulans* NRRL Y-12974 grown in modified PM at day 0, 2, 4, 6, and 8. Bars represent standard error.

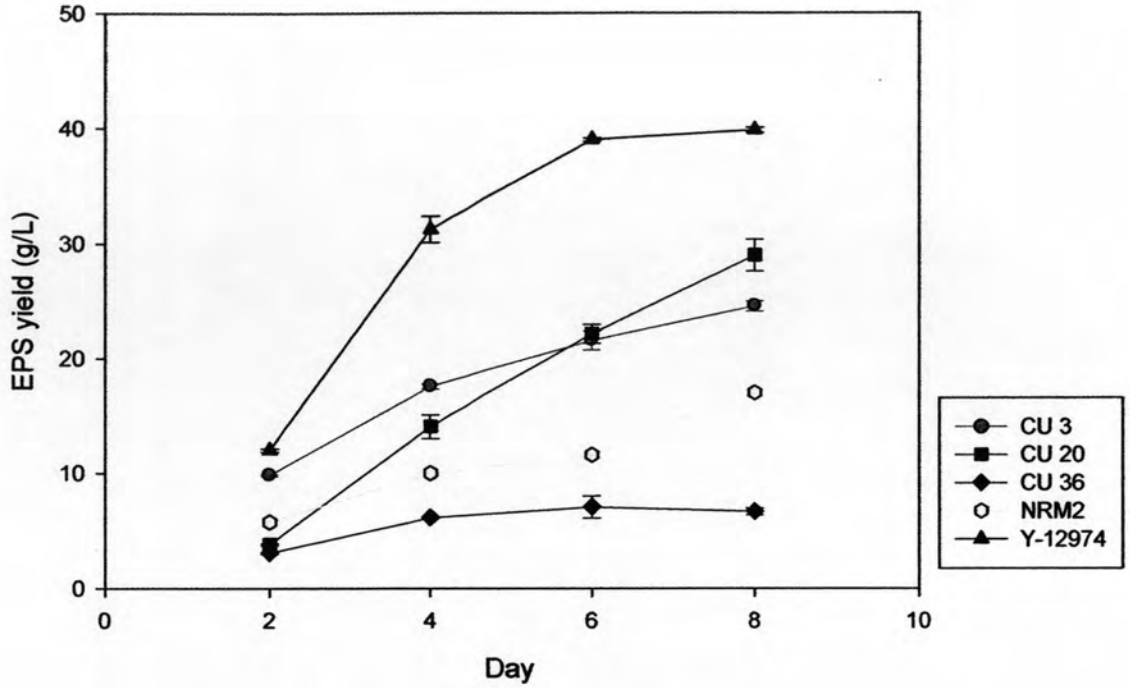

Figure 4.19 EPS yield (g/L) of *A. pullulans* cultured in standard PM (5.0% sucrose and 0.1% N-sources) at day 2, 4, 6, and 8. Bars represent standard error.

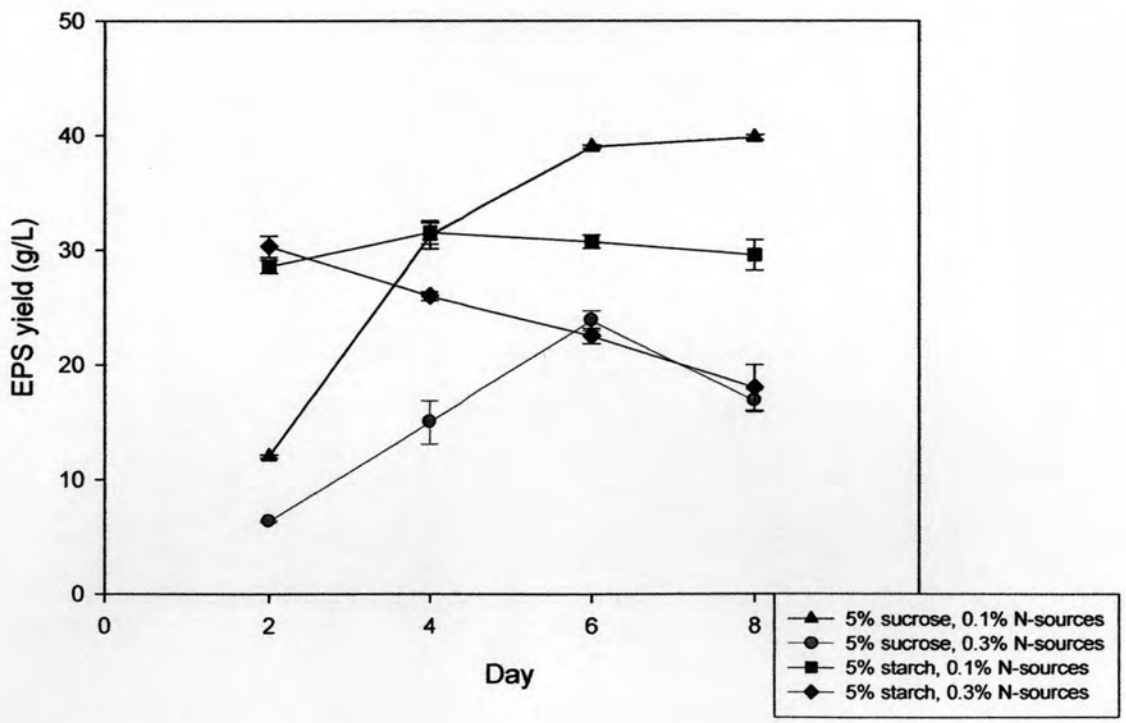

Figure 4.20 EPS yield (g/L) of *A. pullulans* NRRL Y-12974 cultured in modified PM at day 2, 4, 6, and 8. Bars represent standard error.

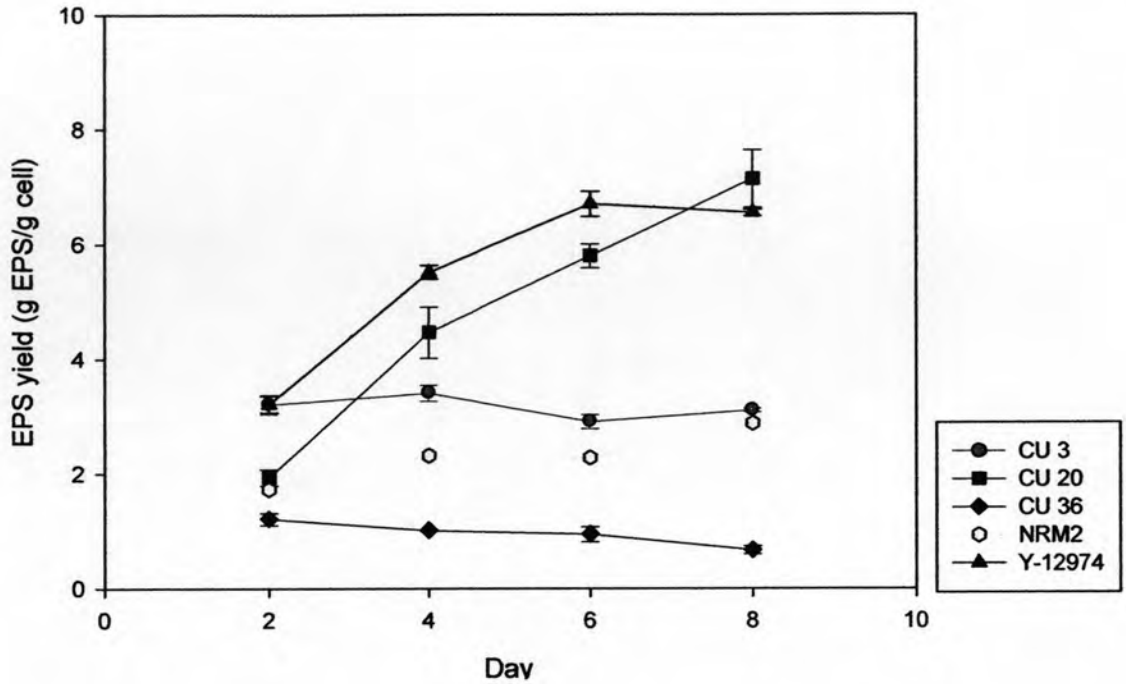

Figure 4.21 EPS yield (g EPS dry weight / g cell dry weight) of *A. pullulans* cultured in standard PM (5.0% sucrose and 0.1% N-sources) at day 2, 4, 6, and 8. Bars represent standard error.

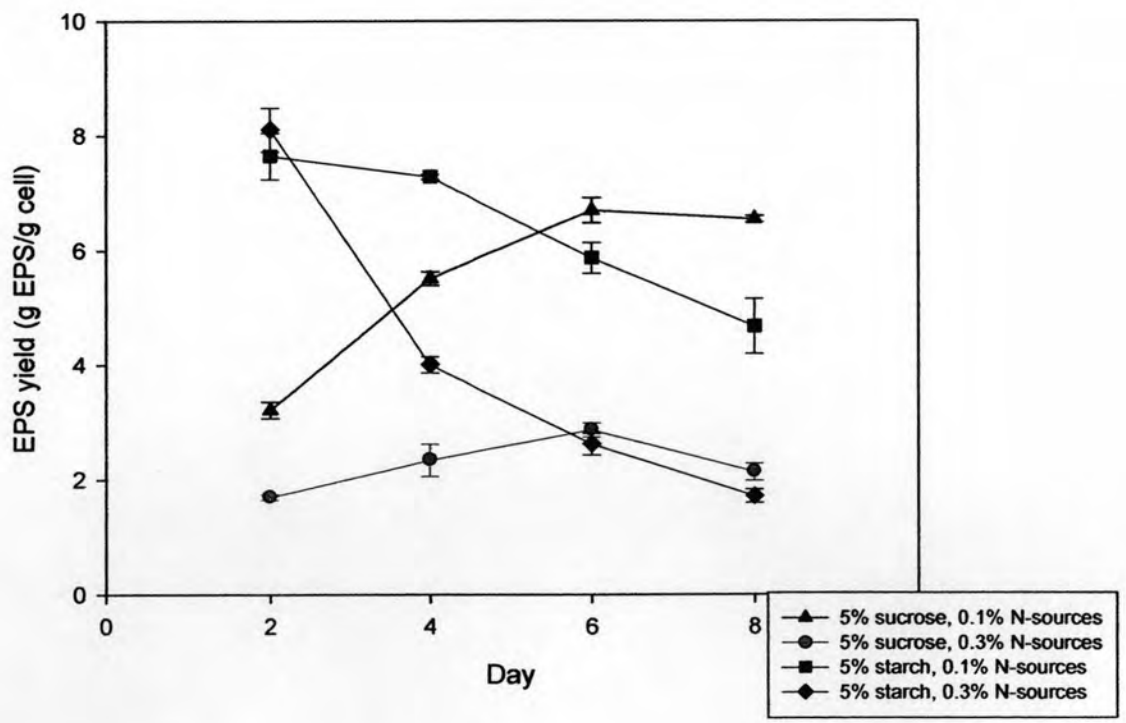

Figure 4.22 EPS yield (g EPS dry weight / g cell dry weight) of *A. pullulans* NRRL Y-12974 cultured in modified PM at day 2, 4, 6, and 8. Bars represent standard error.

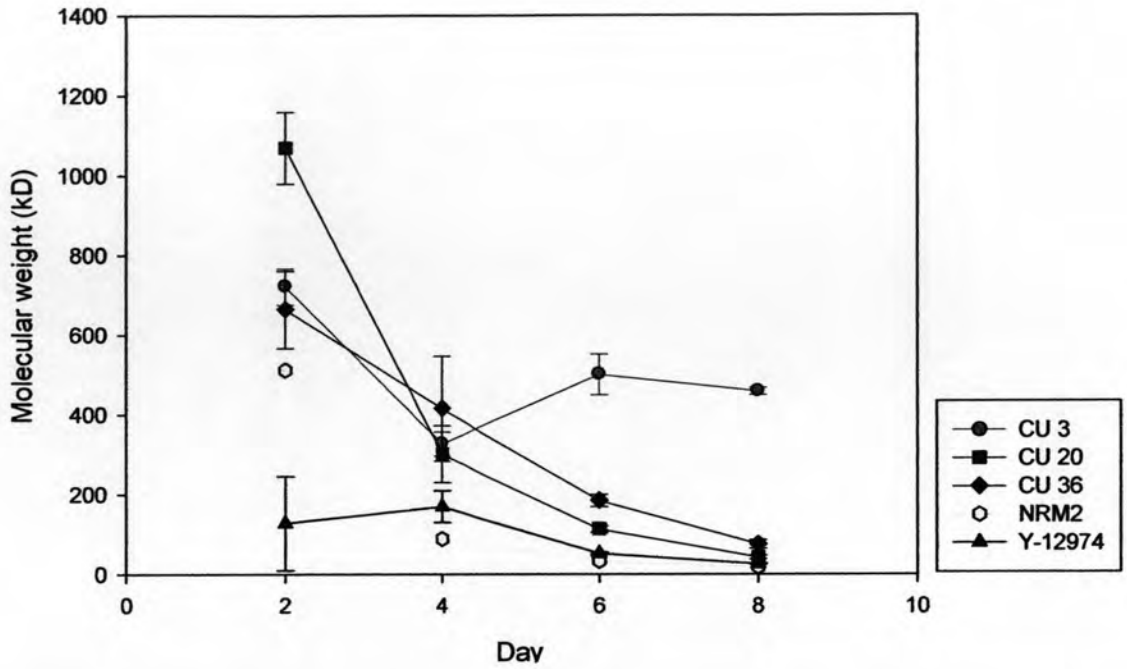

Figure 4.23 Molecular weight (kD) of EPS precipitated from culture supernatants of *A. pullulans* grown in standard PM (5.0% sucrose and 0.1% N-sources) at day 2, 4, 6, and 8. Bars represent standard error.

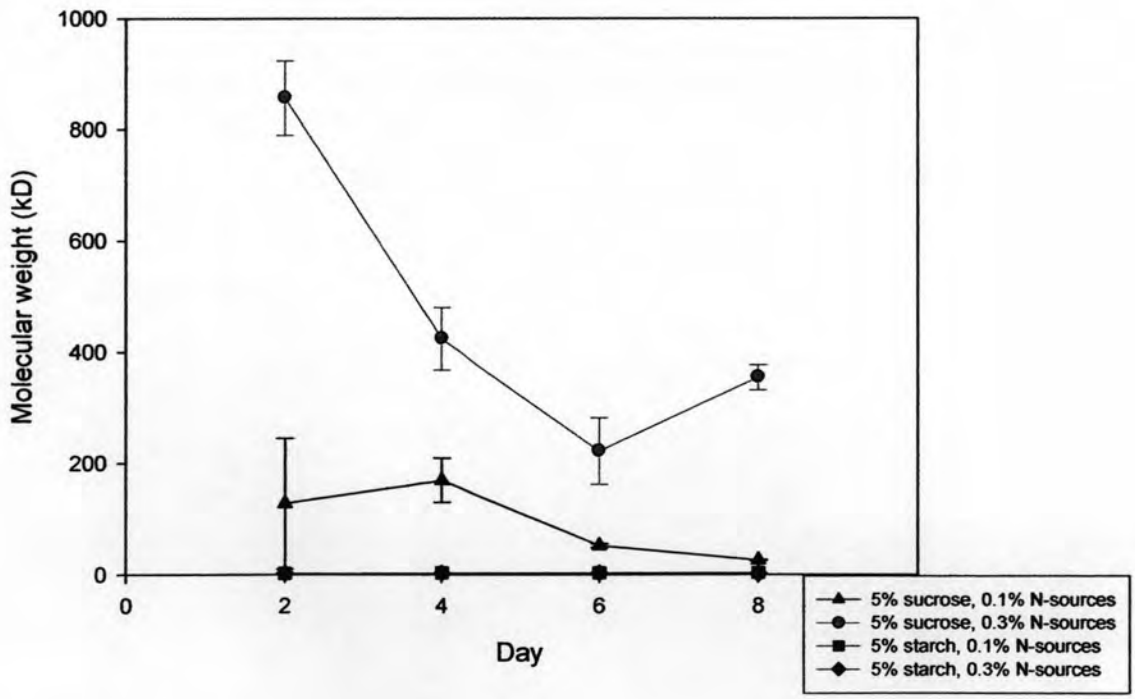

Figure 4.24 Molecular weight (kD) of EPS precipitated from culture supernatants of *A. pullulans* NRRL Y-12974 grown in modified PM at day 2, 4, 6, and 8. Bars represent standard error.

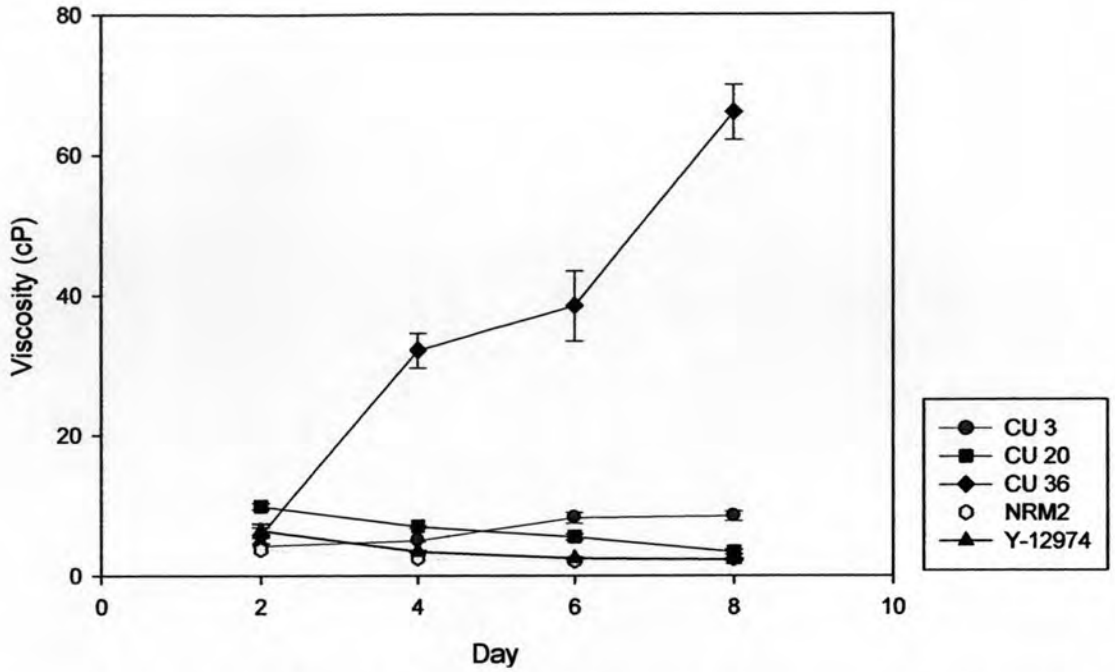

Figure 4.25 Viscosity (cP) of EPS precipitated from culture supernatants of *A. pullulans* grown in standard PM (5.0% sucrose and 0.1% N-sources) at day 2, 4, 6, and 8. Measured at 25°C with a rotation of 30 rpm (shear rate of 39.6 1/S). Bars represent standard error.

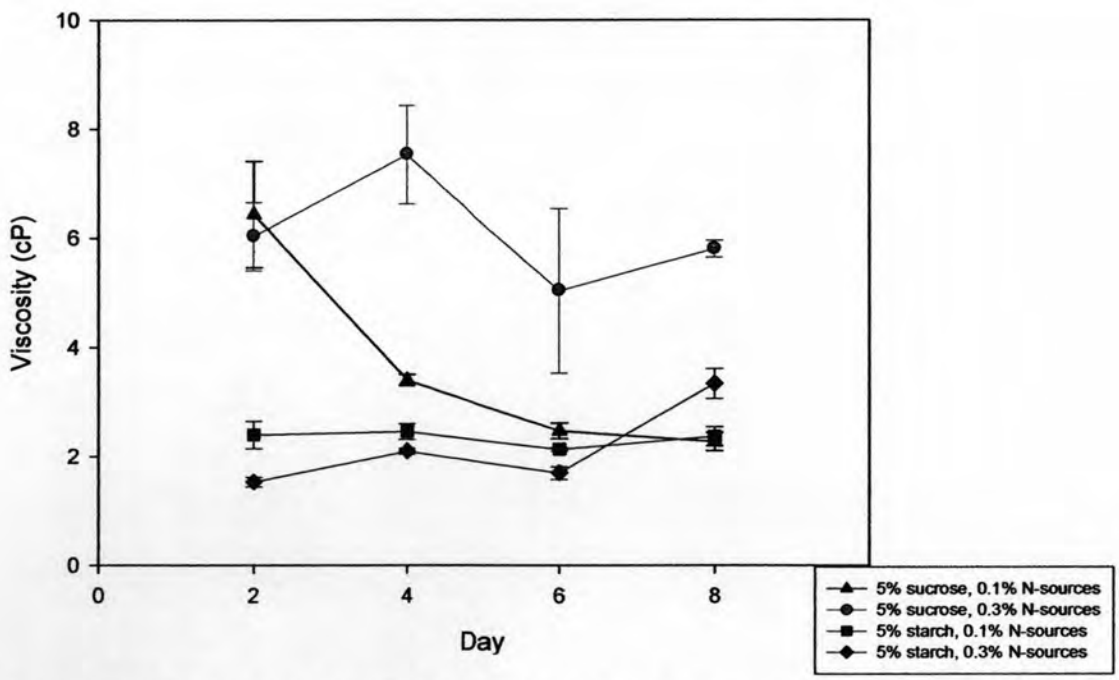

Figure 4.26 Viscosity (cP) of EPS precipitated from culture supernatants of *A. pullulans* NRRL Y-12974 grown in modified PM at day 2, 4, 6, and 8. Measured at 25°C with a rotation of 30 rpm (shear rate of 39.6 1/S). Bars represent standard error.

Table 4.5 The α-amylase activity (U/ml) of *A. pullulans* NRRL Y-12974 grown in modified PM at day 2, 4, 6, and 8 (Numbers are average ± standard error)

Day	5.0% Sucrose, 0.1% N-sources	5.0% Sucrose, 0.3% N-sources	5.0% Starch, 0.1% N-sources	5.0% Starch, 0.3% N-sources
2	0.003 ± 0.002	0.015 ± 0.001	0.005 ± 0.001	0.037 ± 0.003
4	0.000 ± 0.000	0.002 ± 0.000	0.007 ± 0.000	0.026 ± 0.001
6	0.000 ± 0.000	0.001 ± 0.001	0.015 ± 0.003	0.037 ± 0.004
8	0.003 ± 0.001	0.004 ± 0.003	0.026 ± 0.001	0.038 ± 0.005

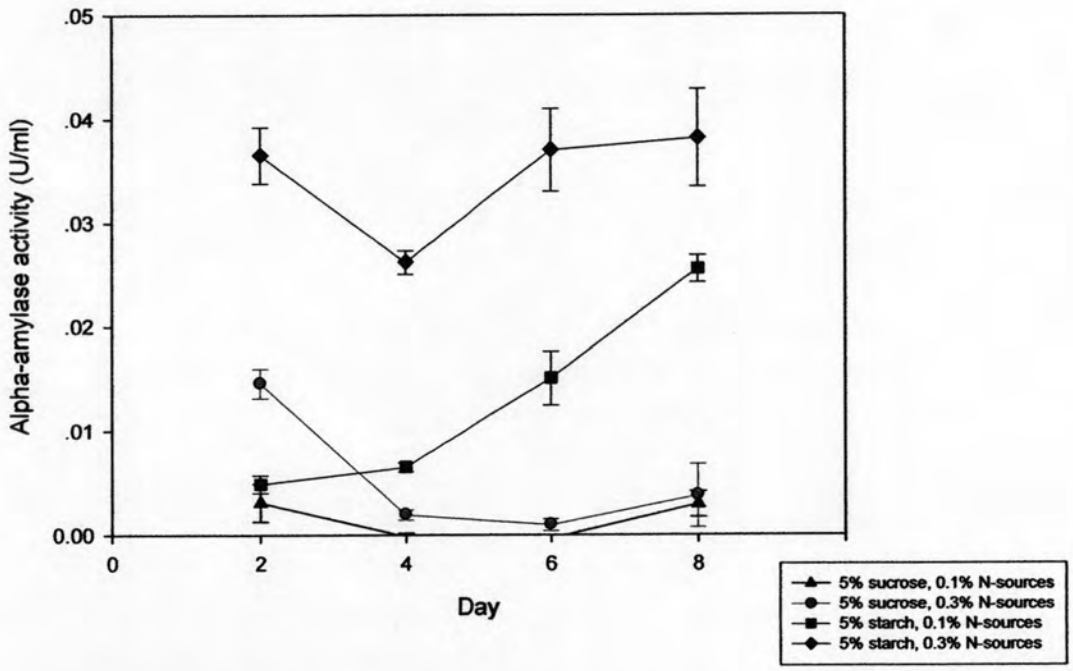

Figure 4.27 The α-amylase activity (U/ml) of *A. pullulans* NRRL Y-12974 grown in modified PM at day 2, 4, 6, and 8. Bars represent standard error.

4.2.2 Other assays for α-amylase and pullulanase

4.2.2.1 Pullulanase and α-amylase sensitivity tests

Compared with standard commercial pullulan, the pullulanase sensitivities of EPS from the five representative strains cultured in standard PM (containing 5.0% sucrose and 0.1% N-sources) were between 41 – 92% (Table 4.9). The highest sensitivity was NRRL Y-12974, followed by CU 3, CU 20, NRM2, and CU 36, respectively. The pullulanase sensitivities of the EPS from NRRL Y-12974 grown in standard and modified PM containing sucrose were rather high (92 and 70%) while those of the cultures grown in modified PM containing starch were very low (16 and 13%).

The α-amylase sensitivity of each EPS produced on sucrose-containing media (Table 4.9) was below the limits of detection because the OD_{540} measurement from the DNS method was below zero. However, the absorbance of NRRL Y-12974 samples cultured in modified PM containing 5.0% starch were approximately 0.17, which was higher than standard pullulan.

Table 4.6 The α-amylase and pullulanse sensitivities (using 0.1% EPS sample at day 2 and 0.1 U/ml of enzymes)

Strain	α-amylase sensitivity (%)[a]	Pullulanase sensitivity (%)[a]
CU 3	ND[b]	72
CU 20	ND	72
CU 36	ND	41
NRM2	ND	57
NRRL Y-12974 (PM; 5.0% sucrose, 0.1% N-sources)	ND	92
NRRL Y-12974 (PM; 5.0% sucrose, 0.3% N-sources)	ND	70
NRRL Y-12974 (PM; 5.0% starch, 0.1% N-sources)	> Std pullulan	16
NRRL Y-12974 (PM; 5.0% starch, 0.3% N-sources)	> Std pullulan	13
Standard pullulan (Sigma)	ND	100

[a] % sensitivity is calculated using the standard pullulan (Sigma) as 100% sensitivity

[b] Not detectable

4.2.2.2 α-amylase screening

After flooded with iodine, the amylase activity could be detected as a clear zone surrounding an individual colony growing on 1% starch agar (Figure 4.28). All selected isolates could produce a clear zone indicating amylase activity. The width of the clear zones was measured and is shown in Table 4.10. Isolates CU 20 and NRRL Y-12974 had the widest clearing zones, followed by CU 3, CU 36, and NRM2.

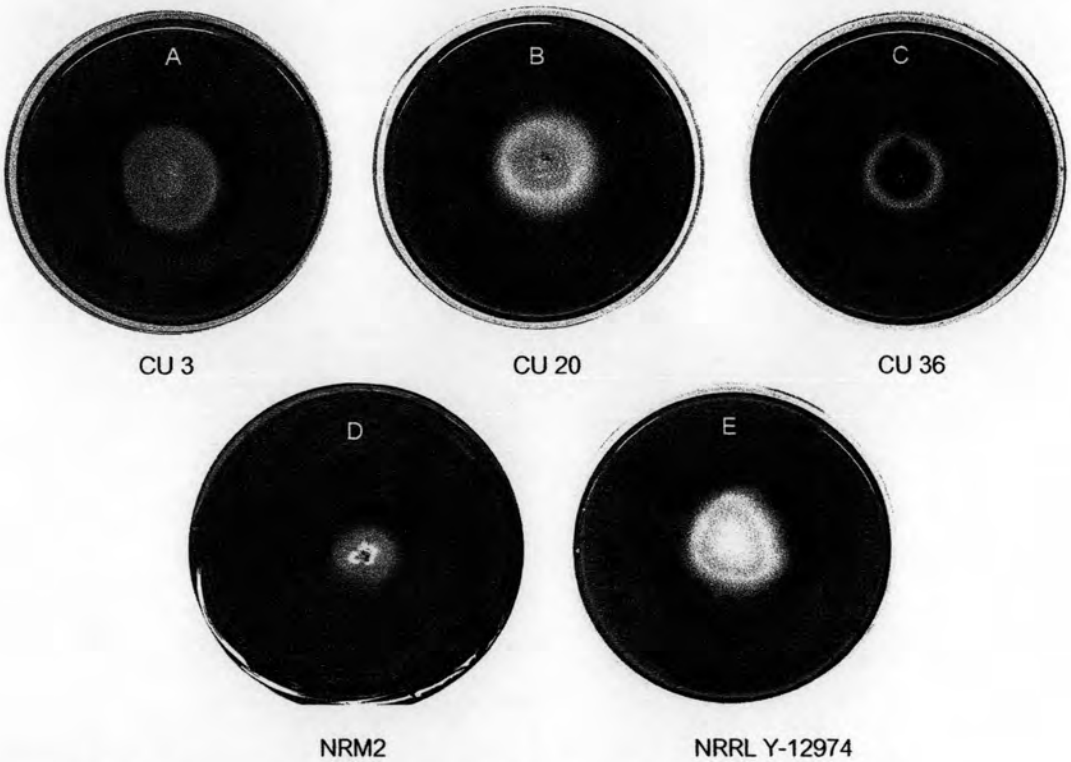

Figure 4.28 The α-amylase screening test of 5 selected *A. pullulans* isolates.

Table 4.7 Width of the clear zones of *A. pullulans* on 1.0% (w/v) starch agar flooded with iodine

Strain	Width of clear zone from the edge of colony (mm)
CU 3	2
CU 20	4
CU 36	0.5
NRM2	0.1
NRRL Y-12974	4

4.2.2.3 Zymogram analysis

Samples of culture supernatants from each representative isolate grown on standard and modified PM were loaded on both a zymogram activity analysis gel (10% SDS-PAGE containing 0.5% (w/v) soluble starch) and a standard 10% SDS-PAGE gel. After soaking with iodine solution, the zymogram analysis gel showed a clear zone only in the control lane containing standard commercial amylase (Sigma) (Figure 4.29 A). The band of amylase was located between 50 kD and 75 kD. Similarly, the Coomassie-stained SDS-PAGE gel showed only a single band in the control lane of standard amylase (Figure 4.29 B). However, the band of the Sigma amylase on the SDS-PAGE analysis gel appeared to be slightly smaller based on migration relative to protein standards.

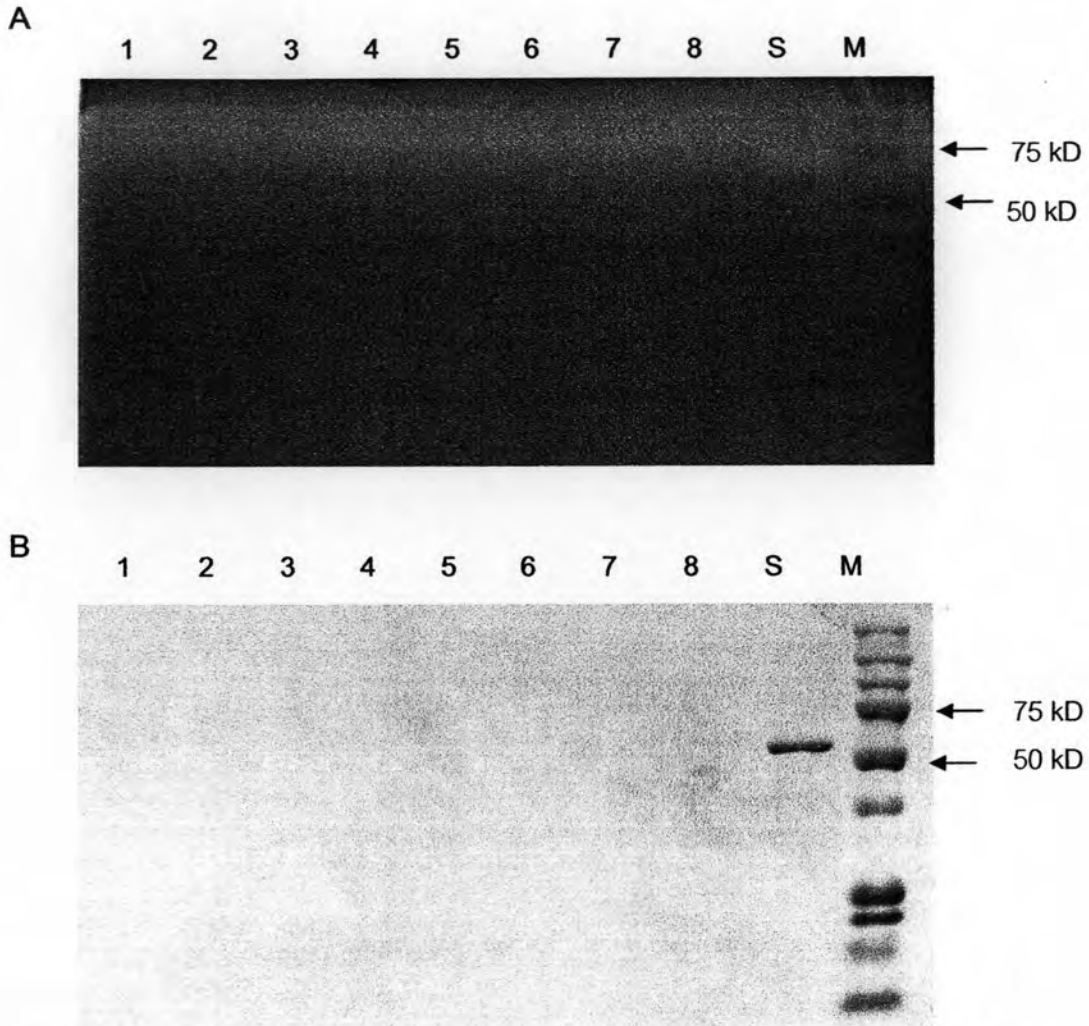

Figure 4.29 (A) Zymogram analysis of α-amylase activity (B) 10% SDS-PAGE gel staining with Coomassie Blue. Lane 1 – 8 represent supernatants from individual cultures collected from the day with the highest amylase activity.

Lane 1: NRRL Y-12974 (PM; 5.0% sucrose, 0.1% N-sources), day 8

Lane 2: NRRL Y-12974 (PM; 5.0% starch, 0.1% N-sources), day 8

Lane 3: NRRL Y-12974 (PM; 5.0% sucrose, 0.3% N-sources), day 2

Lane 4: NRRL Y-12974 (PM; 5.0% starch, 0.3% N-sources), day 8

Lane 5: CU 20, day 2

Lane 6: CU 3, day 8

Lane 7: CU 36, day 2

Lane 8: NRM2, day 8

Lane S: standard α-amylase (Sigma), 2.5 units

Lane M: Precision Plus Protein standards, 23.4 µg

4.2.3 α-amylase gene analyses

4.2.3.1 Genomic DNA sequence analysis

Using the degenerate primers (APamylF1 and APamylR2.2, Table 3.2), the first gDNA fragment was obtained (Fragment 1, Figure 3.2). Flanking regions (both 3' and 5' ends) were further determined using the GenomeWalker™ Universal kit. The adjacent sequences were amplified using the adaptor primer (AP1) and GSP (AP-GSP3f, AP-GSP1r, AP-GSP7f) for both 3' and 5' ends (Figure 3.2 and Table 3.2). Three fragments were obtained (Fragment 2 - 4, Figure 3.2). All fragments were combined and sequences were aligned to obtain the full length of putative gDNA sequence. Similarity to other amino acid sequences in GenBank was determined from the putative gDNA sequence using Blastx. The highest similarity was from *Aspergillus terreus* alpha-amylase (XP_001209405). Using the amino acid sequence of *A. terreus* alpha-amylase (XP_001209405) as the model, the mRNA and amino acid sequences of *A. pullulans* NRRL Y-12974 were predicted by FGENESH+. The putative mRNA was 1,878-bp long encoding an α-amylase of 625 amino acid residues. The sequences of this α-amylase mRNA and its amino acid residues are shown in Figure 4.30.

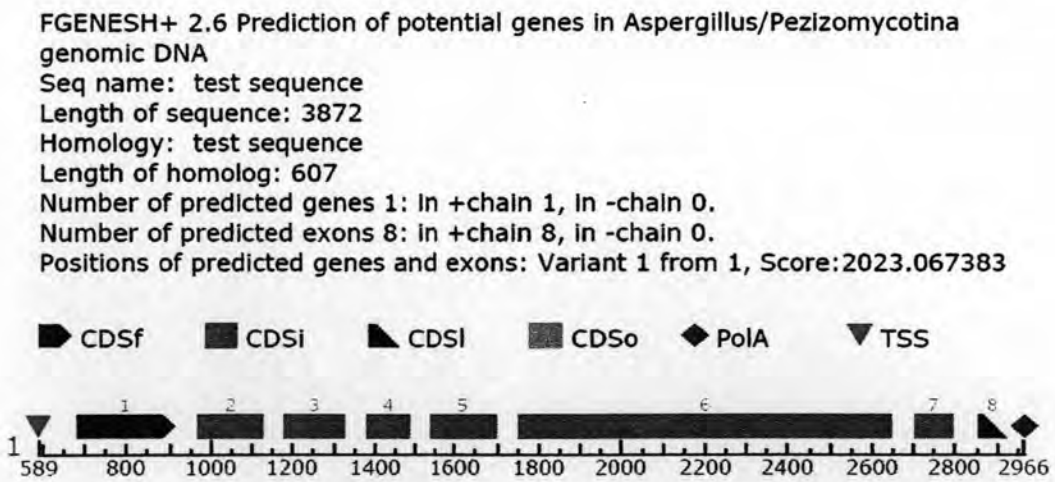

Figure 4.30 Predicted putative mRNA and the deduced amino acid sequences of α-amylase of *A. pullulans* NRRL Y-12974

```
Predicted protein(s):
>FGENESH:[mRNA] 1 8 exon (s) 678 - 2924 1878 bp, chain +
ATGGCAGCCAACTACGTTTCTCGATTGTTGTTCACCCTTCTCTACTTGACCGGGTTGGTGCA
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AATACTTGGCAGGGGTGA

>FGENESH: 1 8 exon (s) 678 - 2924 625 aa, chain +
MAANYVSRLLFTLLYLTGLVQCLTPAQWRSQSIYQVLTDRFARTDGSTTASCDVNKYCGGSF
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FMENHDVARFASLTSDYALAKNAIAFTMLADGIPIIYQGQEQHFSGSSVPNNREALWLSGYP
TSSQLYPFIATVNKIRKQAIKQDTGYLTYKAYPVYSDASTIVMRKGTTGSQVIGVFTNKGSS
GSSSFTLSSSASGFTAGQAVTDVLSCTSYTADSNGNIAININAGAPRVLYPTSKLTGSGLCS
GSSSTSGTPTTIKTSAVSGGCSTPTAVAVTFTDKVTTQYGQTIKLAGSIPQLGSWNAANAVT
LSSAGYTASNPVWSGTVNIPAGQAFSYKFIKVNSDGSVTWESDPNHSYTVPASCGVTTASVS
NTWQG
```

Figure 4.30 (continued)

An alignment of the putative mRNA sequence and the gDNA sequence was performed using Clustal W. The positions of 7 introns, 8 exons, the start codon, and the stop codon on the gDNA sequence were derived as shown in Figure 4.31.

```
CLUSTAL 2.0.10 multiple sequence alignment

mRNA            ------------------------------------------------------------
gDNA            CTGCATTCNTGTGGACGCCAGCCNGGCAGNTTTACTTGTCACAGTCTGTGCTGCCGTCCA 60

mRNA            ------------------------------------------------------------
gDNA            TCACCATNTACCAAGACCAANCNCGAGACTAATANNAGCCGGANAAAGGAGGGGTCANAA 120

mRNA            ------------------------------------------------------------
gDNA            ACTTANTTGTCCAACGACCTGCNAATCCGTTCCCAAAATGCTTCTTGATCCTTATACCAC 180

mRNA            ------------------------------------------------------------
gDNA            ATGGNNGTCGCGCCNAANGCTGNATAACGAGAAGCCNCTCCTTCTTACNCTANTAAGCCT 240

mRNA            ------------------------------------------------------------
gDNA            GCTTCTCGAANCNAANTATTCTGTGCCCGAGTTTCAGTACCTGAATGAGCCGATCGGTAA 300

mRNA            ------------------------------------------------------------
gDNA            AGCCAGCCATTCAAGCCACCGGTGGGTGACCACAGCATGTATTACGCGCTCACAGCTTGT 360

mRNA            ------------------------------------------------------------
gDNA            CACATCTCCTGGTCGGAAACCAGGTCCAACTTCCTGTTTTGGACTCGAGCAGCGGAGATG 420

mRNA            ------------------------------------------------------------
gDNA            AGCACTTGAACAATGAGATTGAATGACTAACGACAACAAACAAGAAACTATCAACACTGA 480

mRNA            ------------------------------------------------------------
gDNA            ATCACTCTATTAGCCTCACCGGTCAAAAGTTCCAAGATATACATGACTTCTTCGAGACAA 540

mRNA            ------------------------------------------------------------
gDNA            GATCTGCTCAGGTCTATTGGTATGGTGATTGTGCCGCGGTAGTAACCAATATAAGAACAT 600

mRNA            ------------------------------------------------------------
gDNA            GCCCATAAGACGCCATTTCCTCAACAATATTATGCTCTACTTTGCAATCTAAAGGATAAC 660

mRNA            ----------------ATGGCAGCCAACTACGTTTCTCGATTGTTGTTCACCCTTCTCT 43
gDNA            GACACAAGCCCGGAGACATGGCAGCCAACTACGTTTCTCGATTGTTGTTCACCCTTCTCT 720
                               ******************************************

mRNA            ACTTGACCGGGTTGGTGCAGTGTCTCACCCCTGCACAATGGAGAAGTCAATCGATCTATC 103
gDNA            ACTTGACCGGGTTGGTGCAGTGTCTCACCCCTGCACAATGGAGAAGTCAATCGATCTATC 780
                ************************************************************

mRNA            AAGTTCTTACCGATCGTTTTGCGAGGACAGATGGTTCTACTACTGCCTCTTGTGATGTCA 163
gDNA            AAGTTCTTACCGATCGTTTTGCGAGGACAGATGGTTCTACTACTGCCTCTTGTGATGTCA 840
                ************************************************************

mRNA            ACAAATATTGTGGCGGATCCTTCCAAGGCATCATCAAGAAGCTGGACTACATCCAGCAGA 223
gDNA            ACAAATATTGTGGCGGATCCTTCCAAGGCATCATCAAGAAGCTGGACTACATCCAGCAGA 900
                ************************************************************
```

Figure 4.31 Alignment of the entire putative gDNA sequence and the putative mRNA of α-amylase of *A. pullulans* NRRL Y-12974. The sequences of introns are indicated.

```
mRNA        TGGGCTTTACTGCG-------------------Intron #1------------------ 237
gDNA        TGGGCTTTACTGCGGTTCGTATTCATGTAGCTGGGCGGACACCATGACAGTCCTAATGTA 960
            **************

mRNA        --------ATCTGGATCAGTCCTGTTGTGAAGAACATCTACAGTTCTGGCCAAGATGGTG 289
gDNA        CACCGCAGATCTGGATCAGTCCTGTTGTGAAGAACATCTACAGTTCTGGCCAAGATGGTG 1020
                   **************************************************

mRNA        ACTCGTATCACGGATACTGGGCTCAAGATATCTACCAAGTCAATACCAACTTCGGTTCTG 349
gDNA        ACTCGTATCACGGATACTGGGCTCAAGATATCTACCAAGTCAATACCAACTTCGGTTCTG 1080
            ************************************************************

mRNA        CTGCCGATCTGGTATCACTGTCCAAGGCTCTCCACGATCGTGGCATG------------- 396
gDNA        CTGCCGATCTGGTATCACTGTCCAAGGCTCTCCACGATCGTGGCATGGTGAGATGATTCT 1140
            *********************************************

mRNA        -------- Intron #2-----------------TATTTGATGGTTGACATTGTCACA 420
gDNA        CTCGCAGGTATACGTGTTTGGGCTTACATTTCTCAGTATTTGATGGTTGACATTGTCACA 1200
                                               ************************

mRNA        AACCATATGGGATACAATGGCTGTGGCAATTGCGTCGACTACTCCATATACAACCCGTTC 480
gDNA        AACCATATGGGATACAATGGCTGTGGCAATTGCGTCGACTACTCCATATACAACCCGTTC 1260
            ************************************************************

mRNA        AACTCGCAGTCATACTATCATCCCTTCTGCCTCATCAACTACAATGATCAGACCAGTGTT 540
gDNA        AACTCGCAGTCATACTATCATCCCTTCTGCCTCATCAACTACAATGATCAGACCAGTGTT 1320
            ************************************************************

mRNA        GAGCAG--------------- Intron #3---------------------------TGC 549
gDNA        GAGCAGGTATGCTTTCAACCTCTTGCCTTTTCGAGGAGGCGCTGATAGACTTCCTAGTGC 1380
            ******                                                   ***

mRNA        TGGGCCGGCGACAACACAGTATCTCTGCCAGACTTGAGAACCGAAGACTCCAACGTACTC 609
gDNA        TGGGCCGGCGACAACACAGTATCTCTGCCAGACTTGAGAACCGAAGACTCCAACGTACTC 1440
            ************************************************************

mRNA        TCCATGTGGAATACCTGGATCAAACAGCTTGTGTTCAACTACACCA-------------- 655
gDNA        TCCATGTGGAATACCTGGATCAAACAGCTTGTGTTCAACTACACCAGTAAGTCGACGAGT 1500
            *********************************************

mRNA        ---------- Intron #4---------------TCGATGGTTTGAGAATCGACTCTGC 680
gDNA        TTCTTGAAGCAAAGACTTTCGCTAACGCTTTCTAGTCGATGGTTTGAGAATCGACTCTGC 1560
                                               ************************

mRNA        CAAGTCCGTTGACAAAGCATTCTATCAACCATTCCAACAAGCCGCCAGTGTCTATGCAGT 740
gDNA        CAAGTCCGTTGACAAAGCATTCTATCAACCATTCCAACAAGCCGCCAGTGTCTATGCAGT 1620
            ************************************************************

mRNA        TGGTGAAGTCTACGATGGCGACCCGAACTACTTCTGCGACTACCAGAACTACCTGGACGG 800
gDNA        TGGTGAAGTCTACGATGGCGACCCGAACTACTTCTGCGACTACCAGAACTACCTGGACGG 1680
            ************************************************************

mRNA        CATGCTCAACTACCCAAC------------- Intron #5------------------- 818
gDNA        CATGCTCAACTACCCAACGTATGAAACGTACTTTTCTGTACGAGAAATGCACAATACTGA 1740
            ******************

mRNA        -----------ATACTACTGGATCACACAAGCCTTCCAGTCCACCTCTGGCAGCATCAGC 867
gDNA        CCACTTGATAGATACTACTGGATCACACAAGCCTTCCAGTCCACCTCTGGCAGCATCAGC 1800
                     *********************************************

mRNA        AATCTCTACAATGGTATCAACACCATGAAATCGACCTGCAAAGACACAACACTCCTAGGC 927
gDNA        AATCTCTACAATGGTATCAACACCATGAAATCGACCTGCAAAGACACAACACTCCTAGGC 1860
            ************************************************************

mRNA        TCGTTCATGGAGAACCATGATGTAGCACGCTTCGCATCATTGACTTCCGATTACGCACTT 987
gDNA        TCGTTCATGGAGAACCATGATGTAGCACGCTTCGCATCATTGACTTCCGATTACGCACTT 1920
            ************************************************************

mRNA        GCCAAAAATGCTATTGCGTTTACAATGCTCGCCGACGGTATCCCAATCATCTACCAAGGC 1047
gDNA        GCCAAAAATGCTATTGCGTTTACAATGCTCGCCGACGGTATCCCAATCATCTACCAAGGC 1980
            ************************************************************
```

Figure 4.31 (continued)

```
mRNA        CAAGAACAGCACTTCTCAGGCTCTAGTGTGCCTAACAACAGAGAAGCACTTTGGCTATCT 1107
gDNA        CAAGAACAGCACTTCTCAGGCTCTAGTGTGCCTAACAACAGAGAAGCACTTTGGCTATCT 2040
            ************************************************************

mRNA        GGCTACCCTACCTCTTCACAACTGTATCCGTTTATTGCTACAGTCAATAAGATCAGAAAG 1167
gDNA        GGCTACCCTACCTCTTCACAACTGTATCCGTTTATTGCTACAGTCAATAAGATCAGAAAG 2100
            ************************************************************

mRNA        CAAGCCATCAAGCAAGATACGGGATATCTCACATACAAGGCCTATCCCGTCTACTCTGAC 1227
gDNA        CAAGCCATCAAGCAAGATACGGGATATCTCACATACAAGGCCTATCCCGTCTACTCTGAC 2160
            ************************************************************

mRNA        GCCTCAACTATCGTCATGAGGAAGGGCACGACAGGATCCCAAGTCATCGGTGTGTTCACC 1287
gDNA        GCCTCAACTATCGTCATGAGGAAGGGCACGACAGGATCCCAAGTCATCGGTGTGTTCACC 2220
            ************************************************************

mRNA        AACAAAGGCTCCTCCGGAAGTAGTTCATTCACACTTTCCTCATCCGCGAGTGGCTTTACA 1347
gDNA        AACAAAGGCTCCTCCGGAAGTAGTTCATTCACACTTTCCTCATCCGCGAGTGGCTTTACA 2280
            ************************************************************

mRNA        GCTGGTCAAGCCGTTACTGATGTCTTGTCCTGCACTTCCTACACTGCCGACTCGAATGGC 1407
gDNA        GCTGGTCAAGCCGTTACTGATGTCTTGTCCTGCACTTCCTACACTGCCGACTCGAATGGC 2340
            ************************************************************

mRNA        AATATTGCTATCAATATCAATGCTGGTGCTCCTAGAGTTCTTTACCCGACCTCAAAGCTG 1467
gDNA        AATATTGCTATCAATATCAATGCTGGTGCTCCTAGAGTTCTTTACCCGACCTCAAAGCTG 2400
            ************************************************************

mRNA        ACTGGCAGTGGTCTTTGTAGTGGCTCGAGTTCTACATCAGGTACACCAACCACGATCAAA 1527
gDNA        ACTGGCAGTGGTCTTTGTAGTGGCTCGAGTTCTACATCAGGTACACCAACCACGATCAAA 2460
            ************************************************************

mRNA        ACATCTGCGGTATCTGGTGGCTGCAGTACTCCTACCGCAGTTGCTGTCACCTTTACCGAC 1587
gDNA        ACATCTGCGGTATCTGGTGGCTGCAGTACTCCTACCGCAGTTGCTGTCACCTTTACCGAC 2520
            ************************************************************

mRNA        AAAGTGACTACTCAATATGGTCAGACCATCAAGCTCGCAGGCTCGATCCCTCAGCTCGGG 1647
gDNA        AAAGTGACTACTCAATATGGTCAGACCATCAAGCTCGCAGGCTCGATCCCTCAGCTCGGG 2580
            ************************************************************

mRNA        TCTTGGAATGCTGCAAACGCCGTTACCTTGTCTTCTGCTGGGTATACGGCTAGTAATCCT 1707
gDNA        TCTTGGAATGCTGCAAACGCCGTTACCTTGTCTTCTGCTGGGTATACGGCTAGTAATCCT 2640
            ************************************************************

mRNA        GTCTGGT------------------ Intron #6------------------------- 1714
gDNA        GTCTGGTAATTCCACCTGCATTGAAGAATATTCTGATCGAGGTTGCTGACGATAATACAG 2700
            *******

mRNA        --CTGGTACCGTCAATATTCCCGCTGGCCAAGCTTTCAGCTACAAGTTCATCAAAGTGAA 1772
gDNA        GTCTGGTACCGTCAATATTCCCGCTGGCCAAGCTTTCAGCTACAAGTTCATCAAAGTGAA 2760
              ************************************************************

mRNA        CTCCGATGGAAGCGTGACTTGGGAGTCCGATCC--------------------------- 1805
gDNA        CTCCGATGGAAGCGTGACTTGGGAGTCCGATCCGTAAGTAACATTCAGTCGTTTTTTTAA 2820
            ********************************

mRNA        ---- Intron #7-----------------AAATCACAGTTATACGGTTCCTGCGAGCT 1834
gDNA        GGAGCACATACGCTGACGTGCTTTTGTAAAGAAATCACAGTTATACGGTTCCTGCGAGCT 2880
                                          ****************************

mRNA        GCGGTGTGACGACTGCATCGGTCAGCAATACTTGGCAGGGGTGA---------------- 1878
gDNA        GCGGTGTGACGACTGCATCGGTCAGCAATACTTGGCAGGGGTGAGCATGCCGGAAGTTCG 2940
            ******************************************

mRNA        ------------------------------------------------------------
gDNA        TGGAAGGTTGGGGAGGGAGACAGGTATAAAATTCGCACACTTTCGGAAGTGTGACAGGGC 3000

mRNA        ------------------------------------------------------------
gDNA        GCAGCTGGACTGCTCCTCCTATGGTTGTAAGAGACAAAAGCGATAGCAAAACAGGGCAGA 3060
```

Figure 4.31 (continued)

```
mRNA        ------------------------------------------------------------
gDNA        CAATCCAAAGTGCTTGTAGACAGAATTTTTGAGACCTCGGAGCTCTAGTGGTGTATGCAC 3120

mRNA        ------------------------------------------------------------
gDNA        TTCGTGCTCCATATGGAAATTTTCGTTTTAAGAGTGGAGGGCCAGCTTTTTGAGCGTCGG 3180

mRNA        ------------------------------------------------------------
gDNA        TCTAGGCCCCAGCGTCATGTACGAGATATTTCTGAGAGGGTTGTCGTGGAGACAACAACG 3240

mRNA        ------------------------------------------------------------
gDNA        AGACCTTGACCGAGATGAAACTCTTCCGCGTGGGTGCTGTAATGGTGTGTTGAACAGATC 3300

mRNA        ------------------------------------------------------------
gDNA        TTTGCGTGTGCTATGTCTCGAAAGTACGATAACCACAAACCTTCCAAACAATTTACATCA 3360

mRNA        ------------------------------------------------------------
gDNA        TTTGAAGGTCTCTGCTTCGCACCAGTGTTCTCTCAAGGGTTCGTTGTCTTTTTCTTGGTA 3420

mRNA        ------------------------------------------------------------
gDNA        AGCCGTTCTTTGCCATCCTTGTGAGTGGTGTATTCATAATCAAGAGGTAATCCTTCAGGC 3480

mRNA        ------------------------------------------------------------
gDNA        CATCGCTTAGCGAAACACGCACGGCAAAGCTGTTTTCCAGGATCTTGTATGTCGTGCCAC 3540

mRNA        ------------------------------------------------------------
gDNA        TGTGTTGTAACAAACGGTCAGCATTAAAGTCTGATAGTAGTTATTTGTTGAGTATGGCAG 3600

mRNA        ------------------------------------------------------------
gDNA        CTTGAATCAGCCTCTCGGCTCGTTGAGTACATACCCATAACCTAGCCTTTCTAAAGGATT 3660

mRNA        ------------------------------------------------------------
gDNA        CGCGAGTACAATCCTCGCAGCGAAGGCCAACTTTCTCCGGGTGCATTATCGGTTCGTGTG 3720

mRNA        ------------------------------------------------------------
gDNA        TAGTCCATTGTACAGGCTTGTCATGTAAAACTCTGGCCCATTCGCTTATTTTGTGATTAA 3780

mRNA        ------------------------------------------------------------
gDNA        GCTTGAACAGAGGATCTTCCATGCGTCTTTGTCGCTTTACGGAAATGTTATGTTGGTTGT 3840

mRNA        --------------------------------
gDNA        ACTTCTTCTTGGCATCATCCCTAAGGTTTGAT 3872
```

Figure 4.31 (continued)

Finally, the complete gDNA sequence of the alpha amylase of *Aureobasidium pullulans* strain NRRL Y-12974 was obtained. It contains 2,247 bp, including 7 introns and 8 exons. The sequence is shown in Figure 4.32.

Complete sequence of the alpha amylase gDNA of *A. pullulans* (2,247 bp)

ATGGCAGCCAACTACGTTTCTCGATTGTTGTTCACCCTTCTCTACTTGACCGGGTTGGTGCA
GTGTCTCACCCCTGCACAATGGAGAAGTCAATCGATCTATCAAGTTCTTACCGATCGTTTTG
CGAGGACAGATGGTTCTACTACTGCCTCTTGTGATGTCAACAAATATTGTGGCGGATCCTTC
CAAGGCATCATCAAGAAGCTGGACTACATCCAGCAGATGGGCTTTACTGCGGTTCGTATTCA
TGTAGCTGGGCGGACACCATGACAGTCCTAATGTACACCGCAGATCTGGATCAGTCCTGTTG
TGAAGAACATCTACAGTTCTGGCCAAGATGGTGACTCGTATCACGGATACTGGGCTCAAGAT
ATCTACCAAGTCAATACCAACTTCGGTTCTGCTGCCGATCTGGTATCACTGTCCAAGGCTCT
CCACGATCGTGGCATGGTGAGATGATTCTCTCGCAGGTATACGTGTTTGGGCTTACATTTCT
CAGTATTTGATGGTTGACATTGTCACAAACCATATGGGATACAATGGCTGTGGCAATTGCGT
CGACTACTCCATATACAACCCGTTCAACTCGCAGTCATACTATCATCCCTTCTGCCTCATCA
ACTACAATGATCAGACCAGTGTTGAGCAGGTATGCTTTCAACCTCTTGCCTTTTCGAGGAGG
CGCTGATAGACTTCCTAGTGCTGGGCCGGCGACAACACAGTATCTCTGCCAGACTTGAGAAC
CGAAGACTCCAACGTACTCTCCATGTGGAATACCTGGATCAAACAGCTTGTGTTCAACTACA
CCAGTAAGTCGACGAGTTTCTTGAAGCAAAGACTTTCGCTAACGCTTTCTAGTCGATGGTTT
GAGAATCGACTCTGCCAAGTCCGTTGACAAAGCATTCTATCAACCATTCCAACAAGCCGCCA
GTGTCTATGCAGTTGGTGAAGTCTACGATGGCGACCCGAACTACTTCTGCGACTACCAGAAC
TACCTGGACGGCATGCTCAACTACCCAACGTATGAAACGTACTTTTCTGTACGAGAAATGCA
CAATACTGACCACTTGATAGATACTACTGGATCACACAAGCCTTCCAGTCCACCTCTGGCAG
CATCAGCAATCTCTACAATGGTATCAACACCATGAAATCGACCTGCAAAGACACAACACTCC
TAGGCTCGTTCATGGAGAACCATGATGTAGCACGCTTCGCATCATTGACTTCCGATTACGCA
CTTGCCAAAAATGCTATTGCGTTTACAATGCTCGCCGACGGTATCCCAATCATCTACCAAGG
CCAAGAACAGCACTTCTCAGGCTCTAGTGTGCCTAACAACAGAGAAGCACTTTGGCTATCTG
GCTACCCTACCTCTTCACAACTGTATCCGTTTATTGCTACAGTCAATAAGATCAGAAAGCAA
GCCATCAAGCAAGATACGGGATATCTCACATACAAGGCCTATCCCGTCTACTCTGACGCCTC
AACTATCGTCATGAGGAAGGGCACGACAGGATCCCAAGTCATCGGTGTGTTCACCAACAAAG
GCTCCTCCGGAAGTAGTTCATTCACACTTTCCTCATCCGCGAGTGGCTTTACAGCTGGTCAA
GCCGTTACTGATGTCTTGTCCTGCACTTCCTACACTGCCGACTCGAATGGCAATATTGCTAT
CAATATCAATGCTGGTGCTCCTAGAGTTCTTTACCCGACCTCAAAGCTGACTGGCAGTGGTC
TTTGTAGTGGCTCGAGTTCTACATCAGGTACACCAACCACGATCAAAACATCTGCGGTATCT
GGTGGCTGCAGTACTCCTACCGCAGTTGCTGTCACCTTTACCGACAAAGTGACTACTCAATA
TGGTCAGACCATCAAGCTCGCAGGCTCGATCCCTCAGCTCGGGTCTTGGAATGCTGCAAACG
CCGTTACCTTGTCTTCTGCTGGGTATACGGCTAGTAATCCTGTCTGGTAATTCCACCTGCAT
TGAAGAATATTCTGATCGAGGTTGCTGACGATAATACAGGTCTGGTACCGTCAATATTCCCG
CTGGCCAAGCTTTCAGCTACAAGTTCATCAAAGTGAACTCCGATGGAAGCGTGACTTGGGAG
TCCGATCCGTAAGTAACATTCAGTCGTTTTTTTAAGGAGCACATACGCTGACGTGCTTTTGT
AAAGAAATCACAGTTATACGGTTCCTGCGAGCTGCGGTGTGACGACTGCATCGGTCAGCAAT
ACTTGGCAGGGGTGA

Figure 4.32 Complete sequence of α-amylase genomic DNA of *A. pullulans* NRRL Y-12974.

The α-amylase amino acid sequences of *A. pullulans* strain NRRL Y-12974 were used to search for homologous sequences from other species in the GenBank database using Blastp. Putative conserved domains were detected as an alpha-amylase superfamily. Using DNA star program, the closely related sequences were aligned. The percent identity indicated that the α-amylase amino acid sequence of *A. pullulans* is similar to those of *Neosartorya fischeri* (XP_001265628) (93.7%) and

Aspergillus kawachii (BAA22993) (95.8) in the GenBank database (Figure 4.33). The multiple alignments are shown in Figure 4.34.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	
1		54.0	52.3	52.3	52.2	51.0	51.2	51.8	50.2	49.8	50.1	47.2	58.7	1
2	69.7		85.0	85.0	73.9	69.7	69.5	78.7	74.2	75.0	69.5	67.1	60.5	2
3	73.8	16.8		93.7	75.0	67.7	67.6	76.5	71.7	72.3	70.1	67.7	59.4	3
4	73.8	16.8	6.5		73.8	67.4	68.0	75.5	71.9	72.2	69.9	67.6	58.7	4
5	74.2	32.1	30.4	32.2		64.7	68.0	72.5	68.0	68.2	64.4	64.8	57.8	5
6	77.2	38.7	42.1	42.7	47.4		58.7	65.2	66.2	65.9	57.7	57.6	58.4	6
7	76.8	39.2	42.3	41.6	41.6	59.2		67.7	64.9	65.5	63.2	72.4	56.7	7
8	75.1	25.1	28.2	29.8	34.2	46.6	42.1		67.6	67.3	63.3	63.3	56.4	8
9	79.4	31.6	35.5	35.1	41.6	44.7	47.1	42.3		95.8	60.1	61.9	56.6	9
10	80.3	30.4	34.6	34.8	41.2	45.2	46.1	42.8	4.4		60.4	63.0	56.8	10
11	79.6	39.1	38.1	38.5	48.0	61.3	50.3	50.0	56.4	55.7		62.3	52.4	11
12	87.5	43.2	42.2	42.4	47.3	61.7	34.5	50.0	52.7	50.7	52.1		52.4	12
13	59.3	55.6	57.8	59.2	61.3	60.0	63.5	64.2	63.9	63.4	73.6	73.6		13
	1	2	3	4	5	6	7	8	9	10	11	12	13	

1 XP_001908940_Podospora anserina.pro
2 XP_001209405_Aspergillus terreus.pro
3 XP_001265628_Neosartorya fischeri.pro
4 XP_749208_Aspergillus fumigatus.pro
5 CAP92733_Penicillium chrysogenum.pro
6 XP_002145753_Penicillium marneffei.pro
7 XP_001272245_Aspergillus clavatus.pro
8 ABF72529_Ophiostoma floccosum.pro
9 BAA22993_Aspergillus kawachii.pro
10 BAD06003_Aspergillus awamori.pro
11 XP_001273134_Aspergillus clavatus.pro
12 XP_661006_Aspergillus nidulans.pro
13 PenAmy Pro

Figure 4.33 Percent identity of α-amylases amino acid sequences closely related to *A. pullulans* NRRL Y-12974 (named as PenAmy in this figure).

Alignment Report of Amy alignment wo leader v2.meg ClustalW (Slow/Accurate, Gonnet)
Wednesday, January 28, 2009 3:25 PM

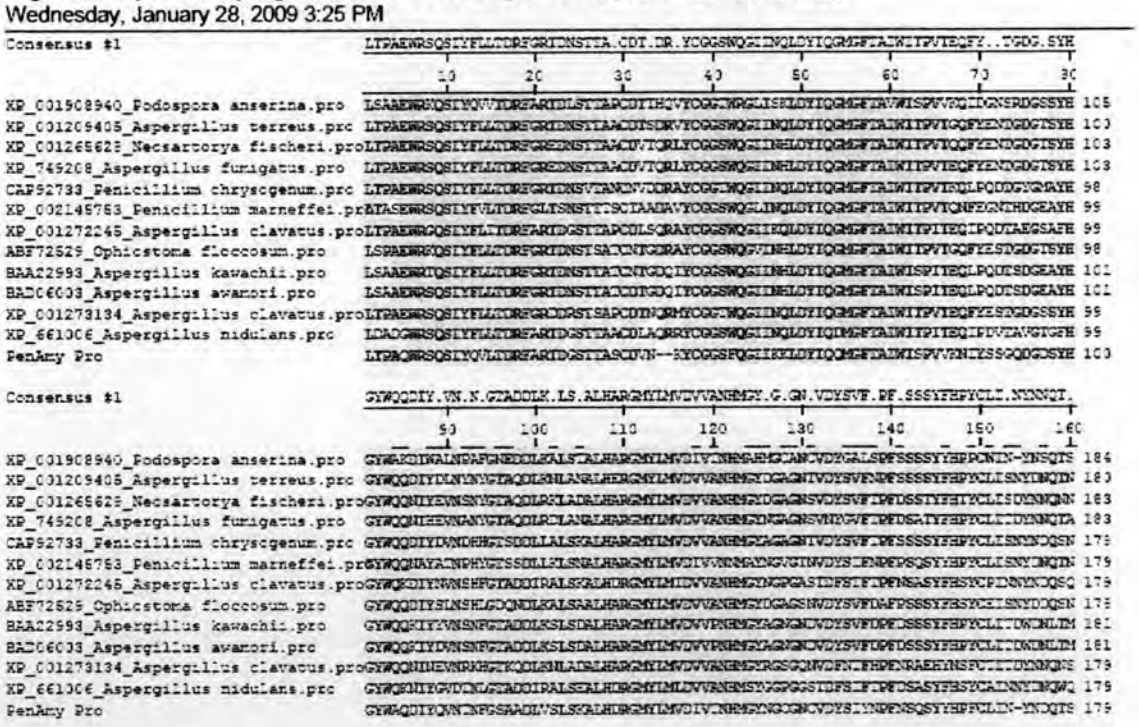

Figure 4.34 Alignment of amino acid sequences of several α-amylases closely related to *A. pullulans* NRRL Y-12974 (named as PenAmy in this figure).

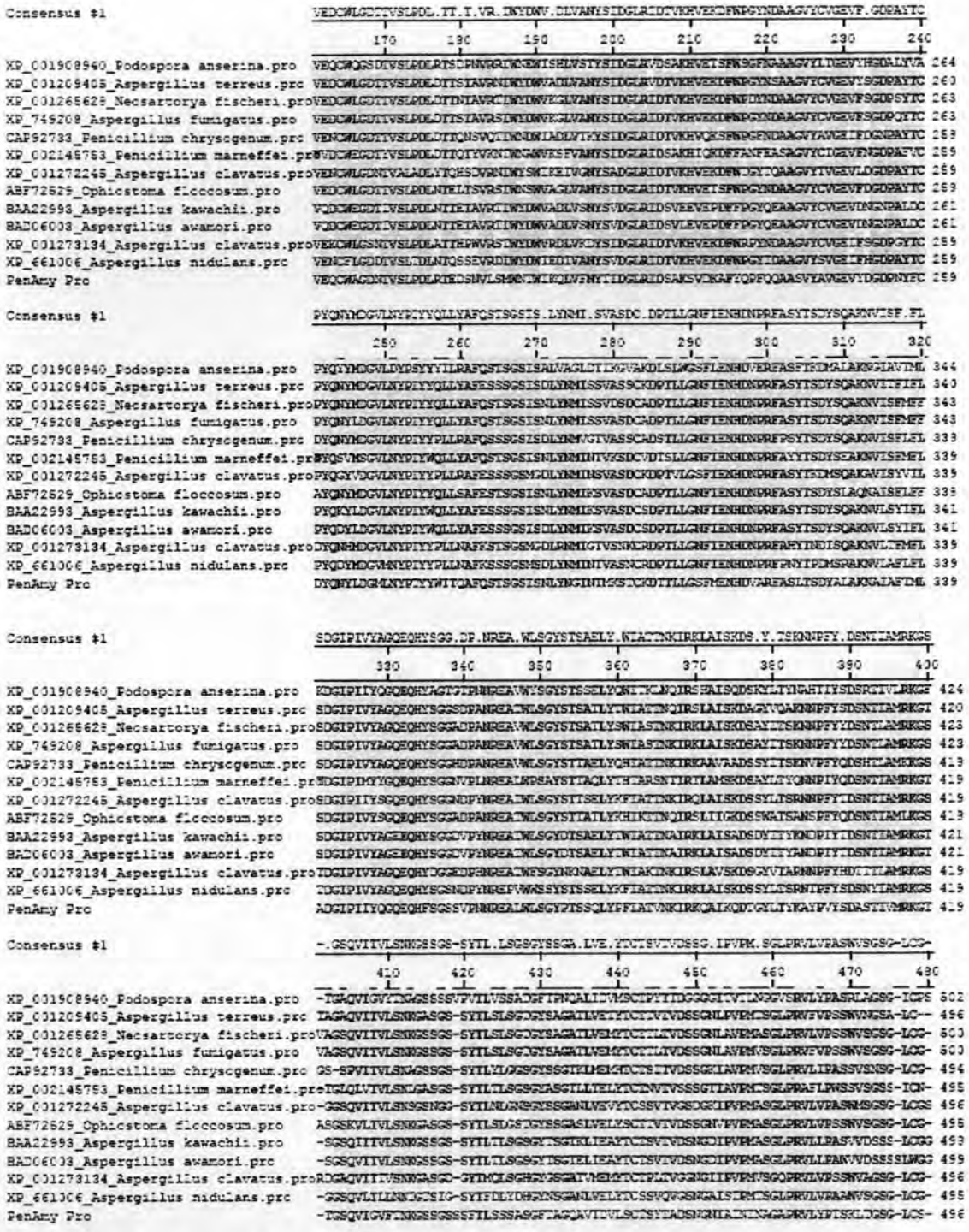

Figure 4.34 (continued)

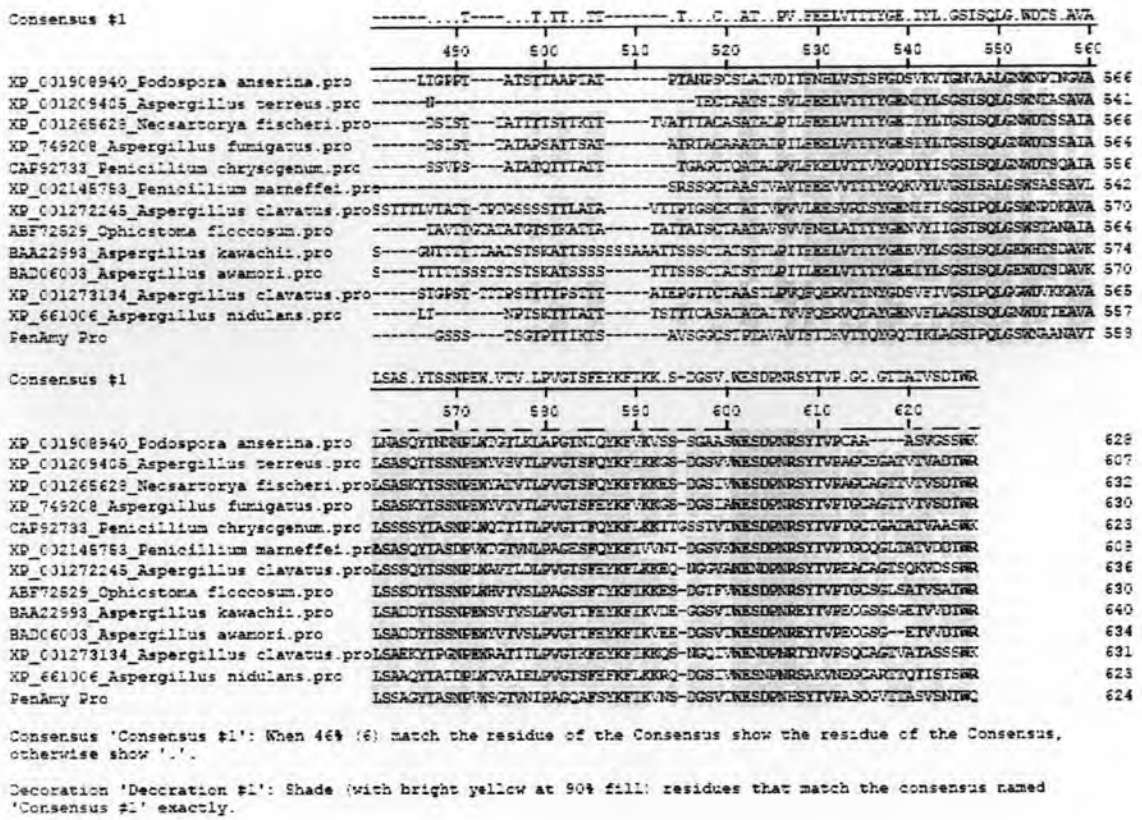

Figure 4.34 (continued)

4.2.3.2 Southern blot hybridization

The total DNA from strain NRRL Y-12974 was completely digested with five restriction enzymes (*Xba*I, *Kpn*I, *Pst*I, *Pvu*II, and *Eco*RI) and samples were separated in a 0.7% agarose gel. The digested DNAs was transferred to a positively charged nylon membrane and hybridized with a DIG-labeling probe (PCR product of cloned fragment 3 (Figure 3.2)). The α-amylase gene was detected using Anti-DIG-AP and CDP star. The Southern blot analysis showed only a single band of α-amylase fragment in each digested gDNA (Figure 4.35).

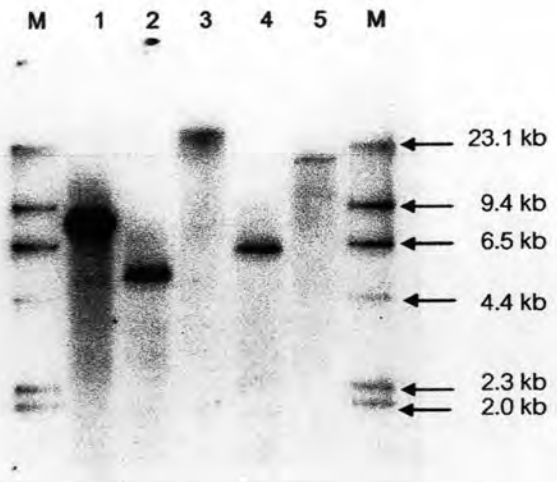

Figure 4.35 Southern blot hybridization on positively charged nylon membrane detected by Anti-DIG-AP and CDP star. Lane 1 – 5 were the total DNA digested with *Xba*I, *Kpn*I, *Pst*I, *Pvu*II, and *Eco*RI, respectively). Lane M was the *Hin*dIII digested Lambda ladder (mixture of DIG labeled *Hin*dIII digested Lambda (20 ng) and unlabeled *Hin*dIII digested Lambda (150 ng)).

4.2.3.3 Detection of α -amylase mRNA

The total RNA from strain NRRL Y-12974 grown on standard and modified PM was treated with DNase I to eliminate gDNA contamination. The DNase I treated-RNA and first strand cDNA derived from this RNA (random hexamers, Invitrogen) were then used as templates to amplify a partial fragment of α-amylase cDNA (fragment 3, Figure 3.2) RT-PCR product could not be amplified from the DNase I treated-RNA. However, a DNA fragment of about 500 bp was amplified from the first strand cDNA of NRRL Y-12974 grown in PM containing either 5.0% starch or 5.0% sucrose in which the PCR products from cells grown in starch PM showed higher concentrations than those from sucrose PM (Figure 4.36 A). Moreover, PCR product was obtained only from cultures grown for 4, 6, and 8 days and not from 2 day old cultures.

An internal control was performed using 26S rDNA from *A. pullulans*. The results showed about 500 bp of PCR products in all samples, with high amounts of DNA from the first strand cDNA (Figure 4.36 B). All 26S rDNA PCR products were similar in quantity indicating that similar amounts of all total RNA tested. However, there were smaller amounts of PCR products from DNase I treated RNA compared to those of

the first strand cDNA. No PCR products was obtained from DNase I-treated RNA from day 2 cultures grown on modified PM (5.0% starch and 0.3% N-sources).

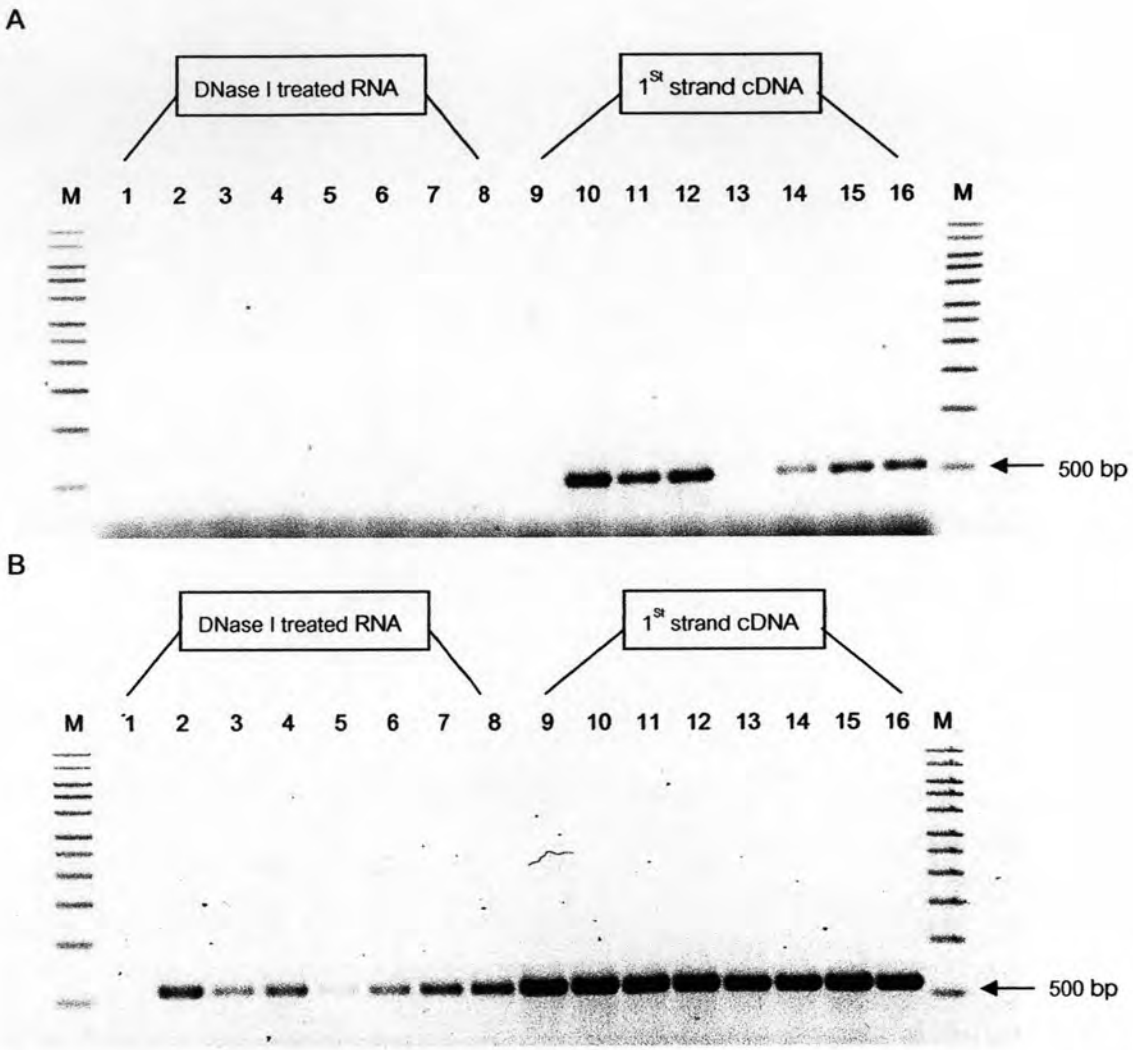

Figure 4.36 Amplification of (A) partial α-amylase and (B) partial 26S rDNA from mRNA and 1st strand cDNA using RT-PCR and PCR, respectively. The PCR products were separated on 1% agarose gel at 100 V for 1 h. Lane 1 – 4 were DNase I treated RNA from NRRL Y-12974 cultured in PM (5.0% starch, 0.3% N-sources) for day 2, 4, 6, and 8, respectively. Lane 5 – 8 were DNase I treated RNA from NRRL Y-12974 cultured in PM (5.0% sucrose, 0.3% N-sources) for day 2, 4, 6, and 8, respectively. Lane 9 – 12 were the 1st strand cDNA from NRRL Y-12974 cultured in PM (5.0% starch, 0.3% N-sources) for day 2, 4, 6, and 8, respectively. Lane 13 – 16 were the 1st strand cDNA from NRRL Y-12974 cultured in PM (5.0% sucrose, 0.3% N-sources) for day 2, 4, 6, and 8, respectively. M was DNA ladder, Directload, 1kB (Sigma), 250 µg.