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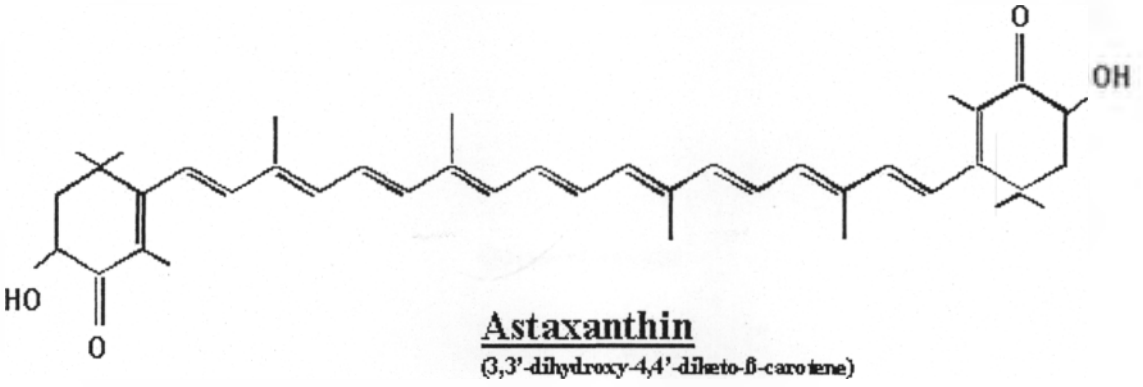
ΑΝΘΟΚΟΜΙΑΣ

"Τροποποίηση του βιοσυνθετικού μονοπατιού καροτενοειδών στη ζύμη *Xanthophyllomyces dendrorhous*"



Astaxanthin
(3,3'-dihydroxy-4,4'-diketo-β-carotene)

Παπαγεωργίου Χρυσοβαλάντης - Ιωάννης

Επιτηρητές: Dr. Παπαδοπούλου Καλλιόπη
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Wageningen 15 / 05 / 2004

Ευχαριστίες

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Με εκτίμηση,

Παπαγεωργίου Χρυσοβαλάντης - Ιωάννης

ΠΕΡΙΕΧΟΜΕΝΑ

ΜΕΡΟΣ 1°: Περίληψη πτυχιακής στα ελληνικά

Εισαγωγή.....1
Υλικά και μέθοδοι.....4
Υλικά.....4
Πλασμίδια και είδη.....4
Μέσα και διαλύματα.....4
Μέθοδοι.....4
Αποτελέσματα.....5
Ανάλυση του αριθμού αντιγράφων.....5
Ανάλυση των πρόσθετων αντιγράφων των *crtE* και *ast* γονιδίων.....6
Κλωνοποίηση του φορέα έκφρασης *cpr*.....7
Επίλογος.....10

ΜΕΡΟΣ 2°: Πτυχιακή στα αγγλικά

ΜΕΡΟΣ 1ο

ΠΕΡΙΛΗΨΗ ΠΤΥΧΙΑΚΗΣ ΣΤΑ ΕΛΛΗΝΙΚΑ

Τίτλος: 'Τροποποίηση του βιοσυνθετικού μονοπατιού καροτενοειδών στη ζύμη *Xanthophyllomyces dendrorhous*'

Εισαγωγή

Η *X. dendrorhous* είναι ζύμη κόκκινου-ροζ χρώματος και απομονώθηκε για πρώτη φορά, στις αρχές του 1970 από τα εκχυλίσματα των φυλλοβόλων δένδρων, στις ορεινές περιοχές της Ιαπωνίας και της Αλάσκας (Phaff. *et al*., 1972; Miller *et al*., 1976). Η ζύμη αυτή, είναι το τελεομορφικό στάδιο της ζύμης *Phaffia rhodozyma*, η οποία ανήκει στην υποκλάση των Ετεροβασιδιομυκήτων (Miller *et al*., 1976; Golubev, 1995).

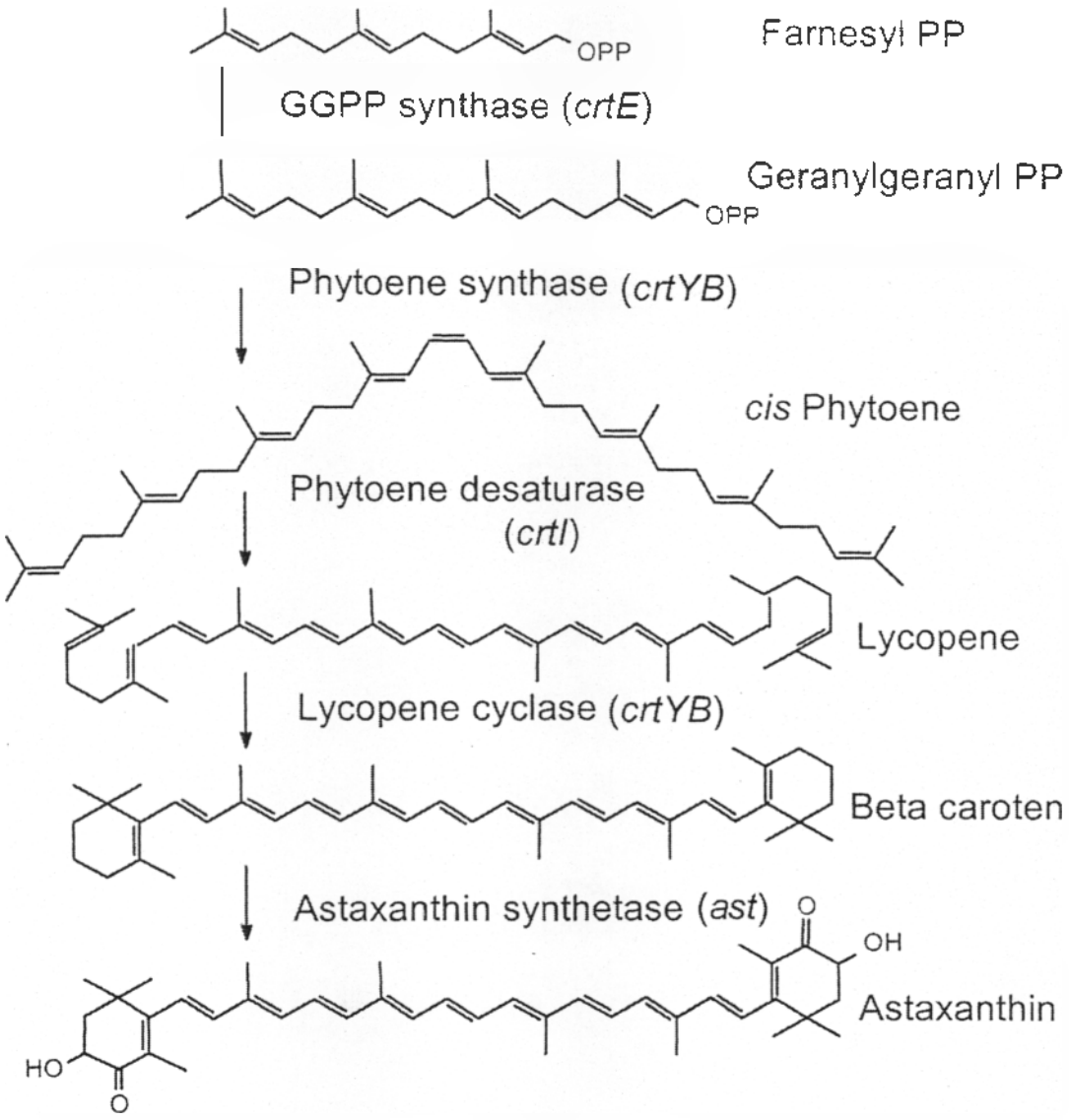
Η *X. dendrorhous* συνθέτει ασταξανθίνη ως το κύριο καροτενοειδές (Εικ. 1) το οποίο και συγκροτεί το 83-87 % του συνολικού περιεχομένου καροτενοειδών (Andrewes *et al*., 1976). Η ασταξανθίνη χρησιμοποιείται στα τρόφιμα και στη βιομηχανία τροφίμων ως χρωστική (π.χ. στη σάρκα του σολομού και της πέστροφας) και ως αντιοξειδωτικό (προστασία από διάφορα είδη καρκίνου) (Johnson *et al*., 1977; Johnson *et al*., 1980; Choubert and Heinrich, 1993).

Ωστόσο, οι καταναλωτές απαιτούν από τις βιομηχανίες να χρησιμοποιούν στα φυσικά προϊόντα, ασταξανθίνη προερχόμενη από φυσικές πηγές και όχι συνθετική ασταξανθίνη. Όπως αναφέρθηκε και προηγουμένως, η ζύμη *X. dendrorhous* είναι φυσική πηγή ασταξανθίνης, αλλά η συγκέντρωση ασταξανθίνης είναι πολύ χαμηλή (0.02-0.03 %) για εμπορική εφαρμογή. Για αυτό το λόγο, έχουν δημιουργηθεί διάφορες μεταλλάξεις, με στόχο την αυξημένη παραγωγή ασταξανθίνης στις ζύμες αυτές.

Στο εργαστήριο μας, υπάρχουν είδη της *X. dendrorhous* τα οποία υπέρ-εκφράζουν τα καροτενογενή (crt) γονίδια *crtE* (συνθάση του πυροφωσφορικού γερανυλγερανυλίου), *crtYB* (συνθάση του φυτοενίου / κυκλάση του λυκοπενίου), *crtI* (αποκορεσμάση του φυτοενίου) και *ast* (συνθετάση της ασταξανθίνης). Η παρουσία και ο αριθμός αντιγράφων των πρόσθετων αντιγράφων καροτενογενών γονιδίων *crtE*,

crtYB, *crtI* και *ast* στα αντίστοιχα μετασχηματισμένα είδη, καθορίστηκε με την ανάλυση κατά Southern.

Ένας επιπλέον στόχος, ήταν η δημιουργία ενός τροποποιημένου είδους της *X. dendrorhous*. Το είδος της *X. dendrorhous*, που υπέρ-παράγει συνθετάση της ασταξανθίνης, το οποίο είναι ένα ένζυμο μονοοξυγενάσης εξαρτώμενης από το κυτόχρωμα P450 (P450 για συντομία) που μετατρέπει το β-καροτένιο σε ασταξανθίνη, παράγει περισσότερη ασταξανθίνη από το είδος μάρτυρα (H. Visser αδημοσίευτα δεδομένα). Ωστόσο, τα ένζυμα P450 χρειάζονται τη συνεργασία άλλων ενζύμων, τα οποία είναι αναγωγάσες εξαρτώμενες από το κυτόχρωμα P450 (*cpr*-P450 αναγωγάσες). Τα P450-αναγωγάσες παρέχουν ένα ηλεκτρόνιο (e^-) στα ένζυμα P450, το οποίο είναι απαραίτητο για να ενεργοποιήσει το οξυγόνο που θα προστεθεί στο μόριο του β-καροτενίου με σκοπό να παραχθεί ασταξανθίνη (van den Brink *et al.*, 1998). Επομένως, ο δεύτερος φορέας θα εισαχθεί σε αυτό το είδος, το οποίο πιθανό θα υπέρ-εκφράσει το P450-αναγωγάση εξίσου καλά. Ιδανικό αποτέλεσμα είναι η αύξηση του συνολικού περιεχομένου ασταξανθίνης.



Εικόνα 1: Μονοπάτι βιοσύνθεσης ασταξανθίνης. Το πυροφωσφορικό ισοπεντενύλιο (IPP) είναι η πρόδρομος ουσία όλων των ισοπρενοειδών. Το πυροφωσφορικό φαρνεσύλιο (FPP) περιέχει 3 μόρια του IPP. Η συνθάση του πυροφωσφορικού γερανυλγερανυλίου (*crtE*) προσθέτει 1 μόριο IPP στο FPP και παράγεται πυροφωσφορικό γερανυλγερανύλιο (GGPP). Η συνθάση του φυτοενίου (*crtYB*), διπλασιάζει το μόριο του GGPP και παράγεται φυτοένιο. Η αποκορεσμάση του φυτοενίου (*crtI*) εισάγει 4 διπλούς δεσμούς στο μόριο του φυτοενίου και παράγεται λυκοπένιο. Η κυκλαση του λυκοπενίου (*crtYB*) κλείνει τα δύο ακραία δακτυλίδια στο μόριο του λυκοπενίου, με 2 κυκλοποιήσεις και παράγεται β-καροτένιο. Τέλος, η συνθετάση της ασταξανθίνης (*ast*) προσθέτει οξυγόνο στο μόριο του β-καροτενίου και παράγεται ασταξανθίνη.

Υλικά και μέθοδοι

Υλικά

Πλασμίδια και είδη

Διαφορετικοί φορείς *Escherichia coli* και *X. dendrorhous* χρησιμοποιήθηκαν στα πειράματα κλωνοποίησης (pGEM-T-Easy, pPRcDNA1 και pPR2TNH).

Το άγριο στέλεχος της *X. dendrorhous* (CBS6938) και οι μετασχηματισμοί αυτής, καλλιεργήθηκαν σε μέσο YEPD στους 21 ^{o}C και χρησιμοποιήθηκαν για τις απομονώσεις χρωμοσωμικού DNA. Τα μετασχηματισμένα είδη παρουσιάζονται στον πίνακα 1.

α/α	Είδος	Τι κάνει;
1	CBS6938 [pPRcDNA1*crtE*] #1, #2, #3	Υπέρ-έκφραση του *crtE* γονιδίου
2	CBS6938 [pPRcDNA1]	Μάρτυρας στο 1
3	CBS6938 [pPR2TN*ast*] #2, #3	Υπέρ-έκφραση του *ast* γονιδίου
4	CBS6938 [pPR*crtI*]	Υπέρ-έκφραση του *crtI* γονιδίου
5	CBS6938 [pPR*crtYB*]	Υπέρ-έκφραση του *crtYB* γονιδίου
6	CBS6938 [pPR2TN]	Μάρτυρας στα 3, 4 και 5

Πίνακας 1: Στον πίνακα παρουσιάζονται τα μετασχηματισμένα είδη που χρησιμοποιήθηκαν σε αυτή την έρευνα.

Μέσα και διαλύματα

Τα διαλύματα που χρησιμοποιήθηκαν σε αυτή την έρευνα παρουσιάζονται στο παράρτημα I (Appendix I).

Μέθοδοι

i) Απομόνωση χρωμοσωμικού DNA

ii) Ανάλυση κατά Southern

iii) Σήμανση του ανιχνευτή με DIG χρησιμοποιώντας PCR

iv) Προ-υβριδισμός και υβριδισμός

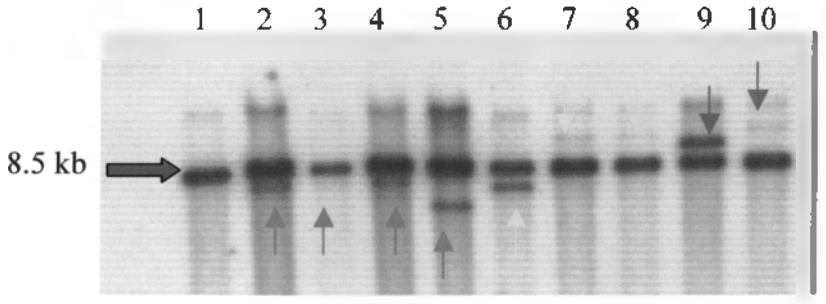
iv) Κλωνοποίηση του γονιδίου *cpr*

Αποτελέσματα

Ανάλυση του αριθμού αντιγράφων

Το χρωμοσωμικό DNA των ειδών της *X. dendrorhous*, υπέστη πέψη με το ένζυμο περιορισμού *SfiI* στους 50 °C για 4 ώρες. Χρησιμοποιήθηκε το *SfiI* γιατί απελευθερώνει τα πλασμίδια από το χρωμοσωμικό DNA. Το χρωμοσωμικό DNA ύστερα από την πέψη, διαχωρίστηκε σε 0.8 % πηχτή αγαρόζης, μεταφέρθηκε σε νάιλον φίλτρο και σχημάτισε υβρίδιο με τον ανιχνευτή rDNA που είχε σημανθεί με DIG.

Όταν εμφανίστηκε το φιλμ, μπορέσαμε να καθορίσουμε τον αριθμό αντιγράφων των πρόσθετων *crtE*, *crtYB*, *crtI* και *ast* γονιδίων στα αντίστοιχα μετασχηματισμένα είδη. Δύο σημάδια ανά μετασχηματισμένο είδος αναμένονταν (Εικ. 2). Ένα από τα ενδογενή μόρια rDNA και ένα από τα απελευθερωμένα πλασμίδια. Από το άγριο στέλεχος αναμενόταν ένα μόνο σημάδι, από τα ενδογενή μόρια rDNA.



Εικόνα 2: Ανάλυση κατά Southern των ειδών της *X. dendrorhous*. Το χρωμοσωμικό DNA ύστερα από την πέψη, διαχωρίστηκε σε πηχτή αγαρόζης, μεταφέρθηκε σε νάιλον φίλτρο και σχημάτισε υβρίδιο με τον ανιχνευτή rDNA που είχε σημανθεί με DIG. 1, CBS6938, 2, CBS6938[pPRcDNA1*crtE*]#1, 3, CBS6938[pPRcDNA1*crtE*]#2, 4, CBS6938[pPRcDNA1*crtE*]#3, 5, CBS6938[pPRcDNA1], 6, CBS6938[pPR2TN], 7, CBS6938[pPR2TN*ast*]#2, 8, CBS6938[pPR2TN*ast*]#3, 9, CBS6938[pPR*crtI*], 10, CBS6938[pPR*crtYB*]. Το παχύ οριζόντιο βέλος υποδεικνύει το σημάδι από τα ενδογενή

μόρια rDNA και τα κάθετα βέλη υποδεικνύουν το σημάδι από τα απελευθερωμένα πλασμίδια.

Συγκρίνοντας την εικόνα από την πηχτή αγαρόζης και του φιλμ, μπορέσαμε να υπολογίσουμε το μέγεθος των ενδογενή rDNA και το μέγεθος των εισαχθέντων πλασμιδίων. Έτσι, βρήκαμε ότι τα ενδογενή μόρια rDNA, των ειδών της *X. dendrorhous* έχουν μέγεθος 8.5 kb, το πλασμίδιο pPRcDNA1 6.6 kb, το pPRcDNA1*crtE* 7.8 kb, το pPR2TN 7.5 kb, το pPR2TN*ast* 11.3 kb, το pPR*crtI* 10.3 kb και το pPR*crtYB* 11.4 kb.

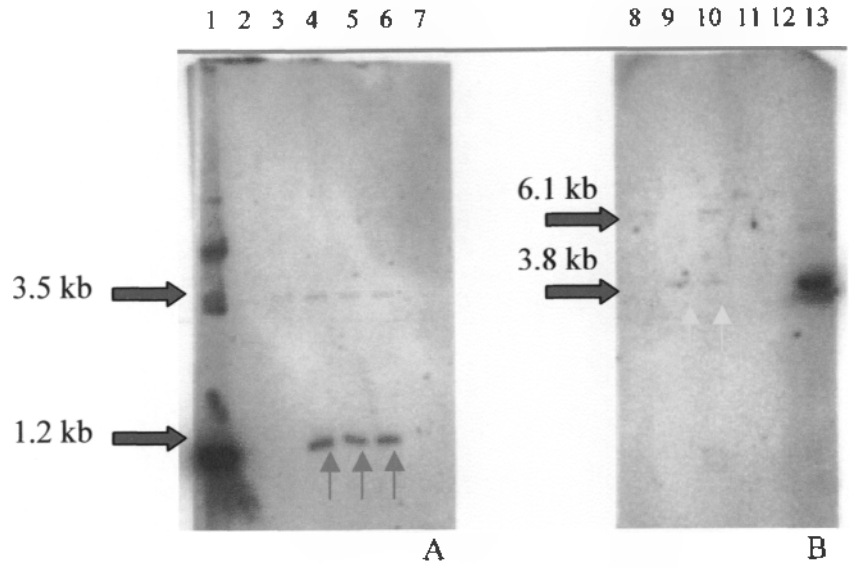
Η ένταση από το σημάδι των ενδογενή μορίων rDNA, αντιστοιχεί σε 61 αντίγραφα (Wery *et al.*, 1997). Συγκρίνοντας την ένταση από το σημάδι αυτό με εκείνο των πλασμιδίων, μπορέσαμε να υπολογίσουμε των αριθμό αντιγράφων. Ο αριθμός αντιγράφων των πρόσθετων καροτενογενών γονιδίων στα αντίστοιχα μετασχηματισμένα είδη είναι 15 αντίγραφα για το *crtE*, 1-5 αντίγραφα για το *crtYB*, 50 αντίγραφα για το *crtI* και 1-5 αντίγραφα για το *ast*.

Ανάλυση των πρόσθετων αντιγράφων των *crtE* και *ast* γονιδίων

Χρησιμοποιώντας τους ανιχνευτές *crtE* και *ast* μπορέσαμε να επιβεβαιώσουμε την παρουσία των πρόσθετων αντιγράφων των *crtE* και *ast* γονιδίων στα αντίστοιχα μετασχηματισμένα είδη.

Το χρωμοσωμικό DNA των *crtE* μετασχηματισμένων ειδών, υπέστη πέψη με τις ενδονουκλεάσεις *EcoRI* και *SstI* και των *ast* μετασχηματισμένων ειδών με τις *NotI* και *HindIII*, στους 37 °C για 2 ώρες. Το άγριο στέλεχος όπως επίσης και τα πλασμίδια που χρησιμοποιήθηκαν για τους μετασχηματισμούς, υπέστησαν πέψη με τις ίδιες ενδονουκλεάσεις σαν θετικοί μάρτυρες. Το χρωμοσωμικό DNA ύστερα από την πέψη, διαχωρίστηκε σε 1 % πηχτή αγαρόζης, μεταφέρθηκε σε νάιλον φίλτρα και σχημάτισε υβρίδια με τους ανιχνευτές *crtE* και *ast*, που είχαν σημανθεί με DIG.

Συγκρίνοντας την εικόνα από την πηχτή αγαρόζης με το φιλμ, μπορέσαμε να υπολογίσουμε το μέγεθος των ενδογενή γονιδίων και των πρόσθετων αντιγράφων γονιδίων. Αποδείξαμε ότι τα μεγέθη των πρόσθετων αντιγράφων των *crtE* και *ast* γονιδίων ήταν τα αναμενόμενα, 1.2 kb και 3.8 kb αντίστοιχα (Εικ. 3).



Εικόνα 3: Ανάλυση κατά Southern των ειδών υπέρ-έκφρασης των *crtE* και *ast* γονιδίων. Το χρωμοσωμικό DNA ύστερα από την πέψη, διαχωρίστηκε σε πηχτή αγαρόζης, μεταφέρθηκε σε νάιλον φίλτρο και σχημάτισε υβρίδιο με τους ανιχνευτές *crtE* (A) και *ast* (B) που είχαν σημανθεί με DIG. 1, pPRcDNA1*crtE* * *EcoRI* * *SstI*, 2, CBS6938 * *EcoRI* * *SstI*, 3, CBS6938[pPRcDNA1] * *EcoRI* * *SstI*, 4, CBS6938[pPRcDNA1*crtE*]#1 * *EcoRI* * *SstI*, 5, CBS6938[pPRcDNA1*crtE*]#2 * *EcoRI* * *SstI*, 6, CBS6938[pPRcDNA1*crtE*]#3 * *EcoRI* * *SstI*, 7, λ DNA * *BstEII* (marker), 8, λ DNA * *BstEII* (marker), 9, CBS6938[pPR2TN*ast*]#3 * *NotI* * *HindIII*, 10, CBS6938[pPR2TN*ast*]#2 * *NotI* * *HindIII*, 11, CBS6938[pPR2T] * *NotI* * *HindIII*, 12, CBS6938 * *NotI* * *HindIII*, 13, pPR2TN*ast* * *NotI* * *HindIII*. Τα άνω παχύ οριζόντια βέλη, υποδεικνύουν το σημάδι από τα ενδογενή γονίδια και τα κάθετα βέλη το σημάδι από τα πρόσθετα αντίγραφα γονιδίων.

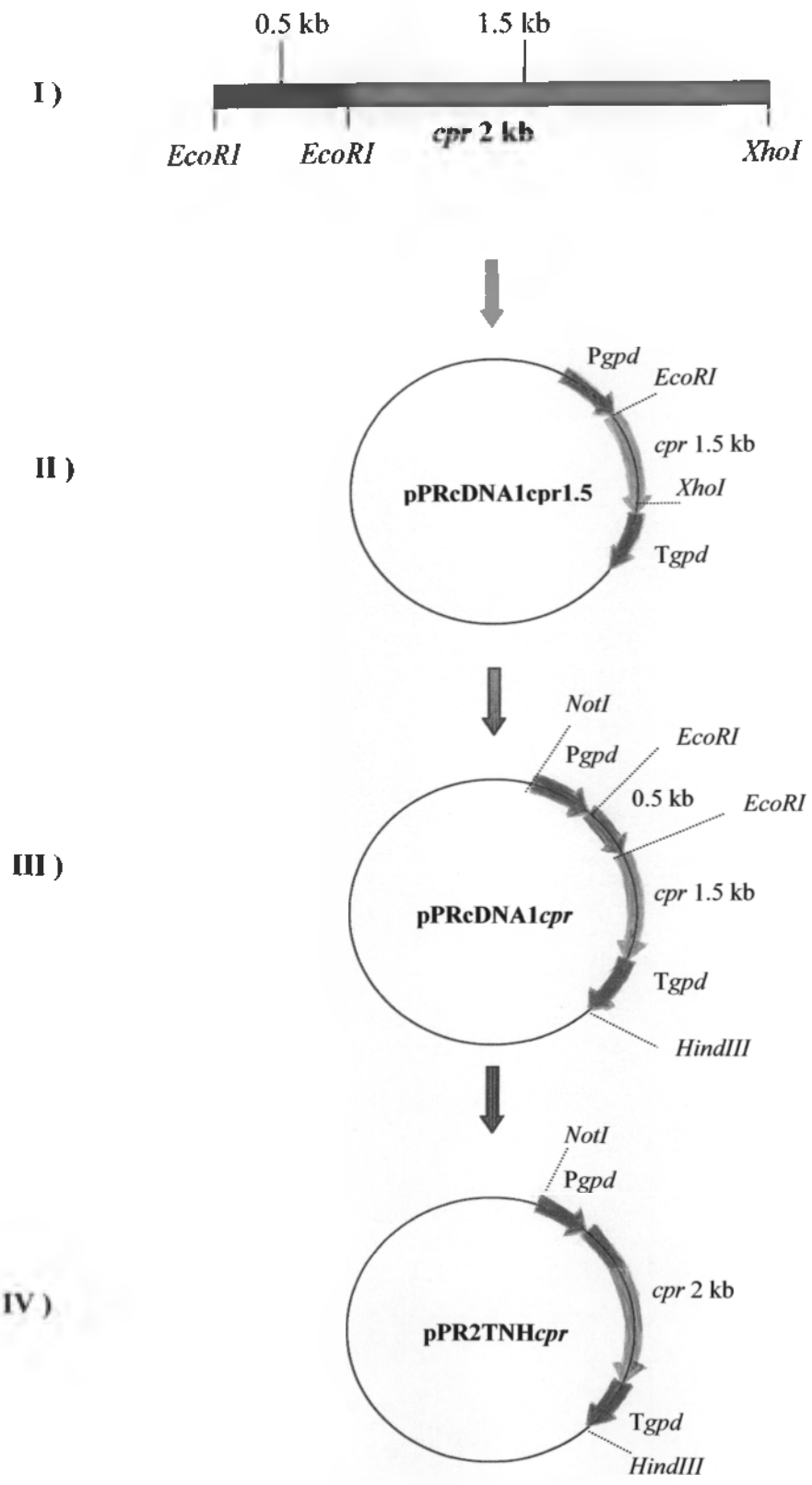
Κλωνοποίηση του φορέα έκφρασης *cpr*

Όπως αναφέρθηκε παραπάνω, ένας άλλος στόχος ήταν η δημιουργία ενός τροποποιημένου είδους της *X. dendrorhous*, με σκοπό να υπέρ-παράγει την P450-αναγωγάση εξίσου καλά με την συνθετάση της ασταξανθίνης.

Αρχικά, το 2 kb κωδική αλληλουχία από τη *Saccharomyces cerevisiae* διευρύνθηκε με αλυσιδωτή αντίδραση της πολυμεράσης (PCR), χρησιμοποιώντας χρωμοσομικό DNA από τη *S. cerevisiae* σαν φόρμα. Το αμπλικόνιο κλωνοποιήθηκε στο φορέα pGEM-T-Easy και η ταυτότητα του επιβεβαιώθηκε με μερικό προσδιορισμό της αλληλουχίας (Εικ. 4, I). Το γονίδιο κόπηκε με τα ένζυμα

περιορισμού *XhoI* και *EcoRI* και εισήχθηκε στο φορέα pPRcDNA1 στις θέσεις μεταξύ του *gpd* υποκινητή και του *gpd* ληκτικού. Ο φορέας pGEM-T-Easy υπέστη πέψη με τα προαναφερθέντα ένζυμα περιορισμού, επειδή οι *EcoRI* και *XhoI* είναι οι μόνες θέσεις κλωνοποίησης στο φορέα pPRcDNA1 (P*gpd* – *EcoRI* – *XhoI* – T*gpd*). Το *cpr* γονίδιο περιέχει τη θέση *EcoRI* και επομένως ήταν αναπόφευκτο να χωριστεί σε δύο τμήματα των 1.5 kb και 0.5 kb. Πρώτα κλωνοποιήθηκε στο φορέα pPRcDNA1 το 1.5 kb τμήμα του *cpr* γονιδίου (Εικ. 4, II) και έπειτα το 0.5 kb (Εικ. 4, III).

Τέλος, ο φορέας pPRcDNA1 υπέστη πέψη με τις ενδονουκλεάσεις *NotI* και *HindIII* και η κασέτα έκφρασης του *cpr* γονιδίου (P*gpd* – *cpr* – T*gpd*) κλωνοποιήθηκε στο φορέα pPR2TNH (Εικ. 4, IV). Ο φορέας pPR2TNH, περιέχει το δείκτη επιλογής υγρομυκίνη για το γονίδιο *cpr* και το μετασχηματισμένο είδος της *X. dendrorhous* CBS6938[pPR2TN*ast*]#3, περιέχει γενετισίνη σαν δείκτη επιλογής για το γονίδιο ast. Με τον τρόπο αυτό, στους καινούριους μετασχηματισμούς θα επιβιώσουν μόνο τα κύτταρα που περιέχουν και τα δύο πλασμίδια (επιλογή).



Εικόνα 4: Σχήμα κλωνοποίησης του γονιδίου *cpr*. I) Το διευρυμένο 2 kb *cpr* γονίδιο κλωνοποιήθηκε στο φορέα pGEM – T – Easy. II) Το 1.5 kb *cpr* (*EcoRI* - *XhoI*) τμήμα κλωνοποιήθηκε στο φορέα pPRcDNA1. III) Το 0.5 kb *cpr* (*EcoRI* - *EcoRI*) τμήμα

κλωνοποιήθηκε στο φορέα pPRcDNA1*cpr*1.5. Σε αυτό το στάδιο, τα δύο τμήματα του γονιδίου *cpr* ενώθηκαν . IV) Η κασέτα έκφρασης του *cpr* (P*gpd* – *cpr* – T*gpd*) κλωνοποιήθηκε στο φορέα pPR2TNH.

Επίλογος

Η *Xanthophyllomyces dendrorhous* είναι ζύμη κόκκινου-ροζ χρώματος και συνθέτει ασταξανθίνη σαν κύρια καροτενοειδή (Phaff *et al.*, 1972; Andrewes *et al.*, 1976). Η ασταξανθίνη χρησιμοποιείται στα τρόφιμα και στη βιομηχανία τροφίμων ως χρωστική και ως αντιοξιδωτικό. Ωστόσο, οι καταναλωτές απαιτούν από τις βιομηχανίες τροφίμων να χρησιμοποιούν φυσικές πηγές ασταξανθίνης. Η *X. dendrorhous* είναι από τις κύριες φυσικές πηγές ασταξανθίνης, αλλά το περιεχόμενο ασταξανθίνης είναι πολύ χαμηλό για εμπορική εφαρμογή. Για αυτό το λόγο, έχουν δημιουργηθεί διάφορες μεταλλάξεις, με στόχο την αυξημένη παραγωγή ασταξανθίνης στις ζύμες αυτές. Στο εργαστήριο μας υπάρχουν είδη της *X. dendrorhous* που υπέρ-εκφράζουν τα καροτενογενή (crt) γονίδια *crtE*, *crtYB*, *crtI* και *ast*.

Χρησιμοποιώντας την ανάλυση κατά Southern, μπορέσαμε να επιβεβαιώσουμε την παρουσία των πρόσθετων καροτενογενών γονιδίων και να καθορίσουμε τον αριθμό αντιγράφων τους. Με τη χρήση του ανιχνευτή rDNA, μπορέσαμε να υπολογίσουμε τον αριθμό αντιγράφων των πρόσθετων *crtE*, *crtYB*, *crtI* και *ast* γονιδίων, στα αντίστοιχα μετασχηματισμένα είδη. Γνωρίζοντας ότι η ένταση από το σημάδι των ενδογενή μορίων rDNA αντιστοιχεί σε 61 αντίγραφα, μπορέσαμε να υπολογίσουμε τον αριθμό αντιγράφων των πλασμιδίων (Wery *et al.*, 1997). Συγκρίνοντας την ένταση από το σημάδι αυτό με εκείνο των πλασμιδίων, δείξαμε ότι ο αριθμός αντιγράφων των πρόσθετων *crtE* γονιδίων είναι 15 αντίγραφα, των *crtYB* 1-5 αντίγραφα, των *crtI* 50 αντίγραφα και των *ast* 1-5 αντίγραφα στα αντίστοιχα μετασχηματισμένα είδη. Ο Verdoes και οι συνεργάτες του, έχουν ήδη δείξει την παρουσία των πρόσθετων αντιγράφων των *crtYB* και *crtI* γονιδίων, στα *crtYB* και

crtI είδη υπέρ-έκφρασης (Verdoes *et al.*, 2003). Οι *crtE* και *ast* ανιχνευτές, χρησιμοποιήθηκαν για να επιβεβαιώσουν την παρουσία των πρόσθετων αντιγράφων των *crtE* και *ast* γονιδίων, στα αντίστοιχα μετασχηματισμένα είδη.

Ένας άλλος στόχος της έρευνας αυτής, ήταν η δημιουργία ενός τροποποιημένου είδους της *X. dendrorhous*. Το είδος της *X. dendrorhous* CBS6938[pPR2TN*ast*]#3, υπέρ-παράγει συνθετάση της ασταξανθίνης, το οποίο είναι ένα ένζυμο μονοοξυγενάσης εξαρτώμενης από το κυτόχρωμα P450 (P450 για συντομία). Ωστόσο, τα ένζυμα P450 χρειάζονται τη συνεργασία άλλων ενζύμων, τα οποία είναι αναγωγάσες εξαρτώμενες από το κυτόχρωμα P450 (*cpr*- P450 αναγωγάσες).

Το 2 kb κωδική αλληλουχία από τη *Saccharomyces cerevisiae* διευρύνθηκε με PCR, χρησιμοποιώντας χρωμοσωμικό DNA από τη *S. cerevisiae* σαν φόρμα. Επιλέχθηκε αυτό το γονίδιο, επειδή η αλληλουχία του είναι γνωστή και δεν περιέχει ιντρόνια, το οποίο διευκολύνει την έκφραση στην *X. dendrorhous*. Η αλληλουχία του γονιδίου *cpr* της *X. dendrorhous* είναι άγνωστη. Το 2 kb αμπλικόνιο κλωνοποιήθηκε στο φορέα pGEM-T-Easy και η ταυτότητα του επιβεβαιώθηκε με μερικό προσδιορισμό της αλληλουχίας. Το γονίδιο *cpr* κλωνοποιήθηκε στο φορέα pPRcDNA1 με σκοπό να υβριδίζουν με τις αλληλουχίες του υποκινητή και της ληκτικής ακολουθίας του γονιδίου που κωδικοποιεί για την αφυδρογονάση της 3 φωσφορικής γλυκεραλδεϋδης (*gpd*), σχηματίζοντας την κασέτα έκφρασης του *cpr*. Τέλος, η κασέτα υπέρ-έκφρασης (P*gpd* – *cpr* – T*gpd*) κλωνοποιήθηκε στο φορέα μετασχηματισμού της *X. dendrorhous* pPR2TNH, ο οποίος παρέχει αντίσταση στην υγρομυκίνη.

Όπως αναφέρθηκε και προηγουμένως, ο φορέας υπέρ-έκφρασης pPR2TNH*cpr* έχει κατασκευασθεί και θα εισαχθεί στο είδος CBS6938[pPR2TN*ast*]#3, που υπέρ-παράγει συνθετάση της ασταξανθίνης. Αυτό αναμένεται να αυξήσει το συνολικό περιεχόμενο ασταξανθίνης του είδους.

ΜΕΡΟΣ 2ο

ΠΤΥΧΙΑΚΗ ΣΤΑ ΑΓΓΛΙΚΑ

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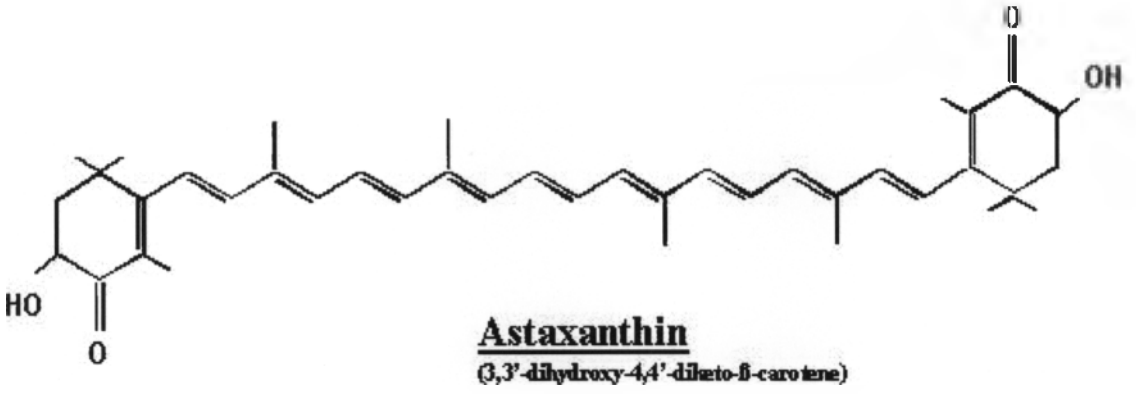
Section of Fungal Genomics

"Metabolic engineering of the carotenoid biosynthetic pathway in *Xanthophyllomyces dendrorhous*"



Astaxanthin
(3,3'-dihydroxy-4,4'-diketo-ß-carotene)

Chrysovalantis – Ioannis Papageorgiou

Supervisors: Dr. Hans Visser
Prof. Dr. Ir. Ab van Ooyen
Dr. Kalliope Papadopoulou

Wageningen 15 / 05 / 2004

Wageningen University & Research Centre, Laboratory of Microbiology, Section of Fungal Genomics

"Metabolic engineering of the carotenoid biosynthetic pathway in *Xanthophyllomyces dendrorhous*"

Report of a 6 months practical training for the period 13/10/2003 - 13/04/2004 in the section of Fungal Genomics, Laboratory of Microbiology, Wageningen University.

Student: Chrysovalantis – Ioannis Papageorgiou

Supervisors: Dr. Hans Visser
Prof. Dr. Ir. Ab van Ooyen
Dr. Kalliope Papadopoulou

Foreword

In this report, the results of my 6 months experiments in project "Metabolic engineering of the carotenoid biosynthetic pathway in *Xanthophyllomyces dendrorhous*" are present. I can look back on a nice and educational period. I have learnt a lot of things which will be very useful afterwards in my profession. I have also a lot of nice moments with the people from the laboratory which I will remember for all my life.

At this point, I would like to thank Prof. Dr. Ir. Johan van den Berg who gave me the opportunity to carry out my practical training in the section of Fungal Genomics, Laboratory of Microbiology in Wageningen University. It was really my honour to work in Fungal Genomics Group.

I would also like to thank my daily supervisor Dr. Hans Visser for letting me to work on this project and for correcting this report. For me, he is not only my supervisor but he is teacher and friend. I have learnt a lot of things relevant with moleculars from him and he was always with me to the success as well as the failure in my experiments.

Furthermore, I would like to thank my supervisor Prof. Dr. Ir. Ab van Ooyen for his valuable advices and for he was always encouraged me to continue my experiments even if I had disappointed results.

Finally, I would like to thank all the people from Fungal Genomics Group for let me feel from the first time like at home and they were always willing to help me when I was asked their help.

I will remember the section of Fungal Genomics and the 1024/1026 laboratory for ever!

Thank you for everything!

Giannis

Contents

Abstract.....6

1 INTRODUCTION.....7
1.1 Carotenoids.....7
1.2 Astaxanthin.....9
1.3 *Xanthophyllomyces dendrorhous*.....11
1.4 Aim of project.....13

2 MATERIALS AND METHODS.....14
2.1 Materials.....14
2.1.1 Plasmids and strains.....14
2.1.2 Mediums and solutions.....14
2.2 Methods.....15
2.2.1 Isolation of chromosomal DNA from *X. dendrorhous*.....15
2.2.2 Southern blot analysis.....16
2.2.2.1 Chromosomal DNA digestion and electrophoresis.....16
2.2.2.1.1 Copy number analysis.....17
2.2.2.1.2 Analysis of extra *crtE* and *ast* gene copies.....17
2.2.2.2 Capillary blotting of DNA.....19
2.2.3 Probe DIG labelling using polymerase chain reaction (PCR).....20
2.2.3.1 Introduction.....20
2.2.3.2 DIG-labelled rDNA probe for the determination of copy number.....21
2.2.3.3 DIG-labelled *crtE* probe for the presence of extra gene copies.....21
2.2.4 Prehybridisation and hybridisation.....22
2.2.5 Cloning of the *cpr* gene.....26
2.2.5.1 Polymerase chain reaction (PCR).....26
2.2.5.2 Isolation of the DNA fragment from the gel.....26
2.2.5.3 Preparation of *E. coli* cells for transformation.....27
2.2.5.4 Ligation.....28

2.2.5.5 Electroporation.....30
2.2.5.6 Isolation of plasmid from *E. coli*.....31

3 RESULTS AND DISCUSSION.....33
3.1 Isolation of chromosomal DNA.....33
3.2 Southern blot analysis.....34
3.2.1 Probe labelling by PCR.....34
3.2.1.1 DIG-labelled rDNA probe.....34
3.2.1.2 DIG-labelled *crtE* probe.....34
3.2.2 Chromosomal DNA digestion and hybridization.....35
3.2.2.1 Copy number analysis.....35
3.2.2.2 Analysis of extra *crtE* and *ast* gene copies.....37
3.3 Cloning of *cpr* expression vector.....39

4 SUMMARY AND CONCLUSION.....47

5 REFERENCES.....49

APPENDIX I.....53

Abstract

Xanthophyllomyces dendrorhous is a red-pink yeast that produces the carotenoid astaxanthin. Astaxanthin is applied in the food and feed industry both as a colorant (pink, e.g. salmon and trout flesh) and as an antioxidant. *X. dendrorhous* is genetically being engineered e.g. over-expression of carotenogenic (crt) genes in order to produce more or different carotenoids. The crt genes are cloned in vectors that integrate in multiple copies in the host's rDNA locus after transformation and they are transcribed from a strong constitutive promoter, thus enabling over-expression. Genes, that are over-expressed this way are, e.g. *crtE* (geranylgeranyl-pyrophosphate synthase), *crtYB* (phytoene synthase / lycopene cyclase), *crtI* (phytoene desaturase) and *ast* (astaxanthin synthetase). The presence and copy number of the extra carotenogenic gene copies in the transformants was determined by Southern analysis. Verdoes *et al.* (2003, *Appl. Environ. Microbiol.* 69:3728-3738) have already shown the presence of extra *crtYB* and *crtI* gene copies in the *crtYB* and *crtI* over-expression strains. By using the *crtE* and *ast* probes we could confirm the presence of extra *crtE* and *ast* gene copies respectively in the corresponding transformants. An rDNA probe was used to determine the copy number of the extra *crtE* (15 copies), *crtYB* (1-5 copies), *crtI* (50 copies) and *ast* (1-5 copies) genes in the respective transformants.

Another item was the construction of an engineered *X. dendrorhous* strain. The *X. dendrorhous* strain that overproduces astaxanthin synthetase, which is a P450 enzyme that converts β-carotene into astaxanthin, produces more astaxanthin than the control strain does. However, P450 enzymes need the cooperation of other enzymes, which are the cytochrome P450-reductases (*cpr*). Therefore, a second vector will be introduced in this strain, which will cause the over-expression of a P450-reductase as well. Ideally, this would result in a further increase in the astaxanthin content. The 2 kb *cpr* coding sequense from *Saccharomyces cerevisiae* was amplified by PCR using chromosomal *S. cerevisiae* DNA as template. The amplicon was cloned in pGEM – T – Easy vector and its identity was confirmed by partial sequencing. Subsequently, the *cpr* gene was flanked by the *X. dendrorhous* glyceraldehyde-3-phosphate dehydrogenase promoter and terminator sequences. This expression cassette was cloned into a *X. dendrorhous* transformation vector, which confers resistance to hygromycin.

1 INTRODUCTION

1.1 Carotenoids

In nature there is a wide range of colours and almost all living organisms, which surround us, have one or more colours. The question is how all these living organisms obtained these colours? With a few exceptions, natural pigments are the answer to this question. The role of natural pigments is not only the coloration, but also a variety of important biological functions. Among the most common and most important natural pigments are the carotenoids.

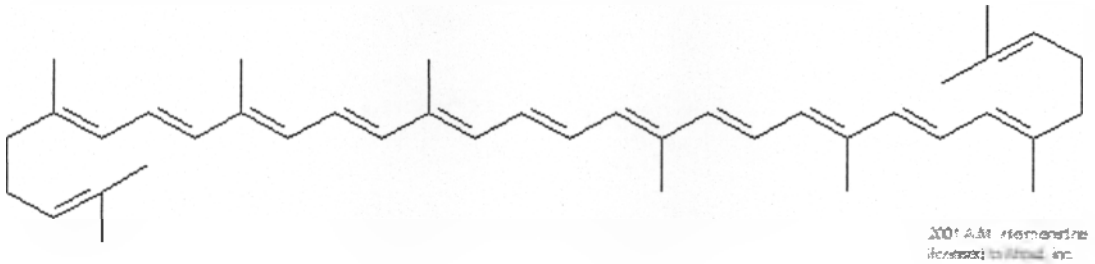
Carotenoids are a class of natural lipid-soluble pigments and are present in plants, algae, bacteria and fungi. They play a critical role in the photosynthetic process and protect organisms against damage by light and oxygen. Since animals are unable to synthesize carotenoids de novo, they incorporate carotenoids from their diet. Carotenoids provide animals mainly with bright coloration; serve both as antioxidants and source for provitamin A activity (Johnson and An, 1991; Lorenz and Cysewski, 2000; Fraser and Bramley, 2004).

More than 600 different carotenoids are known and therefore they are considered to be the most widespread group of pigments in nature. They are responsible for many of the red, orange and yellow hues of fruits and flowers, as well as the colours of some birds, insects, fish and crustaceans (Fraser and Bramley, 2004). Some familiar examples of carotenoid coloration are the red of tomatoes and watermelon and the pink of salmon and flamingoes (Bramley, 2000; Guerin *et al.*, 2003).

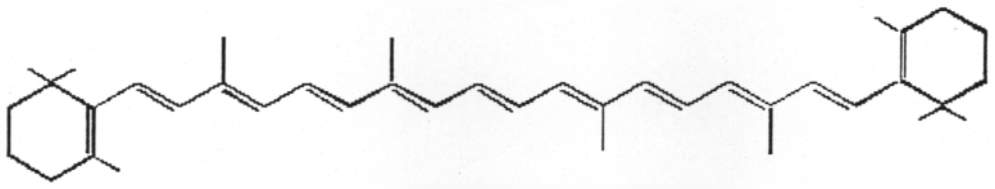
Carotenoids are isoprenoid compounds and are hydrophobic molecules. The majority of carotenoids are derived from a 40-carbon polyene chain. This chain may be modified by

cyclic end-groups and may be complemented with oxygen-containing functional groups. The hydrocarbon carotenoids are known as carotenes, and the oxygenated derivatives of these hydrocarbons are known as xanthophylls. A typical example of a carotene is β-carotene, the principal carotenoid in carrots. Astaxanthin, the major red-pink pigment of salmon flesh is a common xanthophyll. The trivial names which carotenoids have, relate usually with the original source from which they were isolated (Britton, 1995).

Several studies have shown that carotenoids play an important role in human health. Carotenoids due to be strongly antioxidants, prevent human of chronic diseases such as cancer and heart disease (Young and Lowe, 2000; Cooper, 2004). Human get carotenoids mainly through his diet from vegetables and fruits (Fraser and Bramley, 2004). Table 1 shows the typical amounts of carotenoids in crop plants. Some of the most potent antioxidants carotenoids are lycopene, β-carotene and astaxanthin. Lycopene in comparison with β-carotene (Fig. 1) does not have provitamin A activity. Beta-carotene derives from lycopene after cyclisation (Bramley, 2000; Rao and Agarwal, 2000; Arab ant Steck 2000). Astaxanthin is the product of four oxidations of β-carotene.



Lycopene
Molecular Weight: 536.89
Molecular Formula: $C_{40}H_{56}$



Beta-carotene

Figure 1: Chemical structure of lycopene and β-carotene.

Species	Carotenoid (μg/g fresh weight)					
	Total	Zea	Lutein	α–Carotene	β-Carotene	Lycopene
Brussel sprout	1163	-	610	-	553	-
Green bean	940	-	494	70	376	-
Broad bean	767	-	506	-	261	-
Broccoli	2533	-	1614	-	919	-
Green cabbage	139	-	80	-	59	-
Lettuce	201	-	110	-	91	-
Parsley	10,335	-	5812	-	4523	-
Pea	2091	-	1633	-	458	-
Spinach	9890	-	5869	-	4021	-
Watercress	16,632	-	10,713	-	5919	-
Apricot	2196	31	101	37	1766	-
Banana	126	4	33	50	39	-
Carrot (May)	11,427	-	170	2660	8597	-
Carrot (Sept)	14,693	-	283	3610	10,800	-
Orange	211	50	64	Nd	14	-
Pepper	2784	1608	503	167	416	-
Peach	309	42	78	Tr	103	-
Sweet corn	1978	437	522	60	59	-
Tomato	3454	-	78	-	439	2937

Table 1: Carotenoid contents of raw leafy green vegetables, fruits, roots and seeds. Zea: zeaxanthin; Nd: not detected; Tr: traces (Scott and Hart, 1994).

1.2 Astaxanthin

Astaxanthin is a red-pink pigment found in a wide variety of living organisms. This carotenoid is present in salmon, trout, shrimp, lobster and fish eggs. The colours in various birds like flamingoes, scarlet, ibis, roseate spoonbill and guails, derives from astaxanthin (Kanemitsu and Aoe, 1958; Fox, 1962; Weedon, 1971; Schiedt *et al.*, 1981; Schiedt *et al.*, 1985; Johnson and An, 1991; Johnson and Schroeder, 1995; Guerin *et al.*, 2003). In the marine environment phytoplankton and microalgae form the primary sources of astaxanthin for these animals (Johnson and Schroeder, 1995; Lorenz and Cysewski, 2000).

The molecular formula of astaxanthin (3, 3′ –dihydroxy-β,β-carotene-4,4′-dione) (Fig. 2) is $C_{40}H_{52}O_4$ and the molecular weight is 596.86. Isolated crystalline astaxanthin has a

powder form and a fine dark violet-brown colour. In this form, the melting point is approximately 224 °C. In the benzenoid rings on either end of the molecule of astaxanthin, there are two asymetric carbons located at positions 3 and 3′. Accordingly the way that hydroxyl groups are attached to the carbon atoms at the centres of asymmetry, different enantiomers arise. Thus, the 'R configuration' results when the hydroxyl group is attached to protrude above the plane of molecule and 'S configuration' when the hydroxyl group is attached to protrude below the plane of the molecule. In nature, astaxanthin has three configurational isomers: (3S, 3′ S), (3S, 3′ R) and (3R, 3′ R) (Johnson and An, 1991; Lorenz and Cysewski, 2000).

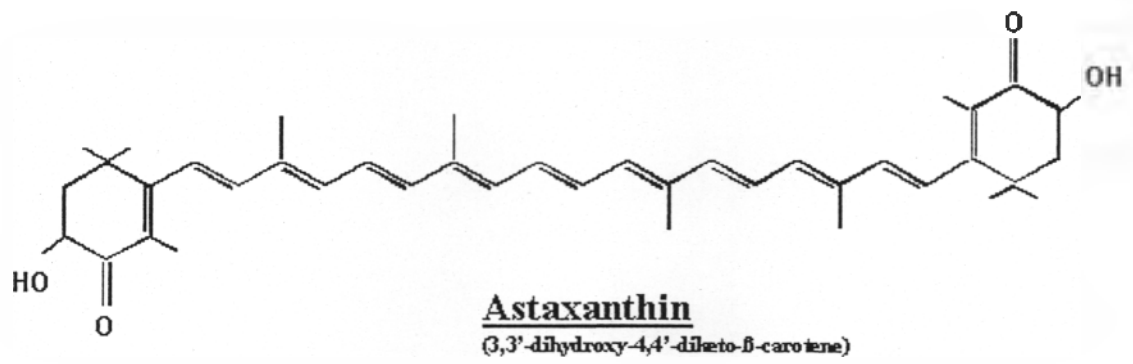


Figure 2: Chemical structure of astaxanthin.

Several studies have shown that astaxanthin has a strong antioxidant activity and it protects the skin from UV-light photooxidation, several kinds of cancer, inflammation, ulcer's *Helicobacter pylorii* infection and ameliorate age-related macular degeneration (Lorenz and Cysewski, 2000; Guerin *et al.*, 2003).

Astaxanthin cannot be synthesized by animals and must be acquired from the diet. Astaxanthin is applied in food and feed industry both as a colorant (e.g. salmon and trout flesh) and as an antioxidant (Johnson *et al.*, 1977; Johnson *et al.*, 1980; Choubert and Heinrich, 1993). However, consumers demand from industries to use astaxanthin derived from natural sources and not synthetic astaxanthin to be applied in natural products. Such natural sources of astaxanthin are the green microalga *Haematococcus pluvialis*, the yeast *Xanthophyllomyces dendrorhous,* krill oil and meal, crawfish and some bacteria like

Brevibacterium sp. and *Mycobacterium laticola* (Johnson and Lewis, 1979; Johnson and An, 1991; Kobayashi *et al.*, 1991; Kobayashi *et al.*, 1993; Lorenz and Cysewski, 2000).

Haematococcus pluvialis produces a high concentration of astaxanthin (0.5 to 4 %) and is the richest natural source of astaxanthin (Steinbrenner and Linden, 2001). *Xanthophyllomyces dendrorhous* has a low concentration level of astaxanthin (0.02 to 0.03 %) but it is preferred in industrial application because it has a rapid heterotrophic metabolism and produces high cell densities in fermentors (Johnson and An, 1991).

1.3 *Xanthophyllomyces dendrorhous*

The yeast *Xanthophyllomyces dendrorhous* has a red-pink colour and it was isolated for the first time in the 1970s by Herman Phaff and colleagues from exudates of deciduous trees in mountainous regions of Japan and Alaska (Phaff *et al.*, 1972). It is a teleomorphic form of the heterobasidiomycetous yeast *Phaffia rhodozyma* (Miller *et al.*, 1976; Golubev, 1995). This heterobasidiomycete is "unique" with regard to its ability of both producing carotenoids and fermenting sugars (Miller *et al.*, 1976).

Astaxanthin is the major pigment in *X. dendrorhous* and comprises 83-87 % of the total carotenoid content (Andrewes *et al.*, 1976). The predominant isomer in *X. dendrorhous* has the (3R, 3'R) configuration (Andrewes and Starr, 1976). *Haematococcus pluvialis* produces astaxanthin the (3S, 3'S) isomer (Guerin *et al.*, 2003). The role of carotenoids in *X. dendrorhous* is to protect it from oxidative damage (Johnson and Schroeder, 1995).

As referred above, there is great interest by the feed industry for *X. dendrorhous.* However, the content of astaxanthin in this yeast is too low for commercial application. Thus, several mutants have been created in order to produce more astaxanthin or different carotenoids. Metabolic engineering in this yeast became easier when Andrewes *et al.* proposed the carotenoid biosynthetic pathway (Fig. 3) (Andrewes *et al.*, 1976). Isopentenyl pyrophosphate (IPP) is the general precursor of all isoprenoids. Farnesyl-pyrophosphate (FPP) contains 3 molecules of IPP. Geranylgeranyl pyrophosphate synthase (*crtE*) adds 1 IPP molecule to FPP and produces geranylgeranyl pyrophosphate (GGPP). Phytoene synthase (*crtYB*) links two GGPP molecules and produces phytoene. Phytoene desaturase (*crtI*) introduces 4 double bonds to the phytoene molecule and produces lycopene. Lycopene

cyclase (*crtYB*) closes the terminal rings of lycopene with 2 cyclizations and produces β-carotene. Astaxanthin synthetase (*ast*) adds oxygen to β-carotene molecule and produces astaxanthin. Four oxidations take place in this step. Astaxanthin is the terminal product in *X. dendrorhous*.

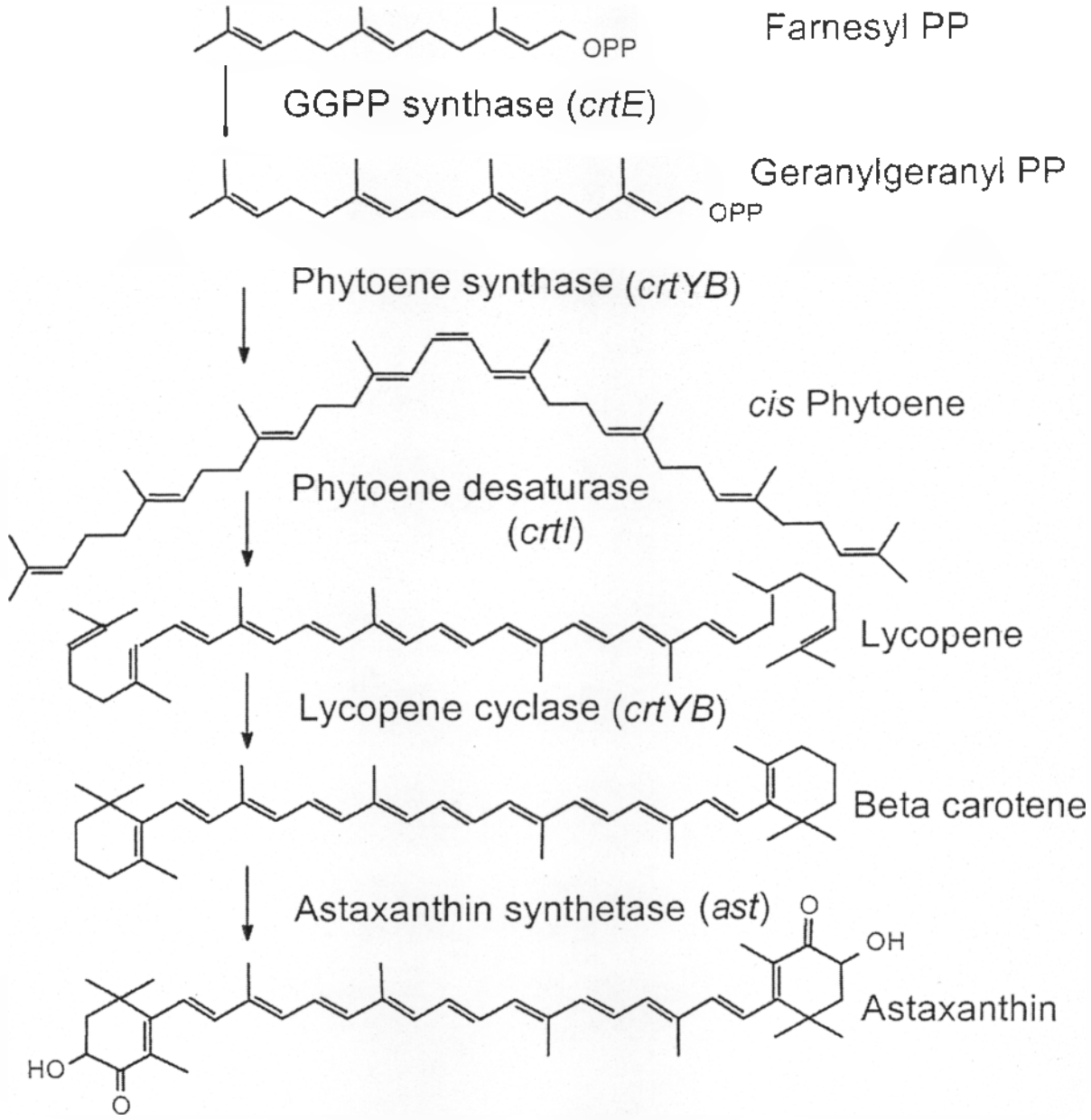


Figure 3: Astaxanthin biosynthetic pathway. Isopentenyl pyrophosphate (IPP) is the general precursor of all isoprenoids. Farnesyl-pyrophosphate (FPP) contains 3 molecules of IPP. Geranylgeranyl pyrophosphate synthase (*crtE*) adds 1 IPP molecule to FPP and produces geranylgeranyl pyrophosphate (GGPP). Phytoene synthase (*crtYB*) links two GGPP molecules and produces

phytoene. Phytoene desaturase (*crtI*) introduces 4 double bonds to the phytoene molecule and produces lycopene. Lycopene cyclase (*crtYB*) closes the terminal rings of lycopene with 2 cyclizations and produces β-carotene. Astaxanthin synthetase (*ast*) adds oxygen to β-carotene molecule and produces astaxanthin.

Several *X. dendrorhous* carotenoid biosynthesis genes have been isolated (Verdoes *et al.*, 1996; Verdoes *et al.*, 1999; Verdoes *et al.*, 1999; Hoshino *et al.*, 2000). Furthermore, a *X. dendrorhous* transformation system has been developed (Wery *et al*, 1998). The carotenogenic (crt) genes are cloned in vectors that integrate in multiple copies in the host's rDNA locus after transformation and they are transcribed from a strong constitutive promoter, thus enabling over-expression. Genes, that are over-expressed this way are, e.g. *crtE* (geranylgeranyl-pyrophosphate synthase), *crtYB* (phytoene synthase / lycopene cyclase), *crtI* (phytoene desaturase) and *ast* (astaxanthin synthetase).

1.4 Aim of project

X. dendrorhous is genetically being engineered e.g. over-expression of carotenogenic (crt) genes in order to produce more or different carotenoids. The presence and copy number of extra carotenogenic gene copies of *crtE, crtYB, crtI* or *ast* in the corresponding transformants was determined by Southern analysis.

Another objective was the construction of an engineered *X. dendrorhous* strain. The *X. dendrorhous* strain that overproduces astaxanthin synthetase, which is a P450 enzyme that converts β-carotene into astaxanthin, produces more astaxanthin than the control strain does (H. Visser unpublished data). However, P450 enzymes need the cooperation of other enzymes, which are the cytochrome P450-reductases (cpr). The cytochrome P450-reductases provide an electron (e^-) to P450 enzymes. This electron is necessary to activate the oxygen that will be added to the β-carotene molecule in order to produce astaxanthin (van den Brink *et al.*, 1998). Therefore, a second vector will be introduced in this strain, which will probably cause the over-expression of a P450-reductase as well. Ideally, this would result in a further increase in the astaxanthin content.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasmids and strains

Different *Escherichia coli* and *X. dendrorhous* vectors were used in cloning experiments (pGEM – T – Easy, pPRcDNA1 and pPR2TNH).

The *X. dendrorhous* wild strain (CBS 6938) and transformants were cultivated in YEPD medium at 21 °C and were used for chromosomal DNA isolations. The transformants are listed in Table 2.

Entry	Strain	What does it do?
1	CBS6938 [pPRcDNA1*crtE*] #1, #2, #3	Over expression of *crtE* gene
2	CBS6938 [pPRcDNA1]	Control to entry 1
3	CBS6938 [pPR2TN*ast*] #2, #3	Over expression of *ast* gene
4	CBS6938 [pPR*crtI*]	Over expression of *crtI* gene
5	CBS6938 [pPR*crtYB*]	Over expression of *crtYB* gene
6	CBS6938 [pPR2TN]	Control to entries 3, 4 and 5

Table 2: In this table the transformants, which have been used in this project, are listed.

2.1.2 Mediums and solutions

The composition of the mediums and solutions, which have been used in this project, are presented in Appendix I.

2.2 Methods

Standard molecular biological methods were used (Sambrook *et al.*, 1986).

2.2.1 Isolation of chromosomal DNA from *X. dendrorhous*

For every strain (wild or transformant), a fresh colony was take from an agar plate and added in a 250 ml erlenmeyer shake flask, which contained 30 ml of YEPD medium and G418 Geneticin as selection marker (in case of transformants). This culture was incubated for 48 hours at 21 °C with 225 rpm. After 48 hours, cells were harvested by centrifugation at 10.000 rpm for 5 min at 4 °C and washed once in cold sterile water. The final cell pellet was resuspended in a minimal volume of cold sterile water. This cell suspension was transferred to a mortar containing liquid nitrogen and 1 gr Alumina (Sigma). Cells were broken by grinding the frozen cell suspension. The broken cells were added to a centrifuge tube containing 5 ml DNA-Extraction buffer. Chromosomal DNA was isolated from this mixture by repeated phenol / chloroform extractions and repeated centrifugations at 15.300 rpm, for 10 min at 4 °C (three times). After the last centrifugation, the volume of the water-phase was measured and 0.1 * V (Volume) of 3M NaAc pH 5.4 was added. This was mixed by vortexing and subsequently 2 * V of EtOH 100 % were added. This was mixed again by vortexing and stored at –20 °C during the night (ethanol precipitation step). The cell pellet was harvested by centrifugation at 15.300 rpm, for 15 min at 4 °C and it was washed in cold 70 % EtOH. The pellet was air dried for 15 minutes and 500 µl sterile water was added and mixed gently. To this solution 10 µl RNAse (1 mg/ml) were added and mixed gently. This was incubated at room temperature for 15 min to degrade co-purified RNA. After 15 min, 250 µl phenol was added. This solution was mixed by vortexing for 20 sec. Then 250 µl chloroform was added and the solution was vortexed again for 20 sec. The liquid-phases were separated by centrifugation for 10 min and 14.000 rpm at room temperature and the upper phase (water

phase) was transferred to a sterile eppendorf tube. The volume was measured and an ethanol precipitation step was performed. Chromosomal DNA was harvested by centrifugation in the cold room (4 °C) for 15 min and 14.000 rpm. After centrifugation the pellet was washed in cold 70 % EtOH and then dried in the air. When the pellet was dry, it was dissolved in 200 µl TE pH 7.5 and stored at – 20 °C. The concentration of DNA was calculated by the formula:

$$[DNA] = A_{260} * 50 * 100$$

Where: A_{260}, the absorbance at 260 nm in the spectrophotometer

50, from $A_{260} = 1 = 50$ µg/ml

100, the dilution factor

The spectrophotometer was set at 260 nm and the UV light was switched on. For each sample 5 µl of chromosomal DNA were diluted in 495 µl sterile water and this solution was added in a quartz cuvette. The spectrophotometer gave the extinction absorbance for each sample.

2.2.2 Southern blot analysis

2.2.2.1 Chromosomal DNA digestion and electrophoresis

For digestion, chromosomal DNA of *X. dendrorhous* was cut with an appropriate restriction enzyme or enzymes in the corresponding reaction buffer in a sterile 1.5 ml eppendorf tube. Restriction enzymes were only removed from the –20 °C freezer for as short as possible periods and always kept on ice. These reactions were carried out at 37 °C for 2 - 4 hours. The different fragments were separated to their size accordingly in agarose gel electrophoresis.

For the preparation of an agarose gel, 1 gr of ultra pure agarose was used and this was dissolved in 100 ml of 1 * TAE by gentle heating until all the agarose was dissolved (in micro-wave oven). The solution was cooled to approximately 60 °C and then 50 µl ethidium bromide (EtBr) were added and gently mixed. The gel was poured in a plastic tray and let to solidify for 1 hour. After 1-hour, the combs and gel dams (tape) were removed from both ends

of the plastic tray. The tray was inserted into the electrophoresis cell. The electrophoresis cell was filled with 1 * TAE buffer. The buffer level was checked to ensure that the gel was covered with about 2 – 3 mm of buffer and the lid put on the electrophoresis cell.

When digestion was completed, 2 μl of loading buffer were added in each sample. Then 20 – 40 μl of each digestion were loaded per well in the agarose gel. The current was regulated at 50 mA till the DNA had entered the gel and then the current was increased to 100 mA. When electrophoresis was completed, the gel was placed in a UV transilluminator to visualize the DNA. The EtBr, which was added in the agarose gel during the preparation of the gel, is the cause that DNA can be visualised in UV light. The gel was photographed before blotting with a ruler alongside the gel.

2.2.2.1.1 Copy number analysis

Five micrograms of chromosomal DNA of *X. denrorhous* strains were digested (per strain) with the restriction enzyme *SfiI*, at 50 °C for 4 hours. *SfiI* releases the plasmids from the chromosomal DNA. In each 1.5 ml eppendorf tube the amounts that are presented below were added.

	x μl DNA (5 μg)
	1 μl *SfiI*
	3 μl NE Buffer 2
	3 μl 10 x BSA
	23 – x μl sterile H_2O
Total volume:	30 μl

The densitometer (BioRad Quantity One software) was used for the estimation of copy number. This software is capable to estimate the signal intensity. Thus, if the signal intensity from the endogenous rDNA units is known that corresponds to x copies, by comparison of this signal intensity to that of the plasmids, the copy number could estimate.

2.2.2.1.2 Analysis of extra *crtE* and *ast* gene copies

The chromosomal DNA of *crtE* and *ast* transformants were digested with the endonucleases *EcoRI* and *SstI* and *NotI* and *HindIII* respectively at 37 °C for 2 hours. The wild strain as well as the plasmids, which were used for the transformants, was digested with the same endonucleases as positive controls. Each sample of strains calculated to have 15 μg DNA and added in 1.5 ml eppendorf tube the amounts that are presented below.

In case of *crtE* strains

	x μl DNA (15 μg)
	4 μl Reaction buffer N° 2
	2 μl *EcoRI*
	2 μl *SstI*
	32 – x μl sterile H_2O
Total volume:	40 μl

	3 μl pPRcDNA1*crtE*
	2 μl Reaction buffer N° 2
	0.5 μl *EcoRI*
	0.5 μl *SstI*
	14 μl sterile H_2O
Total volume:	20 μl

In case of *ast* transformants:

	x μl DNA (15 μg)
	4 μl Reaction buffer N° 2
	2 μl *NotI*
	2 μl *HindIII*
	32 – x μl sterile H_2O
Total volume:	40 μl

	3 µl pPR2TN*ast*
	2 µl Reaction buffer N° 2
	0.5 µl *NotI*
	0.5 µl *HindIII*
	14 µl sterile H_2O
Total volume:	20 µl

2.2.2.2 Capillary blotting of DNA

A picture of the gel, with a ruler alongside the gel, was made. The DNA was denatured in approximately 300 ml (to ensure that solution covers the gel) denaturing solution for 45 minutes with constant gentle agitation. After 45 minutes, the gel was rinsed briefly in demi water and neutralised by soaking the gel in 300 ml neutralising solution for 30 minutes with constant gentle agitation. The solution was refreshed and the gel was incubated for another 15 minutes.

At the same time, a piece of Whatman 3MM paper (12.5 cm x 30 cm) was cut and wrapped around a gel tray. The wrapped support was placed in a large dish and the dish was filled with approximately 600 ml of 10 * SSC (till the liquid reached the top of the support). When the paper was completely wet, all air bubbles were removed using a glass rod. Two pieces of Whatman paper as well as a piece of nylon filter with the same size of the gel were cut. The nylon filter was not touched with bare hands because greasy spots in the filter will inhibit the DNA binding. The nylon filter was placed in a dish with demi water and Whatman paper pieces in a dish with 2 * SSC. It was ensured that liquids covered the filter and the papers. Finally, a stack of paper towels (10 – 12 cm high) with approximately the same size of the gel was cut.

The gel was removed from neutralising solution and inverted, using a glass plate. The inverted gel was placed in the centre of the support and all air bubbles between the paper and the gel were removed. The nylon filter was placed on top of the gel so that the cut corners were aligned, using forceps and the air bubbles between the filter and the gel were smoothed out. The pieces of Whatman paper were placed on the filter, the air bubbles were removed and the stack of paper towels was placed on the 3 MM paper. It was ensured that the paper towels

did not droop over the edge of the gel touching the support. Finally, a glass plate as well as a weight with approximately 500 gr was placed on top of the stack for 8 – 24 hours (Fig. 4).

The next day, the paper towels and 3 MM Whatman papers were removed from the nylon filter. The filter was removed with forceps and placed on a paper towel with DNA side up. The filter was let dry for 30 minutes and then placed in the UV transilluminator with DNA side down and it was exposed for 3 minutes to UV light to cross link the DNA to the filter.

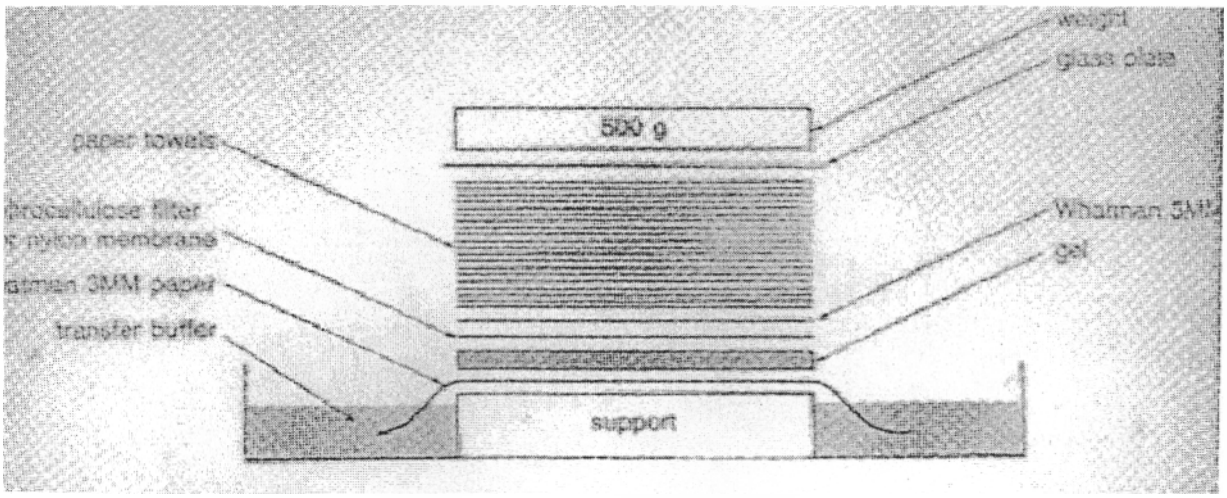


Figure 4: Schematic representation of transfer of DNA to nylon filter.

2.2.3 Probe DIG labelling using polymerase chain reaction (PCR)

2.2.3.1 Introduction

Polymerase chain reaction (PCR) is a laboratory technique, which is used to amplify a specific DNA sequence. PCR requires as starting material very small amounts of DNA that contains the sequence to be amplified. The double stranded DNA molecules separate completely at 94-95 °C for 3-5 minutes. These single strands become the templates for the primers and DNA polymerase. The primers are chosen to flank the region of DNA that is to be amplified so that the newly synthesized strands of DNA starting at each primer extend beyond the position of the primer on the opposite strand. The annealing temperature varies

and depends from the sequence to be amplified. This generates the primed templates for DNA polymerase. After this, the temperature increases to 72 °C for 1 min / 1000 bp for DNA synthesis to proceed. Seventy-two degrees is the optimal temperature for the heat stable *Taq* DNA polymerase. The reaction mixture is again heated to separate the original and newly synthesized strands, which are then available for further cycles of primer hybridization, DNA synthesis and strand separation (Watson *et al.*, 1996).

2.2.3.2 DIG-labelled rDNA probe for the determination of copy number

Polymerase chain reaction (PCR) was carried out in an automated thermal cycler. For the determination of copy numbers of the extra *crtE, crtYB, crtI* and *ast* genes in the respective transformants, an rDNA probe was used. The reaction conditions were as follows: 3 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 52 °C, 2 min at 72 °C, ending with 5 min at 72 °C. A 0.5 kb ribosomal DNA fragment was used as template. The primers Pr DNA1 and Pr DNA4 were used to amplify the DNA. Two PCR tubes were prepared with a total volume of 30 µl in which the amounts that are presented below were added.

Labelling PCR:	Control PCR:
0,5 µl rDNA	0,5 µl rDNA
1 µl Pr DNA1	1 µl Pr DNA1
1 µl Pr DNA 4	1 µl Pr DNA4
3 µl DIG dNTP's	3 µl dNTP's
3 µl 10 x PCR buffer	3 µl 10 x PCR buffer
0,5 µl Super Taq plus	0,5 µl Super Taq plus
21 µl sterile H_2O	21 µl sterile H_2O

The control PCR tube was prepared to check if the DIG was integrated into the rDNA probe.

2.2.3.3 DIG-labelled *crtE* probe for the presence of extra gene copies

For confirmation the presence of extra *crtE* gene copies in the corresponding transformants used the *crtE* probe. The reaction conditions were as follows: 3 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 45 °C, 2 min at 72 °C, ending with 5 min at 72 °C. A *crtE* encoding DNA fragment was used as template and the primers *crtE* R and *crtE* F were used for the amplification. Two PCR tubes were prepared with total volume 30 µl. The amounts that added in each of two tubes are presented below.

Probe lebelling PCR:	Control PCR:
0,5 µl *crtE* frament	0,5 µl *crtE* fragment
1 µl *crtE* R	1 µl *crtE* R
1 µl *crtE* F	1 µl *crtE* F
3 µl DIG dNTP's	3 µl dNTP's
3 µl 10 x PCR buffer	3 µl 10 x PCR buffer
0,5 µl Super Taq plus	0,5 µl Super Taq plus
21 µl sterile H_2O	21 µl sterile H_2O

The control PCR tube was prepared to check if the DIG was integrated into the probe.

For confirmation of extra *ast* gene copies, an *ast* probe was used which was already present in the laboratory.

2.2.4 Prehybridisation and hybridisation

The hybridization oven-rotor was prewarmed at 68 °C and the filters were transferred to sealed glass tubes. Forty millilitres hybridization buffer were added per glass tube and placed for incubation at 68 °C for 4 hours. The rotor was balanced by inserting a dummy tube opposite of the hybridization tube and it was ensured that it turns because the filters may not dry between prehybridization and hybridization.

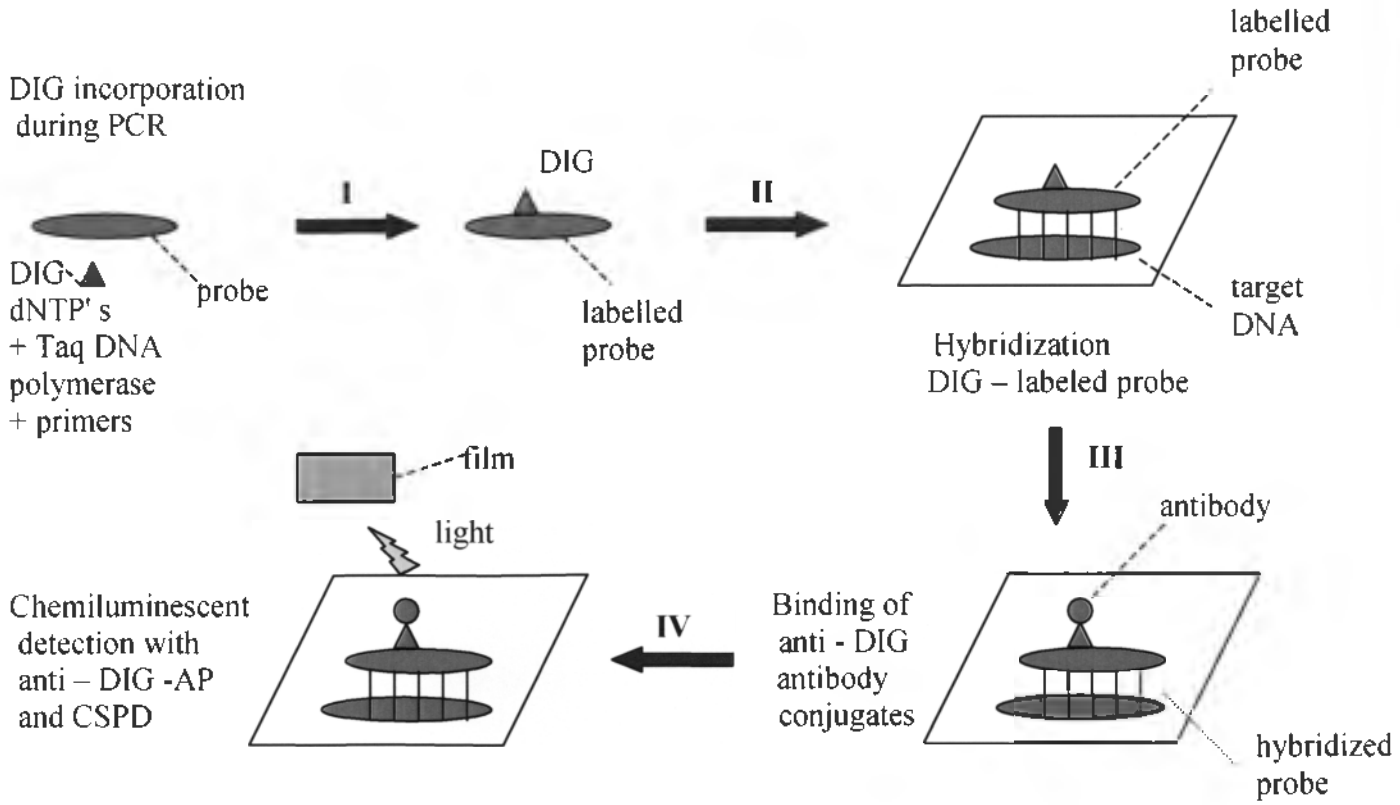
Before hybridization the DNA probe was denatured by boiling at 100 °C for 5 minutes and then immediately stored on ice for further use. After 4 hours the prehybridization solution was replaced with about 20 ml of hybridization buffer containing 1-5 µl denatured probe DNA. The denatured DNA probe was rapidly added to the hybridization buffer. The filter was incubated at 68 °C during the night.

The next day, the hybridization mix was carefully poured out of the tube into a 50 ml plastic tube and after cooling it was stored at – 20 °C. In the hybridisation tube 50 ml of 2 * SSC; SDS 0.1% (w/v) were added and the filters were washed 2 times for 5 minutes at room temperature by gently rolling them manually. The wash buffer was poured into a liquid waste container and in the glass tube 50 ml of prewarmed 0.1 * SSC; SDS 0.1 (w/v) were added. The glass tube was placed into the oven-rotor and washed twice at 68 °C for 15 minutes.

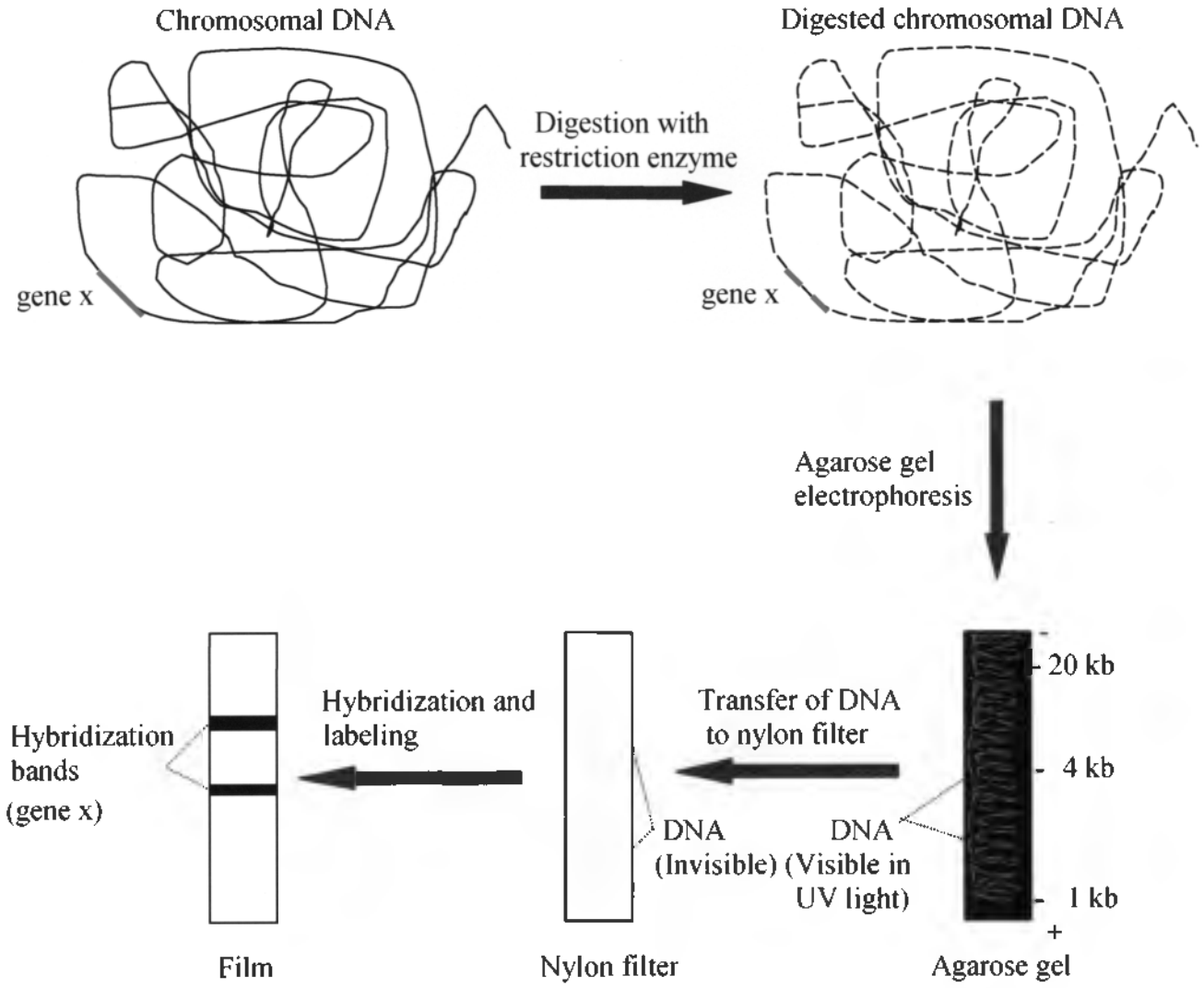
The filter was transferred in a sealed glass tube and 100 ml of blocking solution were added. The glass tube was placed in an oven-rotor and incubated at 20 °C for 30 minutes. After 30 minutes, 80 ml of blocking solution were poured out into the liquid waste container and 2 µl of anti – DIG – AP conjugate were added in Blocking solution. The filter was incubated in 20 ml antibody solution at 20 °C for 30 minutes. Furthermore, the antibody solution was poured into a liquid waste container and in sealed glass tube, 100 ml of washing buffer were added. The glass tube was placed in the oven rotor and the filter was washed 2 times for 15 minutes at 20 °C.

Subsequently the washing buffer was poured into a liquid waste container and the film was equilibrated for 5 minutes in 20 ml detection buffer. At the same time, 2 ml detection buffer were added in 2 ml eppendorf tube and 10 µl of CSPD were diluted in this buffer. Then, the filter was sealed and incubated in a hybridization bag for 5 minutes in 2 ml CSPD solution at room temperature. After 5 minutes, the excess of liquid was dripped off and the filter was blotted briefly with DNA side up on Whatman 3 MM paper. The filter was not completely dried. The filter was placed in a hybridization bag and it was incubated with DNA side up at 37 °C for 15 minutes to enhance the luminescent reaction.

Afterwards, the hybridization bag was placed in Kodak cassette and transferred to a dark room where the Kodak film was added. The film was exposed at room temperature for 25 minutes and then developed in a dark room. If the picture was not clear a new film was added and exposed at room temperature for 3 hours or overnight. After 3 hours or the next day, the film was developed in a dark room.



ure Labelling and Detection by the DIG System. I, DIG is incorporated in probe. II, DIG-labelled probe hybridized to target DNA. III, The anti-DIG antibody conjugates is binded in hybrid. IV, The CSPD reacts with the AP (alkaline phosphatase) and the reaction product is light. The detection accomplishes by capturing the emitted light, from the hybridized probe, on a film.



ure Southern blot analysis. Schematic representation. Chromosomal DNA of *X. dendrorhous* was digested with restriction enzymes in numerous fragments with different sizes. These fragments were separated during agarose gel electrophoresis according to their sizes and transferred to a nylon filter in single stranded (denatured) state. The filter was hybridised to the DIG non-radioactive probe homologous with gene x to form double stranded molecules (hybrids). The detection was accomplished by capturing the emitted light, from the hybridized probe, on a film. The sizes of bands could be estimated from the size marker in the gel.

2.2.5 Cloning of the *cpr* gene

2.2.5.1 Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were carried out in an automated thermal cycler. The 2 kb *cpr* coding sequence from *Saccharomyces cerevisiae* was amplified by PCR using chromosomal *S. cerevisiae* DNA as template. The reaction conditions were as follows: 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 50 °C, 3 min at 72 °C, ending with 5 min at 72 °C. For the amplification the primers PCPRF 5 ' – CTC GAG ATG CCG TTT GGA ATA GAC AAC – 3 ' and PCPRR 5 ' - CTC GAG TTA CCA GAC ATC TTC TTG GTA TC – 3 ' were used. Two PCR tubes were prepared with total volume 30 µl. The amounts that added in each of two tubes are presented below.

In the 1st PCR tube:	In the 2nd PCR tube:
1 µl DNA	5 µl DNA
1 µl PCPR F	1 µl PCPR F
1 µl PCPR R	1 µl PCPR R
3 µl 10 x PCR buffer	3 µl 10 x PCR buffer
5 µl dNTP's	5 µl dNTP's
0.5 µl Super Taq plus	0.5 µl Super Taq plus
18.5 µl sterile H_2O	14.5 µl sterile H_2O

When PCR was completed, the size of the amplicon was checked by agarose gel electrophoresis. When the correct size of the amplicon was ascertained, the DNA fragment was isolated using the QIAEX II Gel Extraction Kit. For cloning experiments, the *cpr* fragment of 5 µl *S. cerevisiae* DNA was isolated from the gel.

2.2.5.2 Isolation of the DNA fragment from the gel

When electrophoresis was completed, the gel was placed in the UV transilluminator and a sharp sterile scalpel was used to cut the piece of gel that contained the correct DNA fragment. The piece of gel was placed in a sterile 1.5 ml eppendorf tube and 300 µl of QXI and 10 µl of Qiaex II suspension were added and this was vortexed. The QXI dissolves the gel and the Qiaex II suspension binds the DNA fragments.

The tube was placed in a water bath for 10 minutes at 50-55 °C and it was mixed every 2 minutes. After this, the tube was placed in a centrifuge and cells were harvested by centrifugation at 14.000 rpm for 10 seconds. The supernatant was discarded and the qiaex pellet was resuspeded in 500 µl of PE buffer. The PE buffer was used to wash the pellet. The qiaex pellet was harvested by centrifugation at 14.000 rpm for 10 seconds. The supernatant was discarded and the cell pellet was resuspeded in 500 µl of PE buffer. The qiaex pellet was harvested by centrifugation at 14.000 rpm for 10 seconds. After centrifugation the supernatant was taken out and the pellet was dried in the air until it becomes white.

When the pellet was completely white 10 µl of EB buffer were added in the tube, this was mixed in the vortex to dissolve the pellet and placed in water bath at 50-55 °C for 5 minutes. The EB buffer releases the DNA from Qiaex II suspension. The qiaex pellet was harvested by centrifugation at 14.000 rpm for 10 seconds and the supernatant was added in a new 1.5 ml eppendorf tube. The pellet was dissolved in 10 µl EB buffer by vortexing and it was placed in a water bath at 50-55 °C for 5 minutes. The qiaex pellet was harvested by centrifugation at 14.000 rpm for 10 seconds and added to the initially eluted DNA solution tube. The tube with DNA solution was stored at –20 °C.

2.2.5.3 Preparation of *E. coli* cells for transformation

Escherichia coli (DH5α) was cultivated at 37 °C in 30 ml LB medium during the night. Two hundred µl from this mixture were added to 50 ml LB medium in shake flask and incubated at 37 °C with 225 rpm for 3 hours. After 3 hours this mixture was divided over two centrifuge tubes. Cells were harvested after centrifugation at 8.000 rpm for 5 minutes and 4 °C. The supernatant was discarded and the pellet was resuspeded in ½ tube volume sterile H_20. In 1 centrifuge tube the content of each tube were combined and cells were harvested by centrifugation at 8.000 rpm, for 5 minutes and 4 °C.

The supernatant was discarded and ½ tube volume of 10 % glycerol / H_2O was added. The tube was mixed by vortexing to resusped the cells and also ½ tube volume of 10 % glycerol / H_2O was added. Cells were harvested by centrifugation at 8.000 rpm for 5 minutes and 4 °C. The supernatant was discarded and the pellet was resuspeded in 250 µl 10 % glycerol / H_2O. The cell suspension was distributed in 1.5 ml eppendorf tube (100 µl per tube) and frozen at –70 °C or used directly.

2.2.5.4 Ligation

The *cpr* gene was cloned in pGEM – T – Easy vector. For the ligation in a 1.5 ml eppendorf tube the amounts that are presented below were added.

	5 µl	2 x Rapid Ligation Buffer
	1 µl	pGEM – T – Easy vector (50 ng)
	3 µl	*cpr*
	1 µl	T4 DNA Ligase (1 unit/µl)
Total volume:	10 µl	

The ligation took place at room temperature for 3 hours. Two microliters from this mixture were used for electro-transformation. One day later, more than 100 colonies were visible at 37 °C on LB medium + Xgal + ampicillin selected agar plate. On this plate there were both blue and white colonies. Ten single white colonies were selected for mini – preps. The plasmid was isolated from *E. coli* cells and digested with the restriction enzyme *XhoI* at 37 °C for 2 hours to check if the *cpr* gene was cloned in pGEM – T – Easy vector.

Due to the *cpr* gene contains the *EcoRI* site, it was unavoidable to cut the *cpr* in two fragments of 1.5 kb and 0.5 kb. Thus, the pGEM – T – Easy*cpr* was digested with endonucleases *XhoI* and *EcoRI* at 37 °C for 2 hours. For this in 1.5 ml eppendorf tube the amounts that are presented below were added.

	3 µl pGEM – T – Easy*cpr*
	2 µl Reaction buffer N^0 2
	0.5 µl *XhoI*
	0.5 µl *EcoRI*
	14 µl H_2O
Total volume:	20 µl

After 2 hours, the DNA was run on 1 % agarose gel electrophoresis. When electrophoresis was completed the *cpr* fragment of 1.5 kb was isolated from the gel by QIAEX.

The isolated *cpr* fragment of 1.5 kb was ligated into the pPRcDNA1 vector resulting in pPRcDNA1*cpr*1.5. The vector was digested before ligation with restriction enzymes *XhoI* and *EcoRI*. For this ligation two 1.5 ml eppendorf tubes were prepared with total volume 10 µl each one. The ligation accomplished at room temperature during the night. The contents of each tube are presented below.

In the 1st tube added:		In the 2nd tube added:	
3 µl	*cpr* fragment (1.5 kb)	-	*cpr* fragment
1 µl	pPRcDNA1	1 µl	pPRcDNA1
2 µl	5 x Reaction buffer	2 µl	5 x Reaction buffer
1 µl	T4 DNA ligase (1 unit / µl)	1 µl	T4 DNA ligase (1 unit / µl)
3 µl	H_2O	6 µl	H_2O

The next day, 2 µl per ligation were used for electo-transformations. Approximately 500 white colonies from first ligation and 50 from the second were grown at 37 °C on LB medium + ampicillin selected agar plates. Ten single white colonies were selected from the first plate for the mini – preps. The plasmid was isolated from *E. coli* cells and digested with the restriction enzymes *EcoRI* and *XhoI* at 37 °C for 2 hours.

When the presence of *cpr* 1.5 kb fragment was proven, which means that the *cpr* (1.5 kb) fragment was integrated into the pPRcDNA1, the plasmid of 10 samples was digested by endonuclease *EcoRI* at 37 °C for 2 hours.

The pPRcDNA1*cpr*1.5 vector was isolated from the gel using the QIAEX gel extraction kit. The plasmid was isolated from the 3rd sample, which had the band with the strongest intensity.

Furthermore, the pGEM – T – Easy*cpr* vector was digested with the same endonuclease as above. The DNA was separated on agarose gel and the *cpr* 0.5 kb fragment was isolated from the gel by QIAEX.

Before 0.5 kb *cpr* fragment was ligated with pPRcDNA1*cpr*1.5 vector, the *EcoRI* ends were necessary to dephosphorylate by CIAP (Calf Intestine Alkaline Phosphatase) to prevent self-ligation (self-closure). For this ligation prepared two 1.5 ml eppendorf tubes with total volume 10 µl. In each epppendorf tube the amounts that are presented below were added.

In the 1st tube:	In the 2nd tube:
2 µl *cpr* 0.5 kb	-
1 µl pPRcDNA1*cpr*1.5	1 µl pPRcDNA1*cpr*1.5
2 µl 5 x Reaction buffer	2 µl 5 x Reaction buffer
1 µl T4 DNA ligase (1 unit / µl)	1 µl T4 DNA ligase (1 unit / µl)
4 µl sterile H_2O	6 µl sterile H_2O

The ligation took place during the night. After electroporation 9 white colonies were visible from the 1st sample and 2 from the 2nd on LB medium + ampicillin selected agar plates. All of 9 colonies were selected for the mini-preps. The pPRcDNA1*cpr* vector was isolated from *E. coli* cells and digested by restriction enzymes *NotI* and *HindIII* at 37 °C for 2 hours.

2.2.5.5 Electroporation

E. coli cells were thawed at room temperature and placed on ice. Two microlitres DNA-ligation product was added to 100 µl cell suspension, mixed by pipetting and stored on ice for 5 minutes. The cell-DNA mix was transferred to a cold electroporation cuvette (0.2 cm), and the suspension was tapped to the bottom of the cuvette. The cuvette was placed in the safety chamber slide. The slide was pushed into the chamber until the cuvette was seated between

the contacts in the base of the chamber. Electroporation was performed on the BioRad electroporator with the following settings:

- 2.500 Volt
- 200 Ohm
- 25 μF

Both red pulse buttons were pressed once and were holding until a continuous tone was sounded. This should produce a pulse with a time constant of 4 to 5 msec. The cuvette was removed from the chamber and immediately 1 ml of SOC medium was added to the cuvette and quickly the cells were resuspended with a pasteur pipette. This rapid addition of SOC medium after the pulse is very important because it maximizes the recovery of transformants. The cell suspension was transferred to a sterile 1.5 ml eppendorf tube and then placed directly in a water bath at 37 °C for 1 hour. Cells were harvested by centrifugation for 25 seconds. Most of the supernatant was discarded, while in the tube approximately 200 μl supernatant remained. The tube was mixed by vortexing to resuspeded the pellet and then the cell suspension was spread on fresh LB + ampicillin + Xgal selective agar plates and these were incubated during the night at 37 °C. After incubation, the single white colonies were selected and each one were added in test tubes which contained 5 ml LB medium + ampicillin. The colonies were picked up with sterile tips using forceps. Forceps were disinfected by flame before use. The test tubes were placed in an incubator at 37 °C and shaken at 225 rpm during the night.

2.2.5.6 Isolation of plasmid from *E. coli*

From each test tube 1.5 ml culture of *E. coli* were added to a 1.5 ml eppendorf tube. Cells were harvested by centrifugation at 14.000 rpm for 1 min. The supernatant was discarded and the pellet was resuspended in 100 μl TGE (P1). TGE makes the cellular membrane permeable. Then 200 μl of NaOH / SDS (P2) were added to the tube and the tube was inverted 5 times to lyse the cells. One hundred fifty microlitres of KAc (P3) were added; the tube was inverted 5 times and placed in a centrifuge at 14.000 rpm for 10 minutes. The P3 precipitates proteins chromosomal DNA. During centrifugation 1 ml of absolute EtOH (100%) was added in a new 1.5 ml eppendorf tube. After centrifugation the supernatant was

added to the EtOH tube. This was mixed by vortexing and incubated at room temperature for 10 minutes.

After 10 minutes, plasmid DNA was harvested by centrifugation at 14.000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 1 ml 70% EtOH. The pellet was dried. When the pellet was completely dry, 50 µl of TE were added to dissolve it and then the plasmid DNA was stored at –20 °C.

3 RESULTS AND DISCUSSION

3.1 Isolation of chromosomal DNA

X. dendrorhous wild strain (CBS6938) and transformants thereof with over-expression of carotenogenic genes were cultivated in 30 ml YEPD medium at 21 °C and 225 rpm for 2 days. After cultivation, the cells were harvested and then the DNA was isolated from each strain. The concentrations of DNA, which was isolated from these strains, are listed in Table 3.

Strain	DNA (µg/µl)
CBS6938	2,7
CBS6938[pPRcDNA1]	1,5
CBS6938[pPRcDNA1*crtE*] #1	1,0
CBS6938[pPRcDNA1*crtE*] #2	2,2
CBS6938[pPRcDNA1*crtE*] #3	1,8
CBS6938[pPR2TN]	2,7
CBS6938[pPR2TN*ast*] #2	3,4
CBS6938[pPR2TN*ast*] #3	2,2
CBS6938[pPR*crtI*]	0,6
CBS6938[pPR*crtYB*]	1,8

Table 3: Concentration of DNA of *X. dendrorhous* strains.

The isolated DNA was used for the determination of the presence as well as the copy number of the extra carotenogenic gene copies in the transformants by Southern analysis.

3.2 Southern blot analysis

3.2.1 Probe labelling by PCR

3.2.1.1 DIG-labelled rDNA probe

For the determination of the number of the extra carotenogenic genes in *X. dendrorhous* transformants, a DNA probe was designed using ribosomal DNA from pPR2TN vector as template. Two PCR tubes were prepared, one with DIG and one without DIG (control). The control PCR tube was prepared to check if the DIG was integrated into the rDNA probe. After 30 cycles, the PCR products were analysed by agarose gel electrophoresis. The picture of the gel after electrophoresis is presented below (Fig. 7).

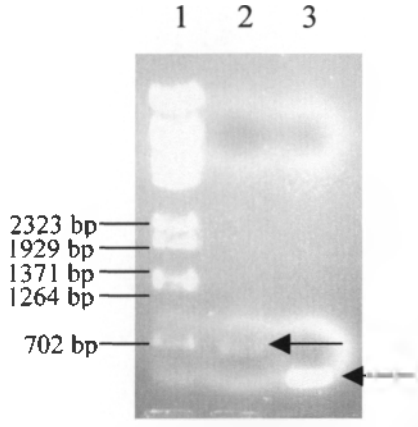


Figure 7: The rDNA probe was analysed by agarose gel electrophoresis. 1, λ DNA * *BstEII* (marker), 2, rDNA with DIG (arrow), 3, rDNA without DIG (discontinuous arrow).

According to the picture from the gel it is clear that the size of rDNA increased due to the integration with DIG by PCR. The size of rDNA fragment is 500 bp (discontinuous arrow) and the size of rDNA with DIG is 700 bp (arrow).

3.2.1.2 DIG-labelled *crtE* probe

The *crtE* and *ast* probes were used to confirm the presence of extra *crtE* and *ast* gene copies in the corresponding transformants respectively. The *crtE* probe was designed by using *crtE* fragment as template. Two PCR tubes were prepared, one with DIG and one without DIG (control). The control tube was prepared as above, to check the presence of DIG into the probe. After 30 cycles, the PCR products were analysed by agarose gel electrophoresis. The picture of the gel after electrophoresis is presented below (Fig. 8). In the picture it is clear that the size of the *crtE* fragment with DIG is higher (700 bp arrow) than that without DIG (500 bp discontinuous arrow). For confirmation of extra *ast* gene copies, an *ast* probe was used which was already present in the laboratory.

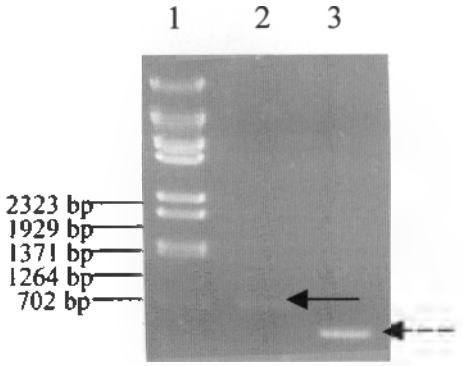


Figure 8: The *crtE* probe was analysed by agarose gel electrophoresis. 1, λ DNA * *BstEII* (marker), 2, *crtE* fragment with DIG (arrow), 3, *crtE* fragment without DIG (discontinuous arrow).

3.2.2 Chromosomal DNA digestion and hybridization

3.2.2.1 Copy number analysis

The chromosomal DNA of *X. dendrorhous* strains were digested (per strain) with the restriction enzyme *SfiI*. The digested chromosomal DNA was separated on a 0,8 % agarose gel (Fig. 9), transferred to nylon filter and hybridised with the DIG-labelled rDNA probe. When the film was developed, we could to determine the copy number of the extra *crtE, crtYB, crtI* and *ast* genes in the respective transformants. Two signals per transformant were expected, 1 from the endogenous rDNA units and 1 from the released plasmids. In the film

both signals were present. In case of the wild strain CBS6938 only one signal from the endogenous rDNA units was expected (Fig. 10).

By comparison of the picture from the gel and the film we could estimate the size of endogenous rDNA and the size of introduced plasmids. Thus, we found that the endogenous rDNA in *X. dendrorhous* strains is 8.5 kb, the pPRcDNA1 6.6 kb, the pPRcDNA1*crtE* 7.8 kb, the pPR2TN 7.5 kb, the pPR2TN*ast* 11.3 kb, the pPR*crtI* 10.3 kb and the pPR*crtYB* 11.4 kb large. According to these results it is clear that the sizes of pPRcDNA1 and pPR2TN vectors were increased when the genes were introduced into them. The increase in the size of vectors could be low or high and it depends from the size of the gene. In case of *crtE* gene, the size of pPRcDNA1 vector was increased by 1.2 kb and in case of *crtYB* gene, the size of pPR2TN vector was increased by 3.9 kb.

The signal intensity from the endogenous rDNA units corresponds to 61 copies (Wery *et al.*, 1997). By comparison of this signal intensity to that of the plasmids, we could estimate their copy number. The estimation was carried out using the densitometer (BioRad Quantity One software). The copy number of the extra carotenogenic genes in the respective transformants is 15 copies of *crtE*, 1-5 copies of *crtYB*, 50 copies of *crtI* and 1-5 copies of *ast*.

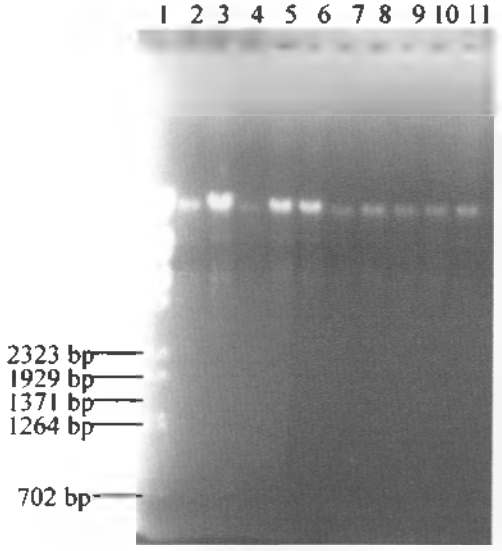


Figure 9: The chromosomal DNA of *X. dendrorhous* strains were digested with endonuclease *SfiI* and separated on 0,8 % agarose gel. 1, λ DNA * *BstE II* (marker), 2, CBS6938, 3, CBS6938[pPRcDNA1*crtE*]#1, 4, CBS6938[pPRcDNA1*crtE*]#2, 5, CBS6938[pPRcDNA1*crtE*]#3, 6, CBS6938[pPRcDNA1], 7, CBS6938[pPR2TN], 8, CBS6938[pPR2TN*ast*]#2 , 9, CBS6938[pPR2TN*ast*]#3, 10, CBS6938[pPR*crtI*], 11, CBS6938[pPR*crtYB*].

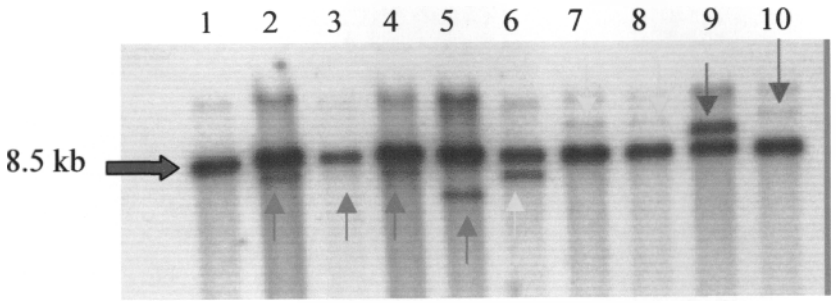


Figure 10: Southern blot analysis of *X. dendrorhous* strains. The digested DNA was transferred from the gel to a nylon filter and hybridised with the DIG-labelled rDNA probe. 1, CBS6938, 2, CBS6938[pPRcDNA1*crtE*]#1, 3, CBS6938[pPRcDNA1*crtE*]#2, 4, CBS6938[pPRcDNA1*crtE*]#3, 5, CBS6938[pPRcDNA1], 6, CBS6938[pPR2TN], 7, CBS6938[pPR2TN*ast*]#2, 8, CBS6938[pPR2TN*ast*]#3, 9, CBS6938[pPR*crtI*], 10, CBS6938[pPR*crtYB*]. The fat horizontal arrow indicates the signal from the endogenous rDNA units and the vertical arrows indicate the signal from the released plasmids.

3.2.2.2 Analysis of extra *crtE* and *ast* gene copies

Verdoes *et al.* have already shown the presence of extra *crtYB* (3.9 kb) and *crtI* (2.8 kb) gene copies in the *crtYB* and *crtI* over-expression strains (Verdoes *et al.*, 2003). By using the *crtE* and *ast* probes we analysed the presence of extra *crtE* and *ast* gene copies respectively in the corresponding transformants.

The chromosomal DNA of *crtE* and *ast* transformants was digested with the endonucleases *EcoRI* and *SstI* and *NotI* and *HindIII* respectively. The wild strain as well as the plsasmids that were used for the transformants, was digested with the same endonucleases as positive controls. The digested chromosomal DNA was separated on 1 % agarose gel (Fig. 11), transferred to nylon filters and hybridised with the DIG-labelled *crtE* and *ast* probes. When the film was developed, we could confirm the presence of extra *crtE* and *ast* gene copies in the corresponding transformants (Fig. 12). By comparison of the picture from the gel to the film, we could estimate the size of the endogenous genes and extra gene copies. In the film it is clear that the copies of the extra *crtE* gene (Fig. 12A arrows) are high and less for the extra *ast* gene (Fig. 12B discontinuous arrows). We proved that the sizes of extra *crtE* and ast gene copies were the expected, 1.2 kb and 3.8 kb respectively. These sizes can also be

confirmed from the film for the copy number analysis (Fig. 10). In that film, the size of pPRcDNA1 vector was increased by 1.2 kb when the *crtE* gene was inserted and the size of pPR2TN vector was increased by 3.8 kb when the *ast* gene was inserted. We have estimated that the copy number of *crtE* gene is 15 and the copy number of *ast* gene 1-5. By comparison the signal intensity of the endogenous *crtE* gene (Fig. 12A, 3.5 kb) to that of extra *crtE* gene copies (Fig. 12A, 1.2 kb) and the signal intensity of the endogenous *ast* gene (Fig. 12B, 6.1 kb) to that of extra *ast* gene copies (Fig. 12B, 3.8 kb) we can ensure that the estimation of copy number was correct. The signal intensity of each endogenous gene has 1 copy so, the signal intensity of extra *crtE* gene is clear that is 15 times to the endogenous gene (Fig. 12A) and the signal intensity of the extra *ast* gene is 1-5 times to the endogenous *ast* gene (Fig. 12B). One signal was expected from the wild strain CBS6938 and the CBS6938[pPRcDNA1] and CBS6938[pPR2TN] transformants (Fig. 12). This signal was from the endogenous gene. The plasmids that were used for the transformants were used as positive controls for the extra gene copies. Thus, one signal was expected with the same size of the genes (Fig. 12).

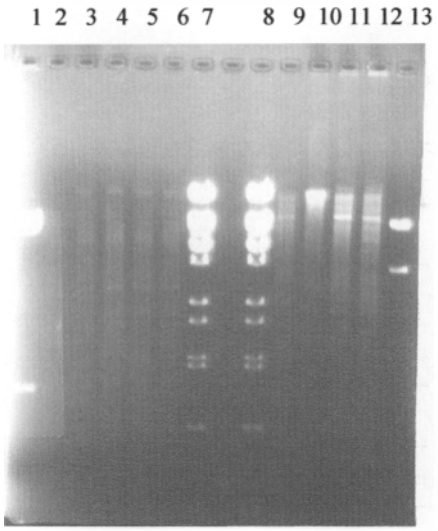


Figure 11: Plasmids and chromosomal DNA of *X. dendrorhous* strains were digested with the endonucleases that were referred above. The DNA separated on 1 % agarose gel and then transferred to nylon filter. 1, pPRcDNA1*crtE* * *EcoRI* * *SstI*, 2, CBS6938 * *EcoRI* * *SstI*, 3, CBS6938[pPRcDNA1] * *EcoRI* * *SstI*, 4,CBS6938[pPRcDNA1*crtE*]#1 * *EcoRI* * *SstI*, 5, CBS6938[pPRcDNA1*crtE*]#2 * *EcoRI* * *SstI*, 6, CBS6938[pPRcDNA1*crtE*]#3 * *EcoRI* * *SstI*, 7, λ DNA * *BstEII* (marker), 8, λ DNA * *BstEII* (marker), 9, CBS6938[pPR2TN*ast*]#3 * *NotI* * *HindIII*,

10, CBS6938[pPR2TN*ast*]#2 * *NotI* * *HindIII*, 11, CBS6938[pPR2T] * *NotI* * *HindIII*, 12, CBS6938 * *NotI* * *HindIII*, 13, pPR2TN*ast* * *NotI* * *HindIII*.

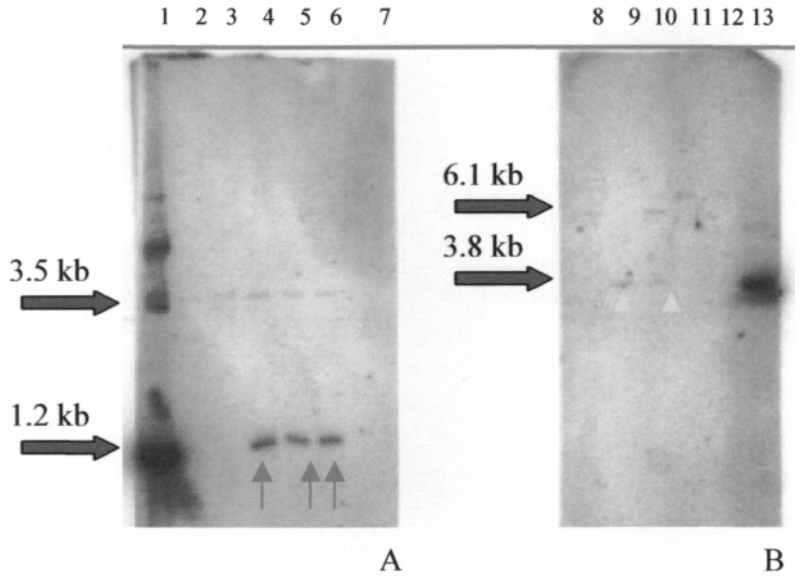


Figure 12: Southern blot analysis of *crtE* and *ast* over-expression strains. The blots were hybridized with a DIG-labelled *crtE* (A) and *ast* (B) probes. 1, pPRcDNA1*crtE* * *EcoRI* * *SstI*, 2, CBS6938 * *EcoRI* * *SstI*, 3, CBS6938[pPRcDNA1] * *EcoRI* * *SstI*, 4, CBS6938[pPRcDNA1*crtE*]#1 * *EcoRI* * *SstI*, 5, CBS6938[pPRcDNA1*crtE*]#2 * *EcoRI* * *SstI*, 6, CBS6938[pPRcDNA1*crtE*]#3 * *EcoRI* * *SstI*, 7, λ DNA * *BstEII* (marker), 8, λ DNA * *BstEII* (marker), 9, CBS6938[pPR2TN*ast*]#3 * *NotI* * *HindIII*, 10, CBS6938[pPR2TNast]#2 * *NotI* * *HindIII*, 11, CBS6938[pPR2T] * *NotI* * *HindIII*, 12, CBS6938 * *NotI* * *HindIII*, 13, pPR2TN*ast* * *NotI* * *HindIII*. The upper fat horizontal arrows indicate the signal from the endogenous genes and the vertical arrows indicate the signal from the extra gene copies.

3.3 Cloning of *cpr* expression vector

As mentioned before, another objective was the construction of an engineered *X. dendrorhous* strain in order to over-produce a P450-reductase as well as astaxanthin synthetase. For this, firstly the *cpr* gene from *Saccharomyces cerevisiae* was amplified and then the amplicon was cloned in pGEM – T – Easy. The gene was cut by adequate restriction enzymes from pGEM – T – Easy and was inserted into pPRcDNA1 in the site between the *gpd* promoter and *gpd* terminator. The *cpr* expression cassette (P*gpd* – *cpr* – T*gpd*) was cloned into pPR2TNH vector (Fig. 12). The pPR2TNH vector includes the selection marker

hygromycine for the *cpr* gene and *X. dendrorhous* transformant strain CBS6938[pPR2TNast]#3 already contains G418 Geneticin as selection marker for the *ast* gene. Thus, new transformants will survive only when the cells contain both plasmids (selection).

S. cerevisiae was selected because its genome includes a *cpr* gene and the sequence is already known. *X. dendrorhous* has also a *cpr* gene but the sequence is unknown yet. Moreover, the *cpr* gene from *S. cerevisiae,* compared to other fungi like *Aspergillus niger*, does not include introns. This makes the transcription of the *S. cerevisiae cpr* in *X. dendrorhous* possible.

The 2 kb *cpr* coding sequence from *S. cerevisiae* was amplified by PCR using chromosomal *S. cerevisiae* DNA as template. Two PCR tubes were prepared, one with 1 µl *S. cerevisiae* DNA and one with 5 µl. After 30 cycles, the PCR products were analysed by agarose gel electrophoresis. The picture of gel when electrophoresis completed is presented below (Fig. 13). For cloning experiments, the *cpr* fragment of 5 µl *S. cerevisiae* DNA was isolated by Qiaex II gel extraction kit.

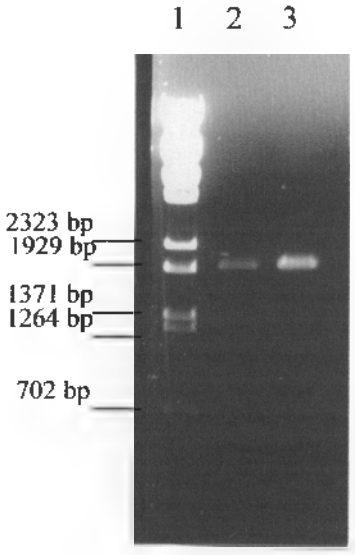


Figure 13: PCR products were analysed by agarose gel electrophoresis. 1, λ DNA * *BstEII* (marker), 2, DNA of *S. cerevisiae* 1 µl, 3, DNA of *S. cerevisiae* 5 µl.

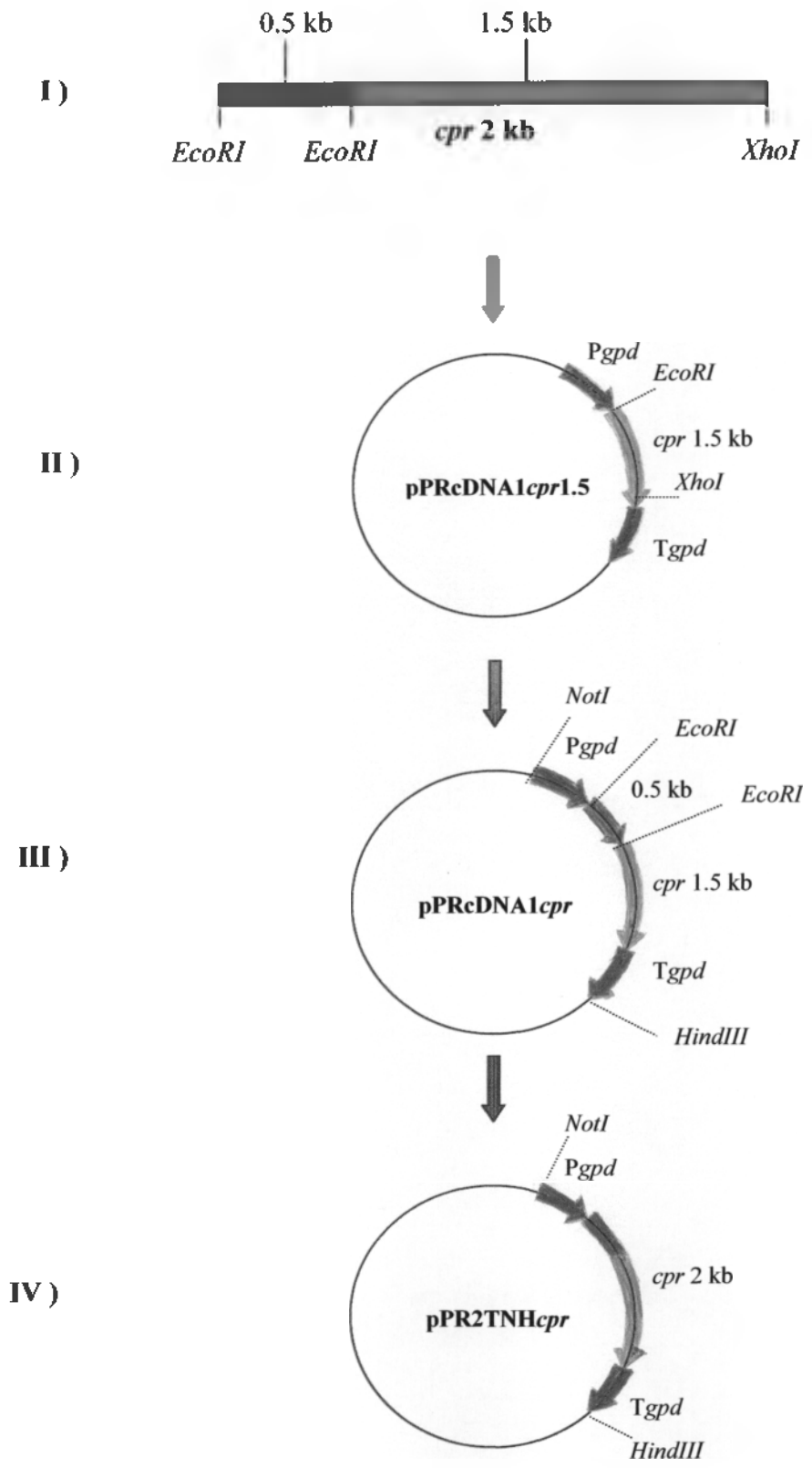


Figure 12: Cloning scheme of *cpr* gene. I) The amplified 2 kb *cpr* gene was cloned in pGEM – T – Easy vector. II) The 1.5 kb *cpr* (*EcoRI* - *XhoI*) fragment was cloned in pPRcDNA1 vector. III) The 0.5 kb *cpr* (*EcoRI* - *EcoRI*) fragment was cloned in pPRcDNA1*cpr*1.5 vector. In this step the two fragments of *cpr* gene were united. IV) The *cpr* expression cassette (P*gpd* – *cpr* – T*gpd*) was cloned into pPR2TNH vector.

The amplicon was cloned in pGEM – T – Easy vector and its identity was confirmed by partial sequencing. After ligation more than 100 colonies were visible on LB medium + Xgal + ampicillin selected agar plate. Ten single white colonies were selected for mini – preps. The pGEM – T – Easy*cpr* vector was isolated from *E. coli* cells and digested with the restriction enzyme *XhoI*. The endonuclease *XhoI* was used to separate the *cpr* gene from pGEM – T – Easy vector. The picture from the gel as it was analysed after electrophoresis is presented in Figure 14.

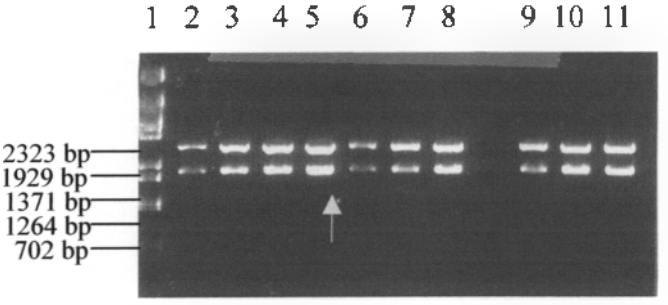


Figure 14: The isolated pGEM – T – Easy*cpr* vector was digested by restriction enzyme *XhoI* and analysed on 1 % agarose gel. 1, λ DNA * *BstEII* (marker), 2-11, pGEM – T – Easy*cpr* * *XhoI*.

According to the picture the pGEM – T – Easy*cpr* vector was digested correct from the restriction enzyme *XhoI*. Two bands were expected, 1 from the 3 kb pGEM – T – Easy vector and 1 from the 2 kb *cpr* gene. The 2 kb *cpr* fragment of the 4th sample (it is indicated with the arrow) was isolated from the gel using the QIAEX gel extraction kit. The *cpr* fragment was selected from the 4th sample because it had the strongest intensity.

Because the *cpr* gene contains the *EcoRI* site, it was unavoidable to cut *cpr* in two fragments of 1.5 kb and 0.5 kb. The pGEM – T – Easy*cpr* vector was digested with endonucleases *XhoI* and *EcoRI*. The vector was digested with these endonucleases, because *EcoRI* and *XhoI* are the only cloning sites in pPRcDNA1 vector (P*gpd* – *EcoRI* – *XhoI*-T*gpd*). The DNA as was analysed by electrophoresis is presented in Figure 15.

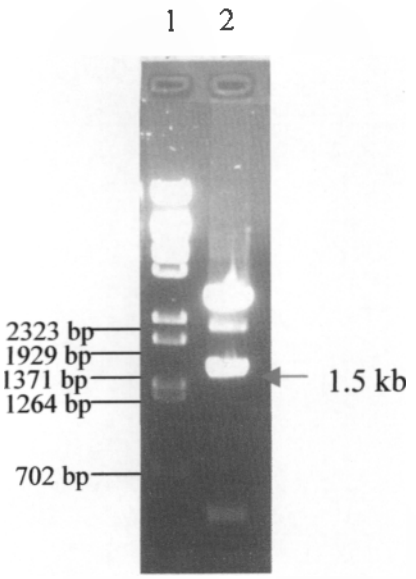


Figure 15: The pGEM – T – Easy*cpr* vector was digested with restriction enzymes *XhoI* and *EcoRI*. The DNA was analysed on 1 % agarose gel and the *cpr* fragment of 1.5 kb was isolated from the gel. 1, λ DNA * *BstEII* (marker), 2, pGEM – T – Easy*cpr* * *XhoI* * *EcoRI*.

The isolated *cpr* fragment of 1.5 kb was ligated into the *EcoRI* and *XhoI* sites of the pPRcDNA1 vector resulting in pPRcDNA1*cpr*1.5 (Fig. 12, II). After ligation, approximately 500 white colonies were observed on LB medium + ampicillin selected agar plates. Ten single white colonies were selected for the mini – preps. The plasmid was isolated from *E. coli* cells and digested with the restriction enzymes *EcoRI* and *XhoI* to check if the 1.5 kb *cpr* fragment was ligated into the pPRcDNA1 vector. Two bands from each sample were expected. One from the 6.6 kb pPRcDNA1 vector and 1 from the 1.5 kb *cpr* fragment. The plasmid DNA fragments were analysed by agarose gel electrophoresis (Fig. 16).

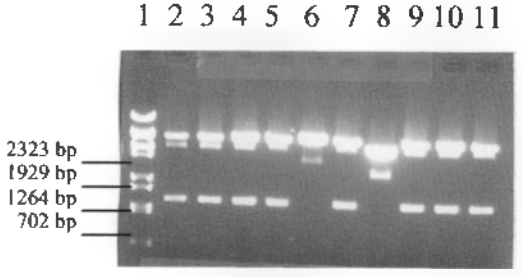


Figure 16: The pPRcDNA1*cpr*1.5 from the 10 samples was digested with the endonucleases *XhoI* and *EcoRI*. The picture from the gel proves that the *cpr* had integrated into pPRcDNA1 vector except for

the lines 6 and 8 because the sizes of the bands were not the expected. 1, λ DNA * *BstEII* (marker), 2-11, pPRcDNA1*cpr*1.5 * *XhoI* * *EcoRI.*

The picture was shown that the *cpr* 1.5 kb fragment had integrated into the pPRcDNA1 vector, except for the samples 6 and 8 because the sizes of the bands were not the expected. The plasmid of 10 samples was digested by endonuclease *EcoRI,* because in this plasmid the 0.5 kb *cpr* fragment will be integrated. (Fig. 17).

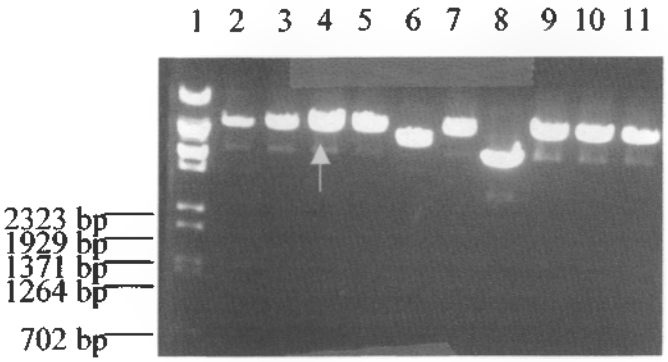


Figure 17: The pPRcDNA1*cpr*1.5 vector from the 10 samples was digested with the restriction enzyme *EcoRI.* The plasmid from the 3rd sample was isolated for further use. 1, λ DNA * *BstEII* (marker), 2-11, pPRcDNA1*cpr*1.5 * *EcoRI.*

The pPRcDNA1*cpr*1.5 plasmid was isolated from the 3rd sample, which had the band with the strongest intensity (arrow).

Furthermore, the pGEM – T – Easy*cpr* vector was digested with *EcoRI* as well and the *cpr* 0.5 kb *EcoRI* fragment was isolated from a gel by QIAEX (Fig. 18).

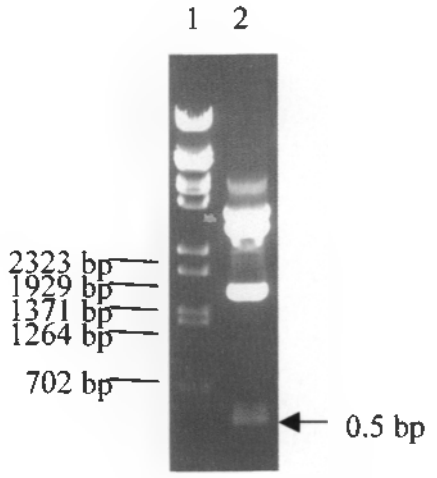


Figure 18: The pGEM – T – Easy*cpr* vector was digested with endonuclease *EcoRI* and analysed on agarose gel. The 0.5 kb *cpr* fragment was isolated from the gel for further use. 1, λ DNA * *BstEII* (marker), 2, pGEM – T – Easy*cpr* * *EcoRI*.

The isolated *cpr* fragment of 0.5 kb was ligated into the pPRcDNA1*cpr*1.5 vector resulting in pPRcDNA1*cpr* (Fig. 12, III). After ligation, 9 white colonies were observed on LB medium + ampicillin selected agar plate. The orientation of the 0.5 kb *EcoRI* fragment in the 9 pPRcDNA1*cpr* vectors were analyzed and the correct plasmid was used and digested with restriction enzymes *NotI* and *HindIII* (H. Visser personal communication). The pPRcDNA1*cpr* vector was digested with these endonucleases because the expression cassette P*gpd* – *cpr* – T*gpd* contains the sites of *NotI* and *HindIII*.

The pPR2TNH vector was digested with the same restriction enzymes as above. The over-expression cassette P*gpd* – *cpr* – T*gpd* was ligated into the pPR2TNH vector (Fig. 12, IV). After ligation, more than 2.000 white colonies were observed on LB medium + ampicillin selected agar plate. One single colony was selected for the mini-prep. The pPR2TNH*cpr* vector was isolated from *E. coli* cells and digested with the restriction enzymes *NotI* and *HindIII* (Fig. 19). Two bands were expected, 1 from the 7.5 kb pPR2TNH vector and 1 from the 2 kb over-expression cassette. According to the picture the pPR2TNH*cpr* vector was digested correct from the endonucleases *NotI* and *HindIII*.

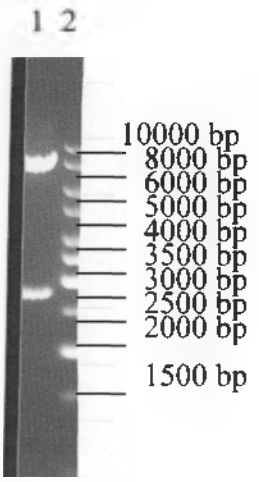


Figure 19: The pPR2TNH*cpr* vector was digested with the restriction enzymes *NotI* and *HindIII* and analysed on agarose gel. 1, λ Gene ruler DNA ladder (marker), 2, pPR2TNH*cpr* * *NotI* * *HindIII*.

The pPR2TNH*cpr* over-expression vector has been constructed and will be introduced in CBS6938[pPR2TN*ast*] #3 *X. dendrorhous* strain, which will probably cause the over-expression of a P450-reductase as well.

4 SUMMARY AND CONCLUSION

Xanthophyllomyces dendrorhous was isolated during the early 1970's from exudates of deciduous trees in mountainous regions of Japan and Alaska (Phaff *et al.*, 1972; Miller *et al.*, 1976). *X. dendrorhous* synthesizes astaxanthin as its principal carotenoid (Andrewes *et al.*, 1976). Astaxanthin is applied in food and feed industry both as a colorant and as an antioxidant. There is a growing demand for astaxanthin from natural sources and not synthetic astaxanthin to be applied in natural products. Therefore, there has been considerable commercial interest in using *X. dendrorhous* as a source for natural astaxanthin. However, *X. dendrorhous* has a too low astaxanthin content (0.02 to 0.03 %) for commercial application. For this reason, several mutants have been created in order to produce more astaxanthin. In our laboratory there are *X. dendrorhous* strains, which over-express the carotenogenic (crt) genes *crtE* (geranylgeranyl-pyrophosphate synthase), *crtYB* (phytoene synthase / lycopene cyclase), *crtI* (phytoene desaturase) and *ast* (astaxanthin synthetase).

The presence and copy number of the extra carotenogenic gene copies in the transformants was determined by Southern analysis. By using the rDNA probe we could to determine the copy number of the extra *crtE*, *crtYB*, *crtI* and *ast* genes in the respective transformants. Knowing that the signal intensity from the endogenous rDNA units is more or less equal to 61 copies we could estimate the copy number of the plasmids (Wery *et al.*, 1997). By comparison of this signal intensity to that of the released plasmids, we shown that the copy number of the extra *crtE* gene is 15 copies, of *crtYB* is 1-5 copies, of *crtI* is 50 copies and of *ast* is 1-5 copies in the respective transformants. Verdoes *et al.* have already shown the presence of extra *crtYB* and *crtI* gene copies in the *crtYB* and *crtI* over-expression strains (Verdoes *et al.*, 2003). The *crtE* and *ast* probes were used to confirm the presence of extra *crtE* and *ast* gene copies respectively in the corresponding transformants.

Another objective in this project was the construction of an engineered *X. dendrorhous* strain. The *X. dendrorhous* strain CBS6938[pPR2TN*ast*]#3 overproduces astaxanthin

synthetase, which is a P450 enzyme. However, P450 enzymes need the cooperation of other enzymes, which are the cytochrome P450-reductases (*cpr*). The 2 kb *cpr* coding sequence from *S. cerevisiae* was amplified by PCR using chromosomal *S. cerevisiae* DNA as template. We have chosen this gene because its sequence is known and it does not contain introns, which facilitates expression in *X. dendrorhous*. The sequence of the *X. dendrorhous cpr* gene is unknown. The 2 kb amplicon was cloned in the pGEM-T-Easy vector and its identity was confirmed by partial sequencing. The *cpr* gene was cloned in the pPRcDNA1 vector in order to flank it with the promoter and terminator sequences of the *X. dendrorhous* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) encoding gene, thereby creating a *cpr* expression cassette. Finally, this over-expression cassette (P*gpd* – *cpr* – T*gpd*) was cloned into a *X. dendrorhous* transformation vector pPR2TNH, which confers resistance to hygromycine.

As mentioned before, the pPR2TNH*cpr* over-expression vector has been constructed and will be introduced in the astaxanthin synthetase overproducing strain CBS6938[pPR2TN*ast*]#3. Analysis of these future transformants will reveal whether the astaxanthin content is further increased by the over-expression of the two genes.

5 REFERENCES

- **Agarwal, S. and Rao A. V.**. 2000. Tomato lycopene and its role in human health and chronic diseases. CMAJ, **163**: 739-744.
- **Andrewes, A. G., Phaff, H. J. and Starr, M. P.**. 1976. Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. Phytochemistry, **15**: 1003-7.
- **Andrewes, A. G. and Starr, M. P.**. 1976. (3R,3'R)-Astaxanthin from the yeast *Phaffia rhodozyma*. Phytochemistry, **15**: 1009-1011.
- **Arab, L. and Steck S.**. 2000. Lycopene and cardiovascular disease. Am. J. Clin. Nutr., **71**: 1691S-5S.
- **Bramley, P. M.**. 2000. Is lycopene beneficial to human health? Phytochemistry, **54**: 233-6.
- **van den Brink, H. J. M., van Gorcom, R. F. M., van den Hondel, C. A. M. J. J. and Punt, P. J.**. 1998. Review. Cytochrome P450 enzyme systems in fungi. Fung. Gen. Biol., **23**: 1-17.
- **Britton, G.**.1995. Structure and properties of carotenoids in relation to function. J. FASEB, **9**: 1551-8.
- **Choubert, G. and Heinrich, O.**. 1993. Carotenoid pigments of the green alga *Haematococcus pluvialis*: assay on rainbow trout *Oncorhynchus mykiss* pigmentation in comparison with synthetic astaxanthin and canthaxanthin. Aquaculture, **112**: 217-226.
- **Cooper, D. A.**. 2004. Carotenoids in health and disease: Recent scientific evaluations, research recommendations and the consumer. J. Nutr., **134**: 221S-4S.
- **Fox, D. L.**. 1962. Metabolic fractionation, storage and display of carotenoid pigments by flamingoes. Comp. Biochem. Physiol., **6**: 1-40.
- **Fraser, P. D. and Bramley, P. M.**. 2004. The biosynthesis and nutritional uses of carotenoids. Progr. Lipid Research, **43**: 228-265.

- **Golubev, W. I.**. 1995. Perfect state of *Rhodomyces dendrorhous* (*Phaffia rhodozyma*). Yeast, **11**: 101-110.
- **Guerin, M., Huntley, M. E. and Olaizola, M.**. 2003. *Haematococcus* astaxanthin: applications for human health and nutrition. TRENDS Biotech., **21**: 210-6.
- **Hoshino, T., Ojima, K. and Setoguchi, Y.**. 2000. Astaxanthin synthetase. European Patent Application EP 1 035 206 A1.
- **Johnson, E. A. and An, G. H.**. 1991. Astaxanthin from Microbial Sources. Critic. Rev. Biotech., **11**: 297-326.
- **Johnson, E. A., Conklin, D. E. and Lewis, M. J.**. 1977. The yeast *Phaffia rhodozyma* as a dietary pigment source for salmonids and crustaceans. J. Fish Res. Board Can., **34**: 2417-2421.
- **Johnson, E. A. and Lewis, M. J.**. 1979. Astaxanthin formation by the yeast *Phaffia rhodozyma*. J. Gen. Microbiol., **115**: 173-183.
- **Johnson, E. A. and Schroeder, W. A.**. 1995. Astaxanthin from the yeast *Phaffia rhodozyma*. Stud. Mycol., **38**: 81-9.
- **Johnson, E. A., Villa, T. G. and Lewis, M. J.**. 1980. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. Aquaculture, **20**: 123-134.
- **Kanemitsu, T. and Aoe, T.**. 1958. Studies on the carotenoids of salmon. I. Identification of the muscle pigments. Bull. Jpn. Soc. Sci. Fish, **24**: 209-215.
- **Kanemitsu, T. and Aoe, T.**. 1958. Studies on the carotenoids of salmon. II. Determination of muscle pigment. Bull. Jpn. Soc. Sci. Fish, **24**: 555-8.
- **Kobayashi, M., Kakizono, T. and Nagai, S.**. 1991. Astaxanthin production by a green alga, *Haematococcus pluvialis* accompanied with morphological changes in acetate media. J. Ferment. Bioeng., **71**: 335-9.
- **Kobayashi, M., Kakizono, T. and Nagai, S.**. 1993. Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. Appl. Environ. Microbiol., **59**: 867-873.
- **Lorenz, R. T. and Cysewski, G. R.**. 2000. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. TIBTECH., **18**: 160-7.
- **Miller, M. W., Yoneyama, M. and Soneda, M.**. 1976. *Phaffia*, a new yeast in the *Deuteromycotina* (*Blastomycetes*). Int. J. Syst. Bacteriol., **26** : 286-291.
- **Phaff, H. J., Miller, M. W., Yoneyama, M. and Soneda, M.**. 1972. A comparative study of the yeast florae associated with trees on the Japanese Islands and on the west

coast of North America. In: Fermentation Technology Today (Terui, G., Ed.), pp. 759-774. Society of Fermentation Technology, Osaka, Japan.

- **Rao, A. V. and Agarwal, S.**. 2000. Role of antioxidant lycopene in cancer and heart disease. J. Am. Col. Nutr., **19**: 563-9.
- **Sambrook, J., Fritsch, E. F. and Maniatis, T.**. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
- **Schiedt, K., Leuenberger, F. J. and Vecchi, M.**. 1981. Natural occurrence of enantiomeric and meso-astaxanthin. V. Wild salmon (*Salmo salar* and *Oncorhynchus*). Helv. Chim. Acta, **64**: 449-457.
- **Schiedt, K., Leuenberger, F. J., Vecchi, M. and Glinz, E.**. 1985. Absorption, retention and metabolic transformation of carotenoids in rainbow trout, salmon and chicken. Pure Appl. Chem., **57**: 685-692.
- **Scott, K. J. and Hart, D. J.**. 1994. The carotenoid composition of vegetables and fruit commonly consumed in the UK. Norwich: IFR.
- **Steinbrenner, J. and Linden, H.**. 2001. Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. Plant Physiol., **125**: 810-7.
- **Verdoes, J. C., Krubasik, P., Sandmann, G. and van Ooyen, A. J. J.**. 1999. Isolation and functional characterisation of a novel type of carotenoid biosynthetic gene from *Xanthophyllomyces dendrorhous*. Mol. Gen. Genet., **262**: 453-461.
- **Verdoes, J. C., Sandmann, G., Visser, H., Diaz, M., van Mossel, M. and van Ooyen, A. J. J.**. 2003. Metabolic engineering of the carotenoid biosynthetic pathway in the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Appl. Environ. Microbiol., **69**: 3728-3738.
- **Verdoes, J. C., Wery, J. and van Ooyen, A. J. J.**. 1996. Improved methods for transforming *Phaffia* strains, transformed *Phaffia* strains so obtained and recombinant DNA in said method. International Patent Application PCT/EP96/05887.
- **Visser, H., van Ooyen, A. J. J. and Verdoes, J. C.**. 2003. Metabolic engineering of the astaxanthin-biosynthetic pathway of *Xanthophyllomyces dendrorhous*. FEMS Yeast Research., **4**: 221-231.
- **Watson, J. D., Gilman, M., Witkowski, J. and Zoller, M.**. 1996. Recombinant DNA. Second Edition. Freeman, W. H. and Company, New York, pp.79-84.

- **Weedon, B. C. L.**. 1971. Carotenoids. *In* O. Isler (ed.), Birkhauser Verlag, Basel. Occurrence, pp. 29-59.
- **Wery, J., Gutker, D., Renniers, A. C. H. M., Verdoes, J. C. and van Ooyen, A. J. J.**. 1997. High copy number integration into the ribosomal DNA of the yeast *Phaffia rhodozyma*. Gene, **184**: 89-97.
- **Wery, J., Verdoes, J. C. and van Ooyen, A. J. J.**. 1998. Efficient transformation of the astaxanthin-producing yeast *Phaffia rhodozyma*. Biotechnol. Techniq., **12**: 399-405.
- **Young, A. J. and Lowe, G. M.**. 2001. Antioxidant and prooxidant properties of carotenoids. Arch. Bioch. Bioph., **385**: 20-7.

APPENDIX I

1)	YEPD	10 gr Yeast Extract, 20 gr Bacto peptone, 20 gr Glycose
2)	DNA Extraction buffer	50 mM Tris, 10 mM $MgCl_2$, 50 mM NaCl, 1 % SDS
3)	3 M NaAc	
4)	70 % EtOH	70 ml EtOH
5)	100 % EtOH	100 ml EtOH
6)	RNAse solution (1 mg/ml)	0.01 M NaAc pH 5.2, 100 mg RNAse A, 1 ml of 1 M Tris pH 7.4
7)	TE	10 mM Tris-Cl, 1 mM EDTA, pH 7.5
8)	6 * Loading buffer	0.25 % Bromophenol blue, 0.25 % Xylene cyanol FF (optional), 40 % Sucrose in water (or 15 % Ficoll in water)
9)	1 % Agarose	1 gr Ultra pure agarose
10)	50 * TAE	242 gr Tris, 57.1 ml Glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0)
11)	Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl, adjusted to pH 7.5 (20 °C) with solid NaOH
12)	Liquid nitrogen	
13)	Alumina (Sigma)	
14)	Phenol	
15)	Chlorophorm	
16)	EtBr	10 mg/ml EtBr
17)	Denaturing solution	1.5 M NaCl, 0.5 N NaOH
18)	Neutralising solution	1 M Tris (pH 7.4), 1.5 M NaCl
19)	DIG Easy hyb solution	
20)	2 * SSC; SDS 0.1 % (w/v)	100 ml of 20 * SSC, 10 ml of 10 * SDS
21)	0.1 * SSC; SDS 0.1 % (w/v)	5 ml of 20 * SSC, 10 ml of 10 * SDS
22)	20 * SSC	175.3 gr NaCl, 88.2 gr Sodium citrate
23)	Washing buffer	Maleic acid buffer with Tween 20, 0.3 % (v/v)
24)	Blocking stock solution (10x conc.)	Blocking reagent, 10 % (w/v) in Maleic acid buffer
25)	Blocking solution	1x conc. Working solution by diluting the stock solution 1:10 in Maleic acid buffer
26)	Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20 °C)
27)	QXI	
28)	Qiaex II	
29)	PE buffer	

30)	EB buffer	10 mM Tris-Cl pH 8.0
31)	TE (P1 : Resuspension buffer)	50 mM Tris-Cl pH 8.0, 10 mM EDTA, 100 µg/ml RNAse A
32)	NaOH / SDS (P2 : Lysis buffer)	200 mM NaOH, 1 % SDS (w/v)
33)	KAc (P3 : Neutralization buffer)	3.0 M Potassium acetate pH 5.5
34)	SOC medium	2 % Bactotryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glycose
35)	LB medium (Luria-Bertani)	10 gr Bacto tryptone, 10 gr NaCl, 5 gr Yeast extract