Agriculture and Agri-Food Canada / Agriculture et Agroalimentaire Canada

Microbial Detoxifications of Deoxynivalenol (DON) and their Potential Applications in Mitigating Mycotoxin Contaminations

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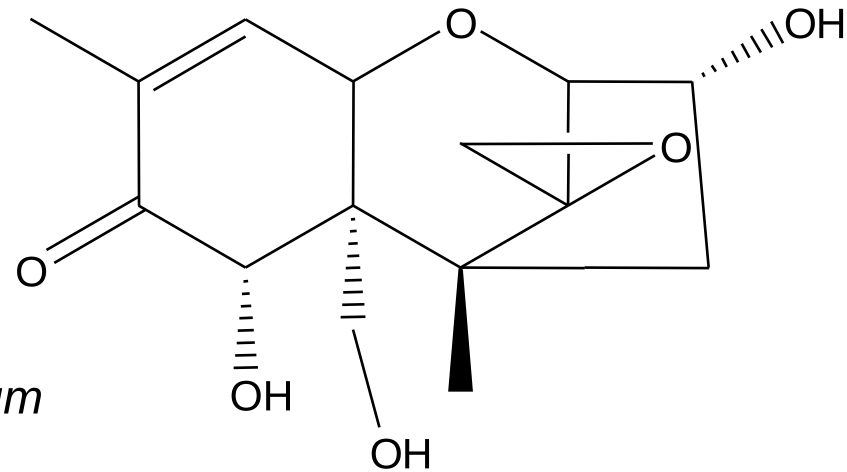
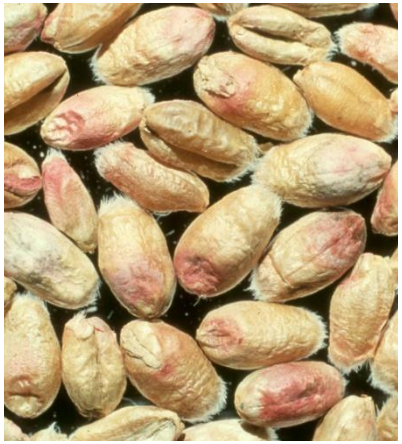
Canada

Deoxynivalenol by species of Fusarium

In 1980, the fungus *Fusarium graminearum* was found to be present in Soft White Winter Wheat in Canada and a mycotoxin, deoxynivalenol (DON) was identified

Fusarium graminearum





4-Deoxynivalenol (DON)
Vomitoxin

A type B Trichothecene

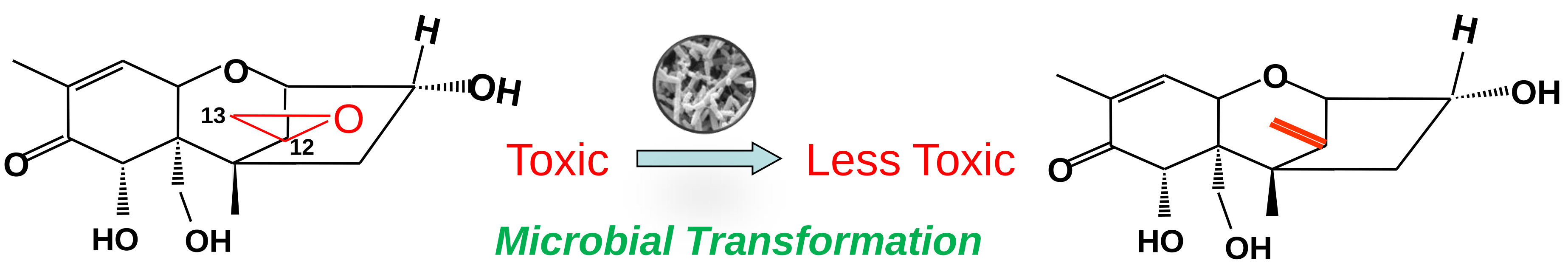
Mycotoxin contaminations

- According to FAO, global losses of foodstuffs due to mycotoxins are in the range of **1000 million tonnes** per year; Fusarium Head Blight is a significant contributor http://www.fao.org/ag/agn/agns/chemicals_mycotoxins_en.asp
- Mycotoxin contamination can not be avoid in the current agricultural practices!
- **Innovative solutions are needed for both reducing mycotoxin production in the field and storage and utilizing already contaminated products**



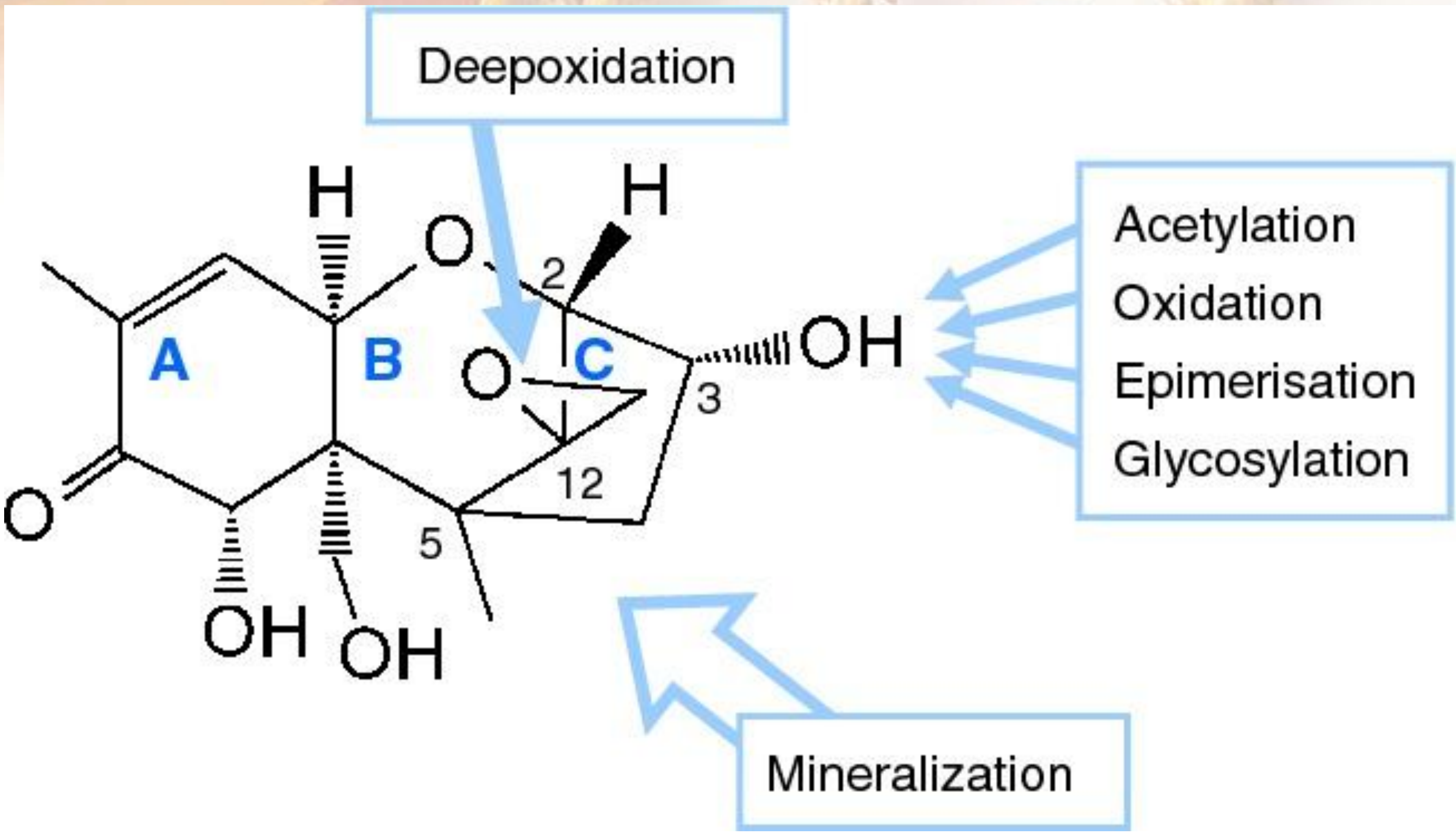

Microbial detoxification

- It is the detoxification of mycotoxins through biotransfomation by microorganisms
- Biotransformation is the ***chemical conversion*** of substances by living organisms or enzyme preparations
- The key for microbial detoxification is the mycotoxin detoxifying microorganisms



Biotransformation reactions and toxicity reductions

Structure of DON and targets for detoxification



Karlovsky, P. Appl Microbiol Biotechnol (2011) 91:491–504

Detoxification by de-epoxidation - DON to dE-DON

deoxynivalenol (DON) **deepoxy DON (dE-DON, DOM-1)**

- The 12,13-epoxy ring in the trichothecene structure (e.g. DON) is an essential functional group for toxicity – inhibition of protein biosynthesis; opening the ring, i.e. de-epoxidation, can result in less toxic product, dE-DON (DOM-1)
- The toxicity of dE-DON is 1/55 of DON determined by the concentration inhibiting 50% of the DNA synthesis (Eriksen *et al.* 2004)

Toxin	IC50 (mM)	IC50 relative to DON
DON	1.5 ± 0.34	1
De-epoxy DON	83.0 ± 8.77	55

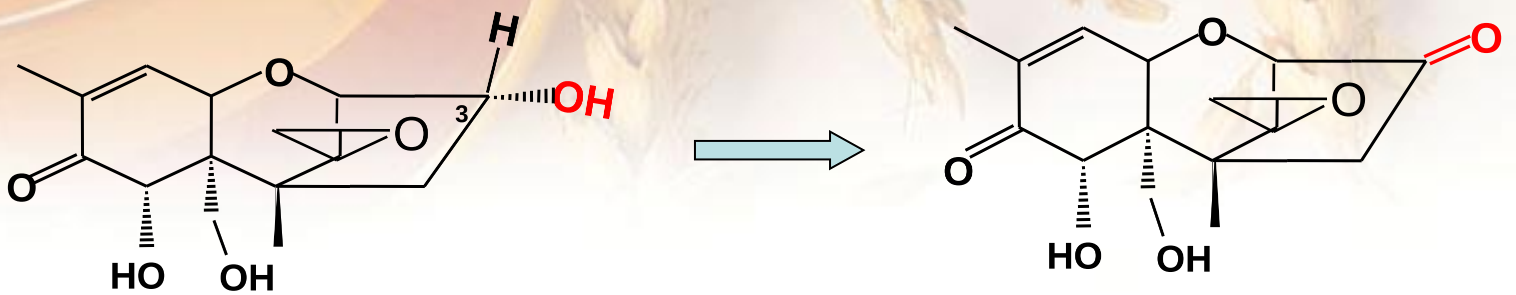
Detoxification by acetylation - DON to 3ADON

deoxynivalenol (DON) — 3-acetyl DON (3ADON)

- The number and position of the acetyl groups also greatly affect the toxicity of trichothecenes. Both acetylation and de-acetylation may reduce toxicity of trichothecenes
- The toxicity of 3ADON is 1/10 of DON determined by the concentration inhibiting 50% of the DNA synthesis (Eriksen *et al.* 2004)

Toxin	IC50 (mM)	IC50 relative to DON
DON	1.5 ± 0.34	1
3-AcDON	14.4 ± 1.59	10

Detoxification by oxidation - DON to 3-keto-DON



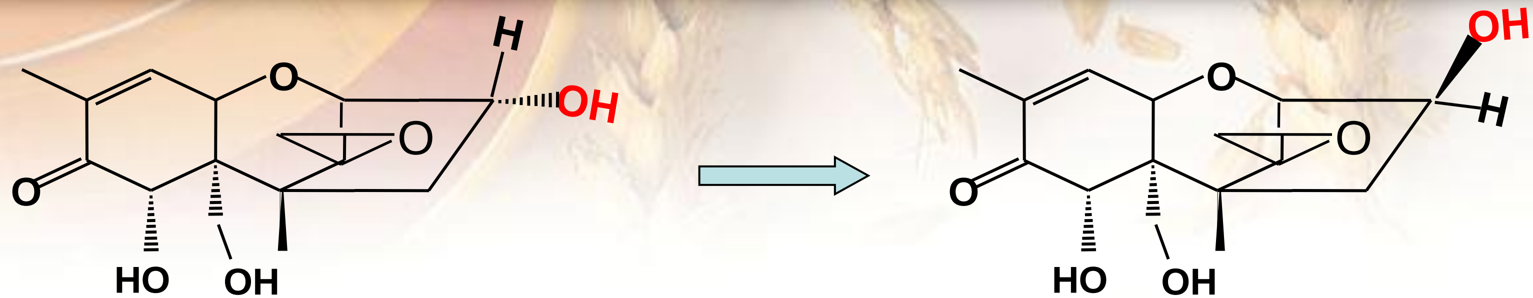
deoxynivalenol (DON)

3-keto DON

➢ The presence and the position of hydroxyl groups on the trichothecene molecules can also influence their toxicity (Betina, 1989).

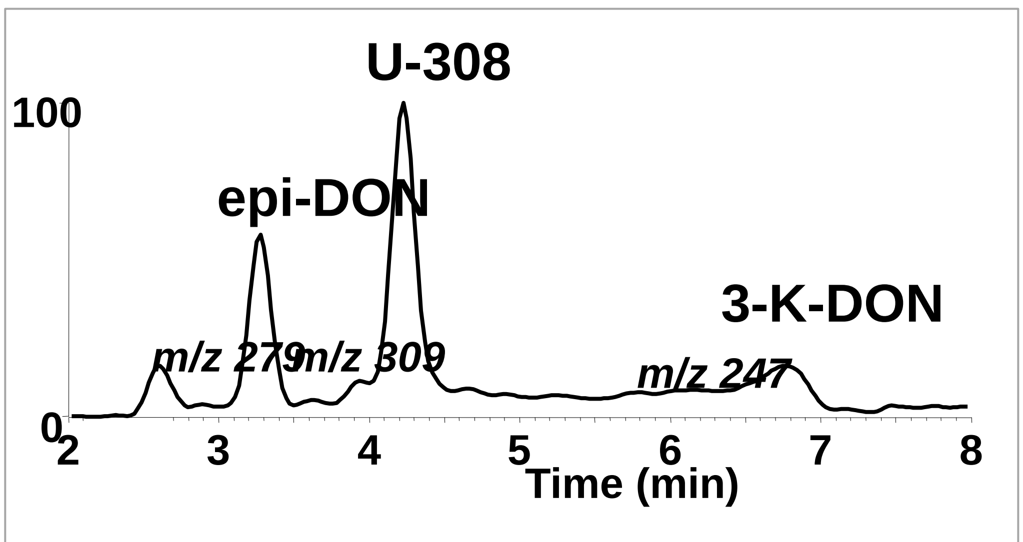
➢ When the C-3 hydroxyl in DON was transformed to oxygen forming 3-keto-DON, the immunosuppressive toxicity of 3-keto-DON decreased remarkably (Shima et al., 1997).

Detoxification by epimerization - DON to 3-*epi*-DON

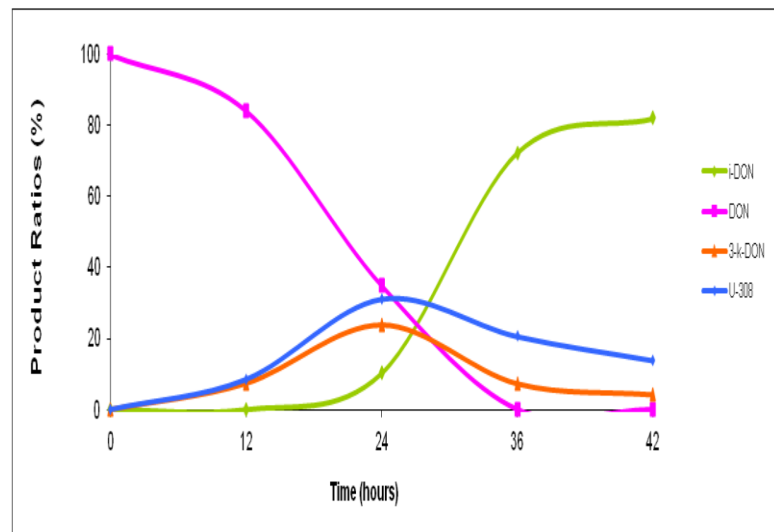


deoxynivalenol (DON) **3-*epi*-DON**

- The epimerization has at least two steps in its pathway

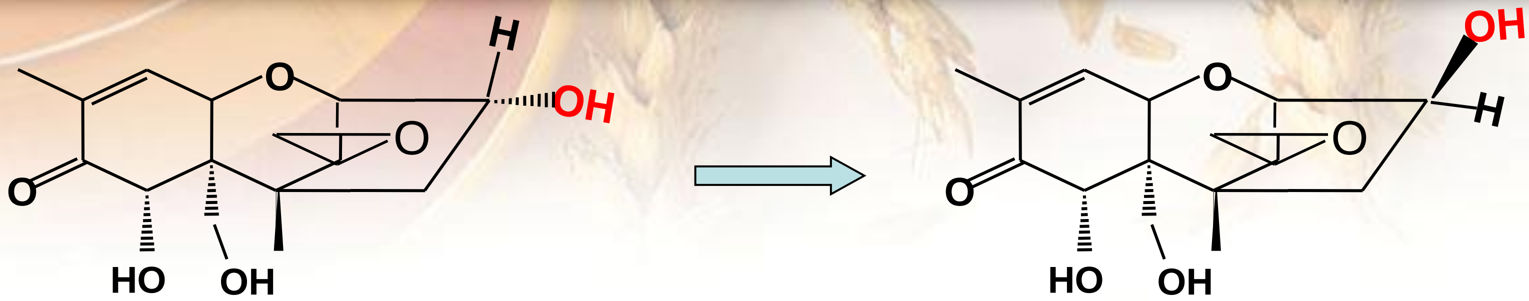


LC-MS analysis of DON transformation by microbial isolate “Barpee”



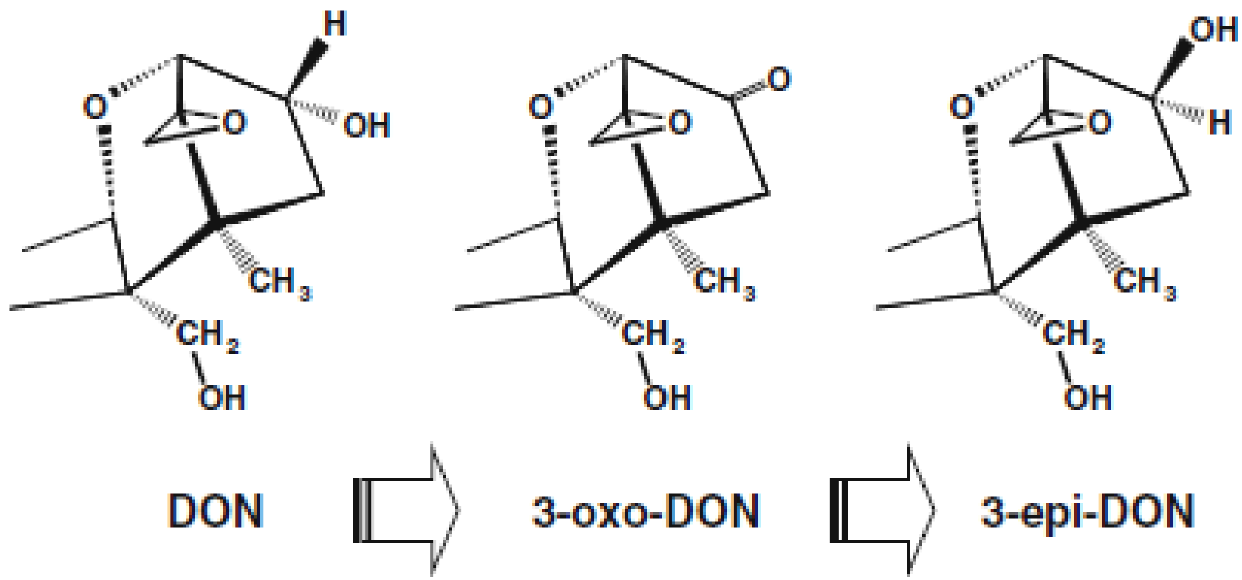
Transformation of DON by “Barpee” under aerobic conditions

Detoxification by epimerization - DON to 3-*epi*-DON



deoxynivalenol (DON) **3-*epi*-DON**

- The epimerization has at least two steps in its pathway

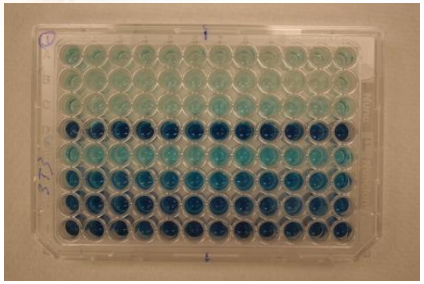


Karlovsky, P. Appl Microbiol Biotechnol (2011) 91:491–504

Toxicity of epi-DON - cytotoxicity

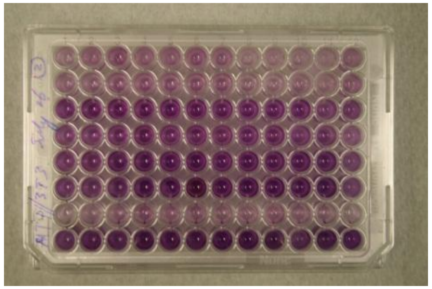
BrdU bioassay: measuring the incorporation of BrdU during DNA synthesis by a cell proliferation ELISA. The IC50 value of epi-DON was 1181 times as that of DON.

Compound	BrdU bioassay using 3T3 fibroblast cells		
	IC_{50}	IC_{50} relative to DON	
	µg/mL (95% Confidence Interval)	Mass concentration	Molar concentration
DON	0.238 (0.162, 0.349)	----	----
epi-DON	281 (156, 505)	1181	1181



MTT bioassay: assessing cell viability on the base of the capability of viable cells to convert soluble MTT (yellow) to purple formazan crystals. The IC50 value of epi-DON was 357 times as that of DON

Compound	MTT bioassay using Caco-2 cells		
	IC_{50}	IC_{50} relative to DON	
	µg/mL (95% Confidence Interval)	Mass concentration[1]	Molar concentration[2]
DON	0.409 (0.324, 0.518)	----	----
epi-DON	146 (100, 212)	357	357



He, J. and Zhou, T. Unpublished data

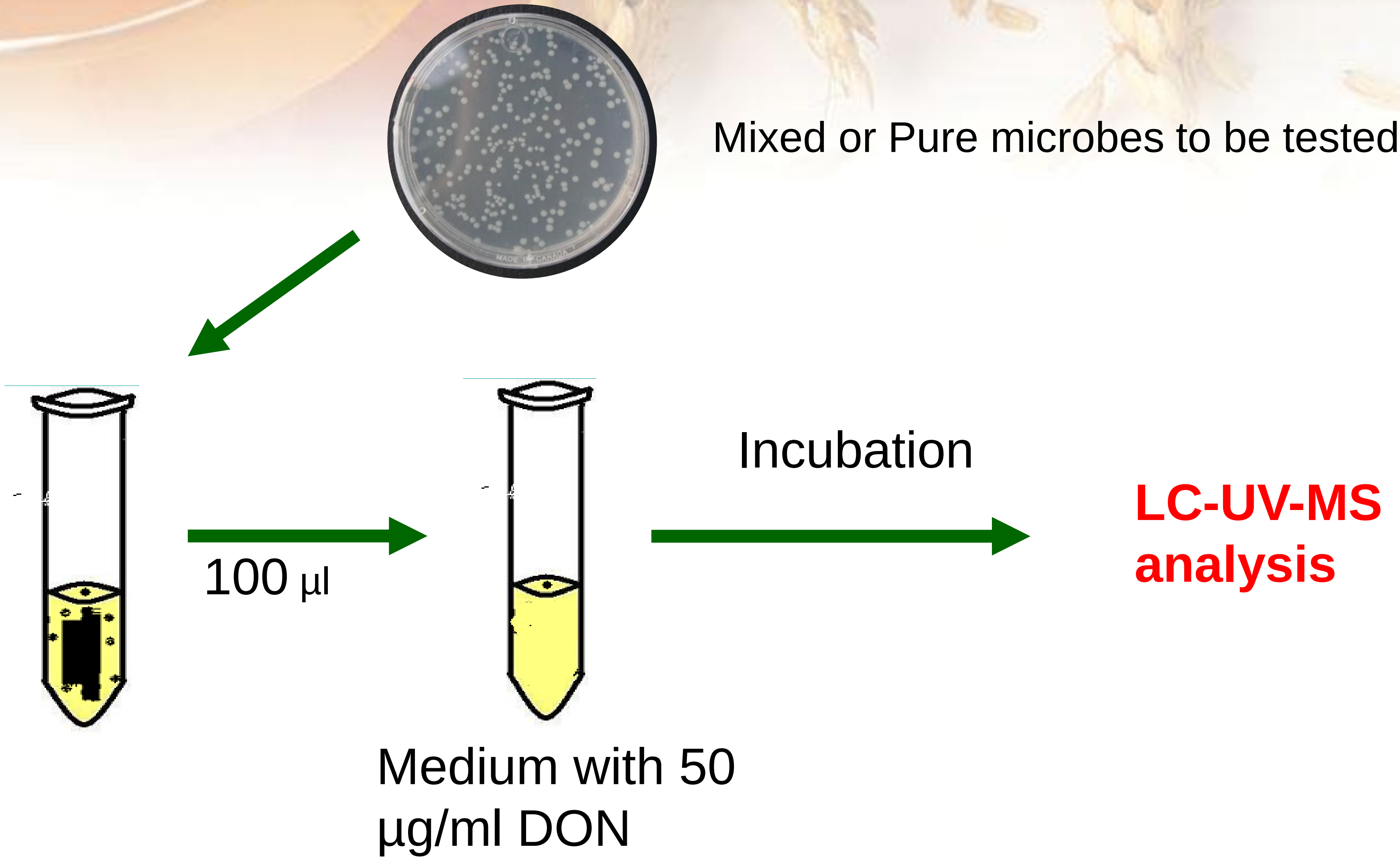
DON detoxifying microorganisms
- Source, efficacy and determining factors

Source of mycotoxin detoxifying microbes - commonly occurring in nature

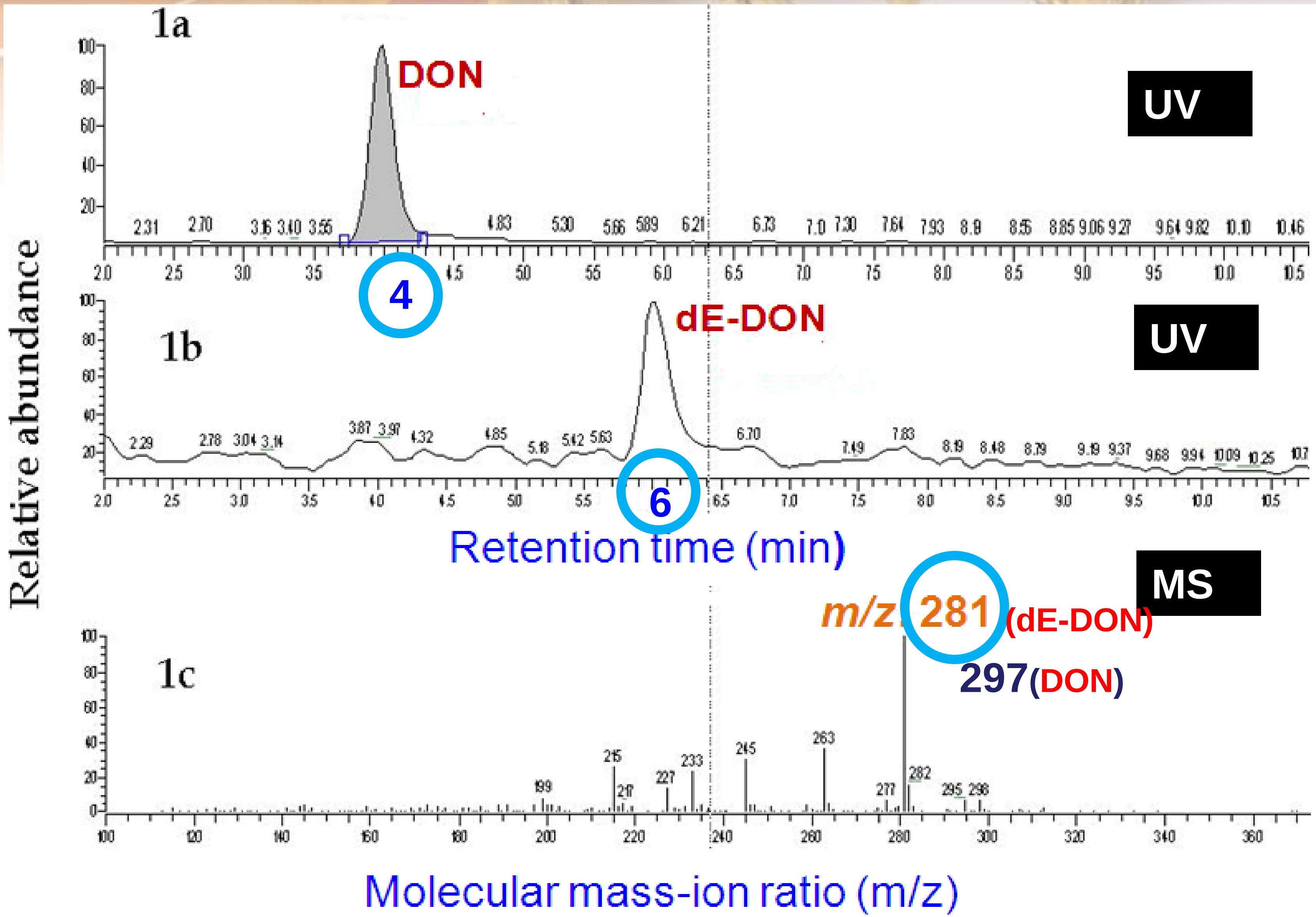
Occurrence /origin	Microorganisms	Transformations
Cow rumen fluid	*Eubacterium* BBSH 797; Rumen microorganisms	Deacetylation; Deepoxidation
Sheep rumen fluid	*Butyrivibrio fibrisolvens, Lactobacillus* sp. ; Rumen bacteria	Deacetylation
Chicken digesta	Gut microbes; *Bacillus* sp. LS100	Deacetylation; Deepoxidation
Rat	Intestinal microorganisms; Faecal microorganisms	Deacetylation; Deepoxidation
Pig	Gut microorganisms	Deepoxidation
Horse	Faecal microorganisms	Deepoxidation
Fish digesta	Intestinal microorganisms	Deacetylation; Deepoxidation
Enhanced soil	*Agrobacterium-Rhizobium* group strain E3-39; *Citrobacter* sp. ADS47; Bacterial strain Barpee	Deepoxidation; oxidation; epimerization
Soil	*Curtobacterium* sp. Strain 114-2	Deacetylation;
Soil /water	Bacterial communities	Deacetylation; Oxidation
Plant pathogen	*Fusarium graminearum; F, nivale; F. solani; F. roseum*	Acetylation; Deacetylation

Zhou, T. et. al 2008 World Mycotoxin Journal 1:23-30

Determination of microbial transformation



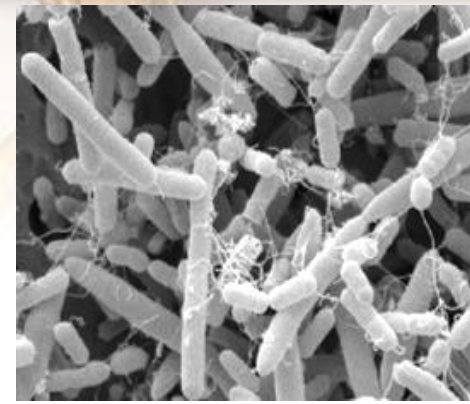
LC-UV-MS for bacterial DON de-epoxydation



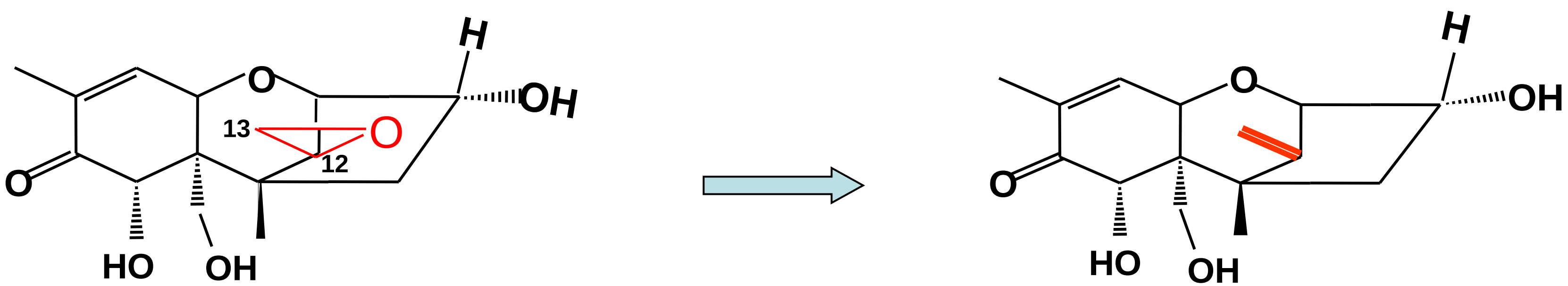
Chicken gut microbes - **De-epoxidation**

Bacillus* sp. *LS100

+ 9 other strains



- Bacterial isolates capable of transforming DON to dE-DON under anaerobic conditions have been identified from chicken digesta (Yu *et al.* 2010)

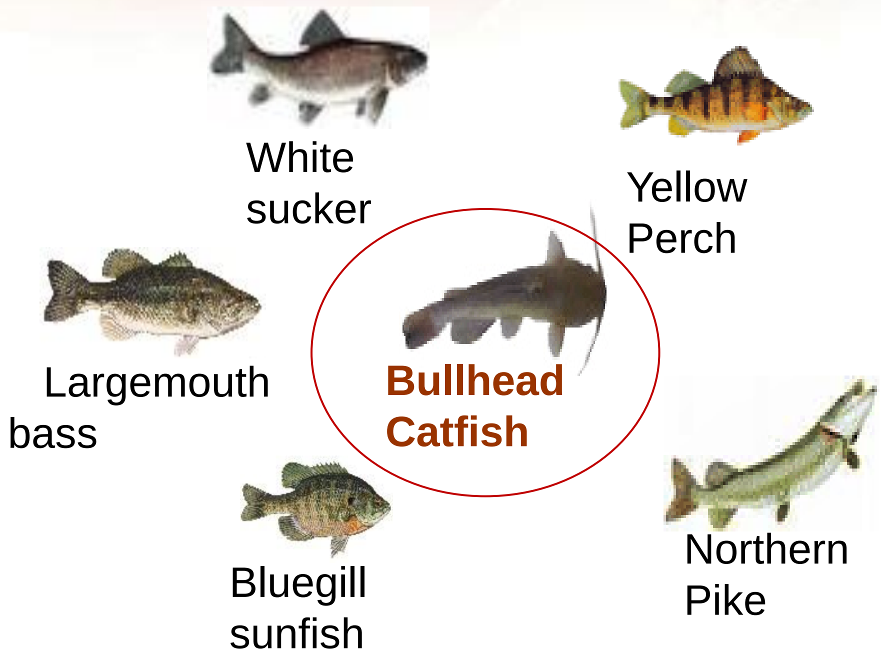


deoxynivalenol (DON)

deepoxy DON (dE-DON, DOM-1)

C133 - Fish gut microbes - **De-epoxidation**

C133

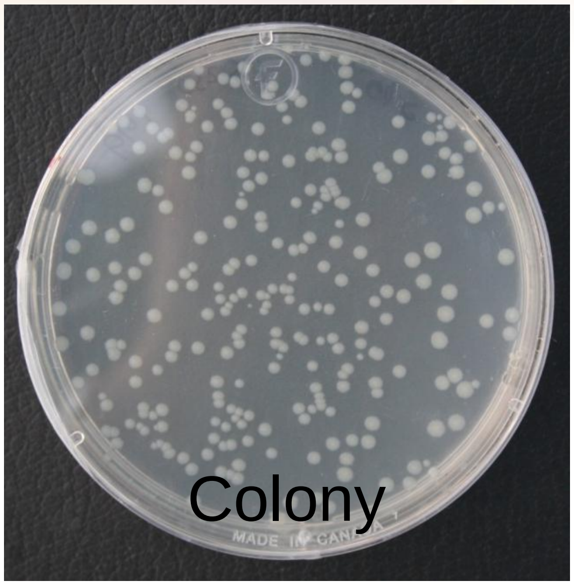


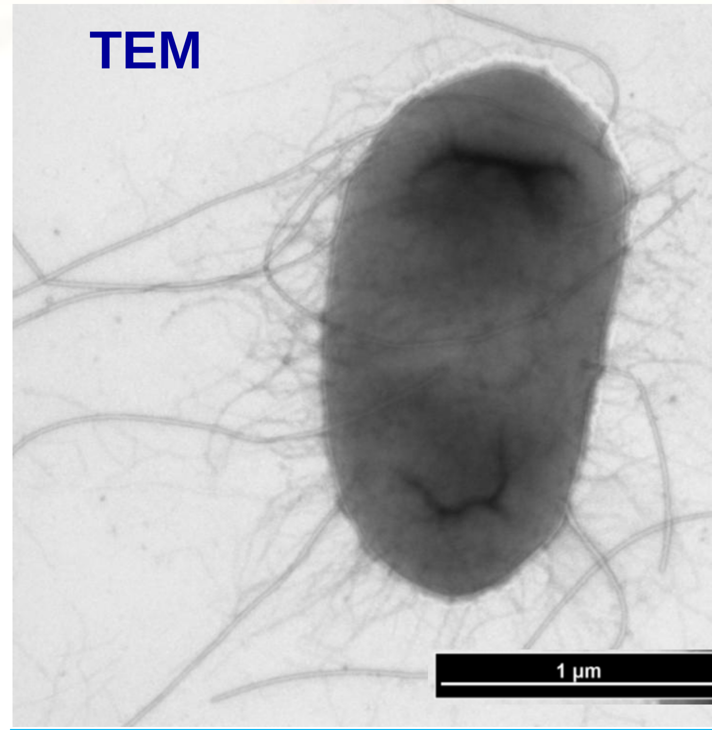
- Microbial community and bacterial isolates capable of transforming DON to deepoxy DON under anaerobic and aerobic conditions have also been identified from fish digesta (Guan, S. *et al.* 2009).

Soil microbe - **De-epoxidation**

Citrobacter **sp. ADS47**

Deepoxidation under both aerobic and anerobic conditions





- Gram negative
- Facultatively anaerobic
- γ proteobacteria

Size: 1.52 x 0.72 µm

Islam, R. *et al.* 2011

Unidentified bacterium from soil-**Epimerization**

Barpee

Bacterial identification by:

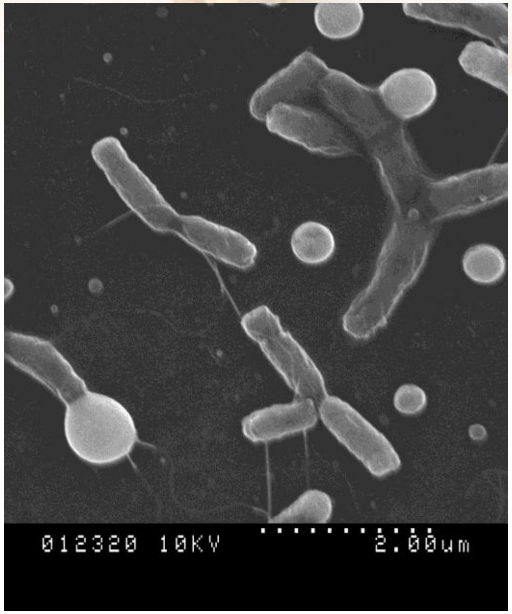
- Gas chromatographic analysis of fatty

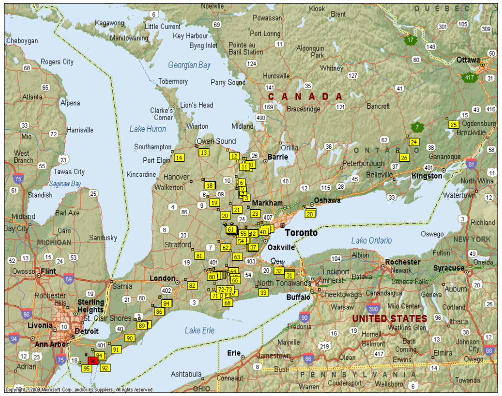
acids methyl esters (GC-FAME)

- Biolog bacterial identification
- 16S rRNA gene sequencing method
- Morphological characterization by

scanning electron microscope (SEM) and transmission electron microscope (TEM)

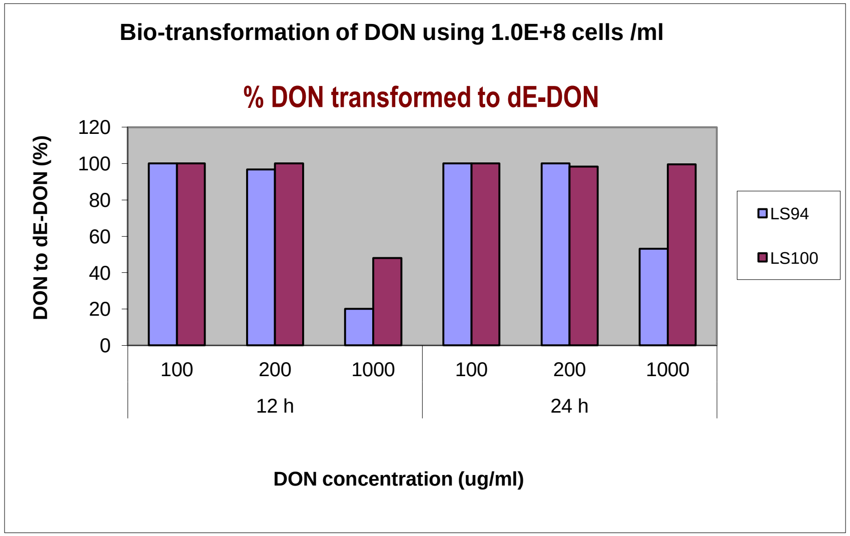
No matched species was found.



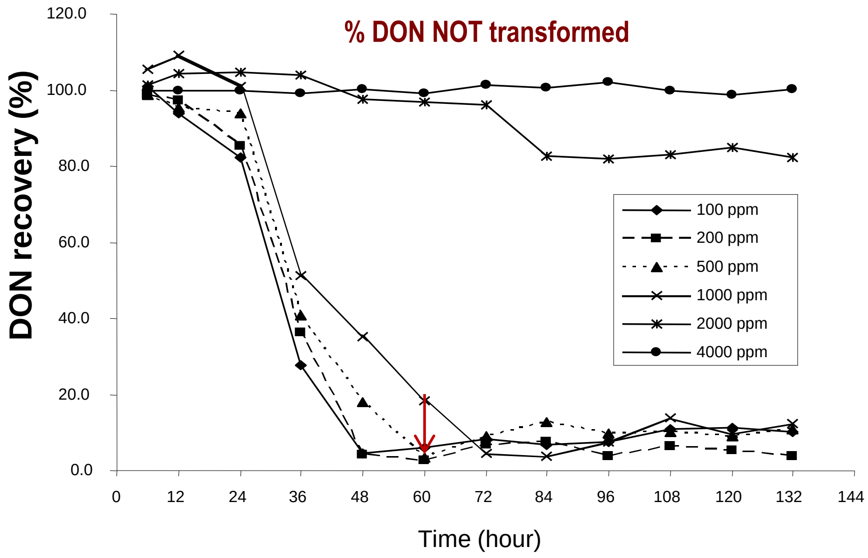


Efficiency of DON detoxifying microbes

Isolates	Efficiency (DON transformation µg / hour / 10^6 cells)	
LS100	8.30	(DON to dE-DON)
ADS47	0.12	(DON to dE-DON)
Barpee	1.39	(DON to 3-epi-DON)



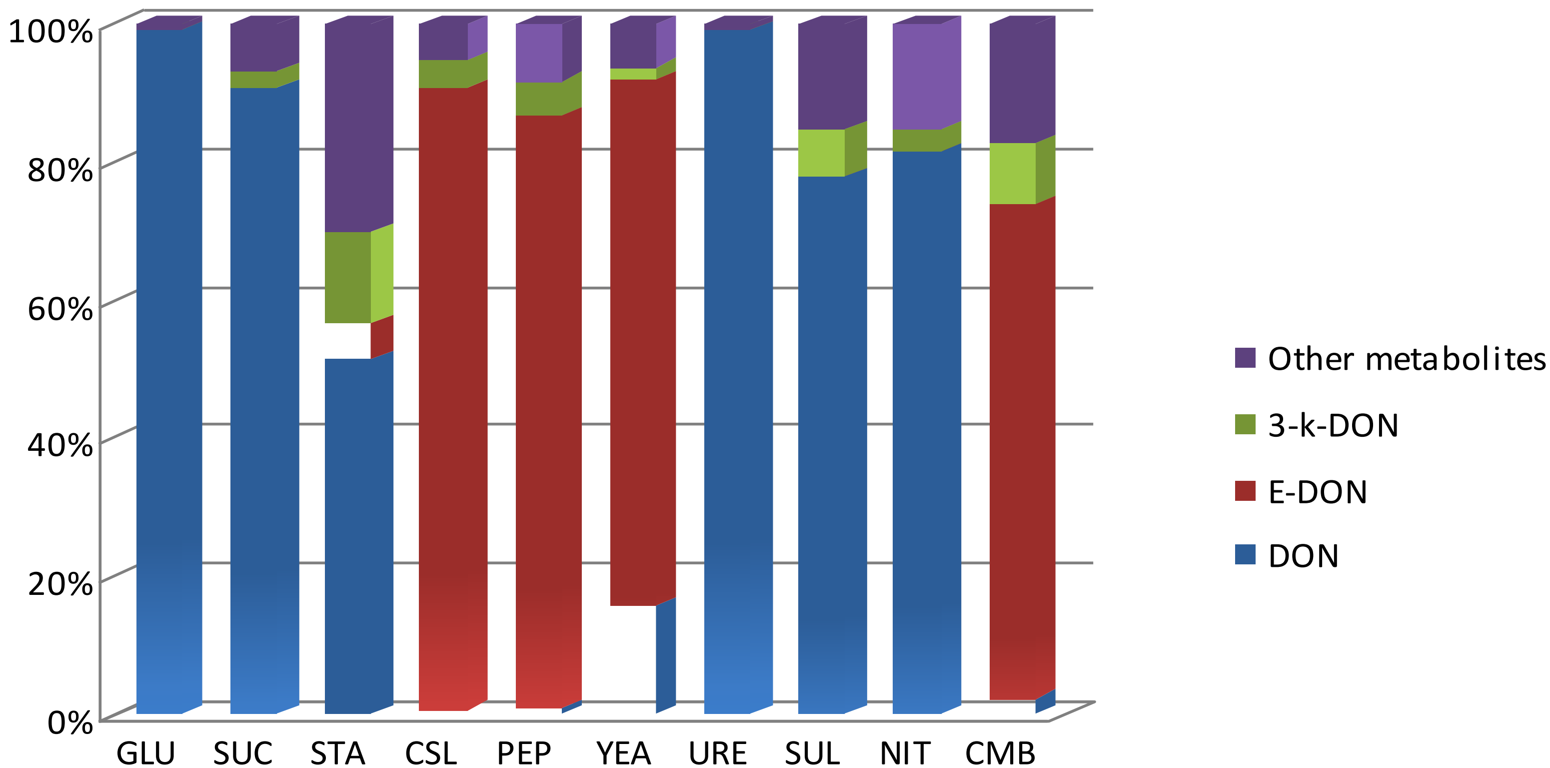
LS100



Barpee

Nutrients (media) for detoxification

Detoxification of DON by bacterial strain Barpee

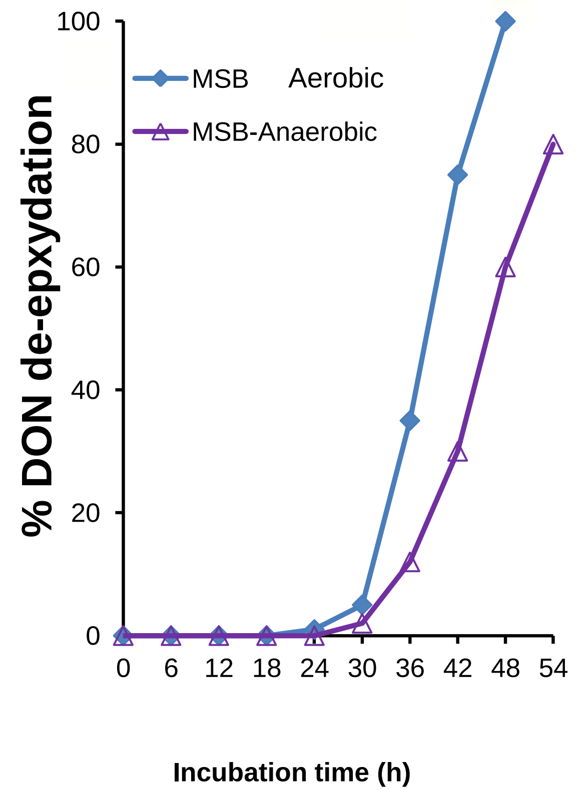


Glucose (GLU); Sucrose (SUC); Corn starch (STA); Corn steep liquor (CSL); Peptone (PEP); Yeast extract (YEA); Urea (URE); Ammonium sulphate (SUL); Ammonium nitrate (NIT); Corn meal broth (CMB)

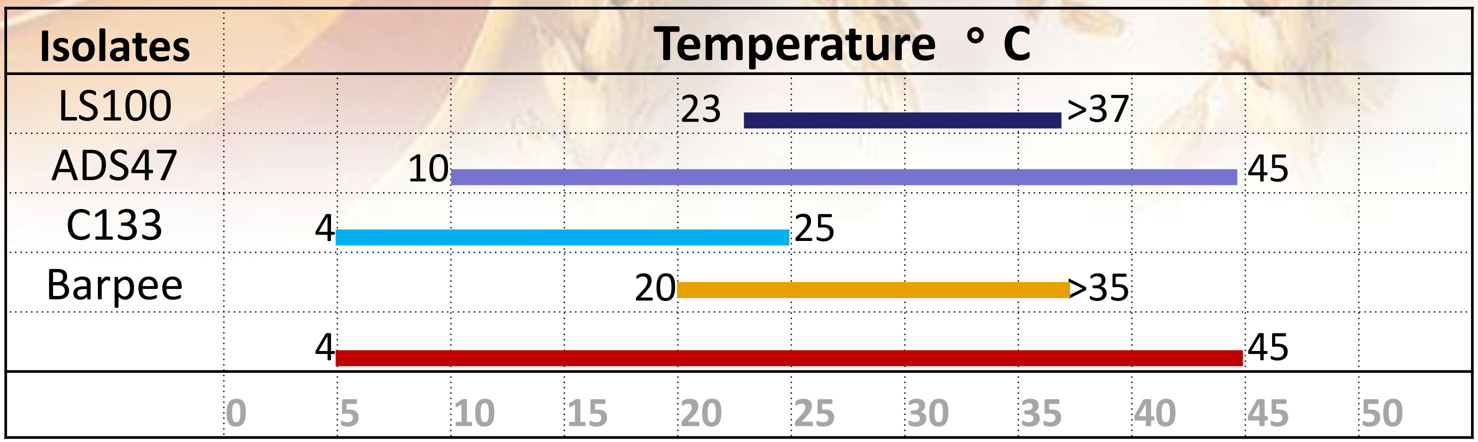
Aerobic vs Anaerobic conditions for detoxification

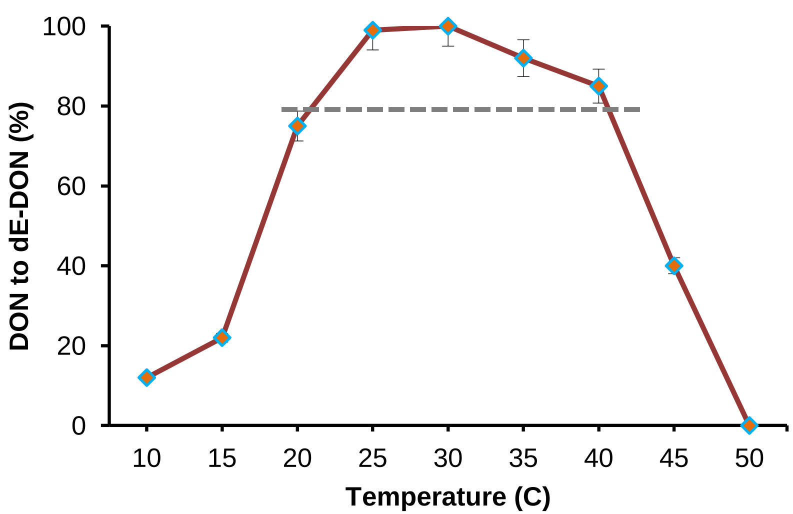
Microbial Isolates	Detoxification	Aerobic	Anaerobic
***Anaerofilum* sp. LS72**	**Deepoxidation**		√
***Bacillus* sp.LS100**	**Deepoxidation**		√
Clostidiales SS-3	**Deepoxidation**		√
***Coriobacterium* sp. LS117**	**Deepoxidation**		√
C133 (mixture from fish)	**Deepoxidation**	√	√
***Citrobacter* sp. ADS47**	**Deepoxidation**	√	√
Barpee (Bacterial strain from soil)	**Epimerization/ oxidation**	√	

Citrobacter sp. ADS47

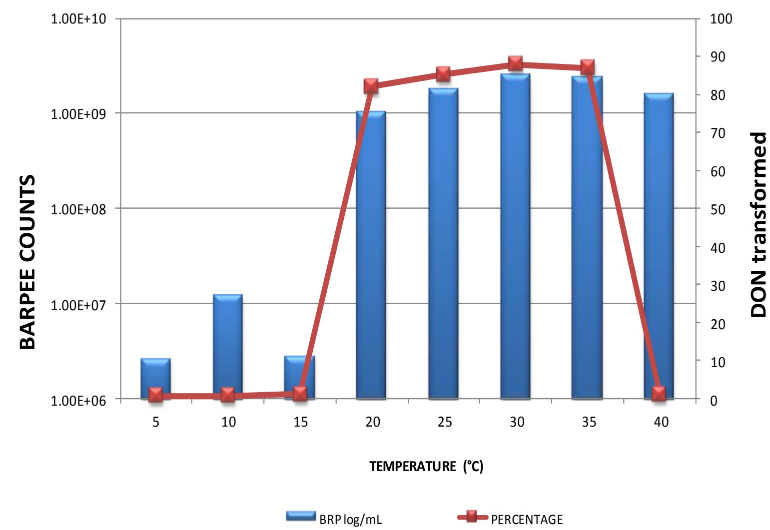


Temperature for DON detoxifying microbes



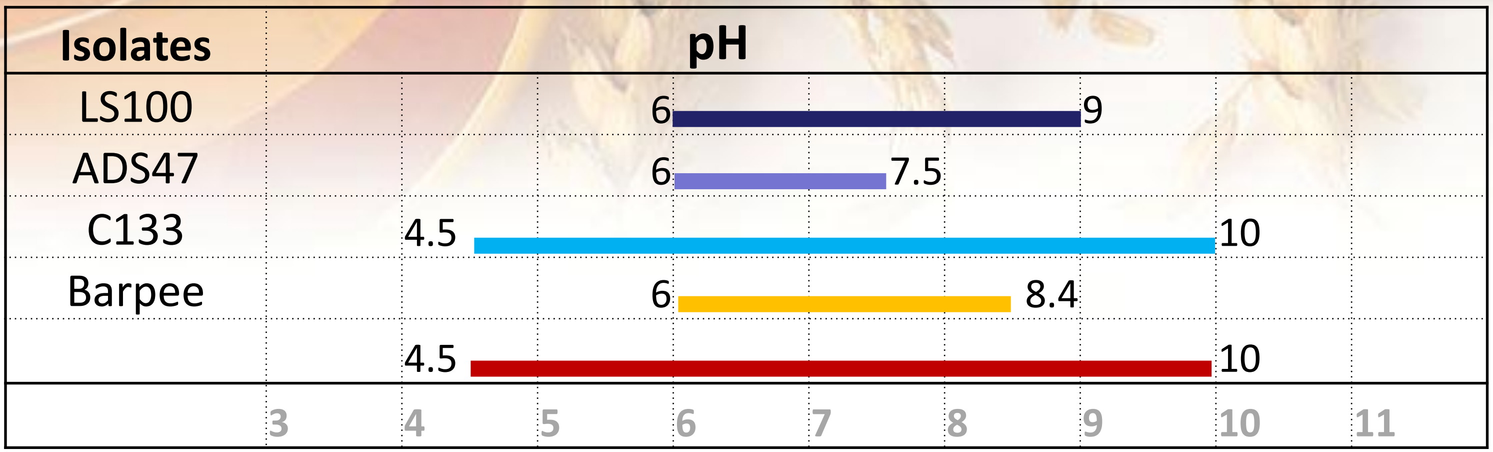


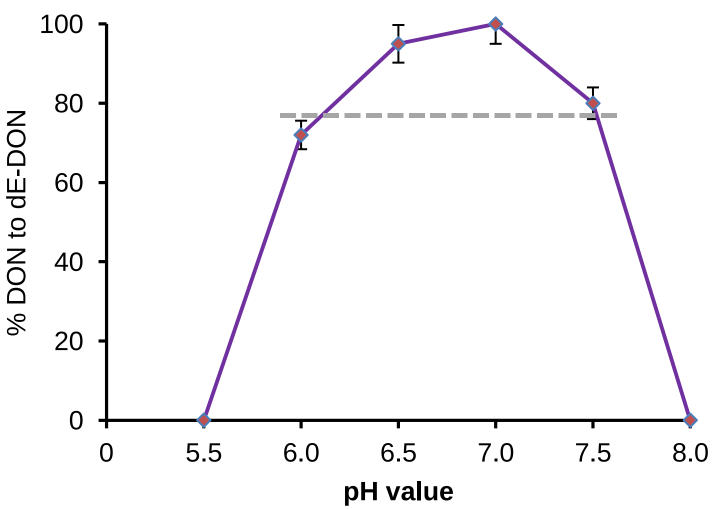
ADS47



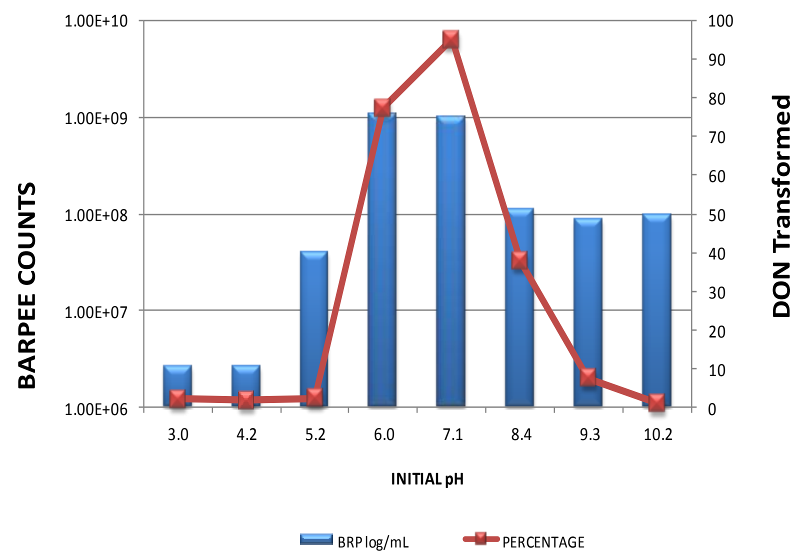
Barpee

pH for DON detoxifying microbes





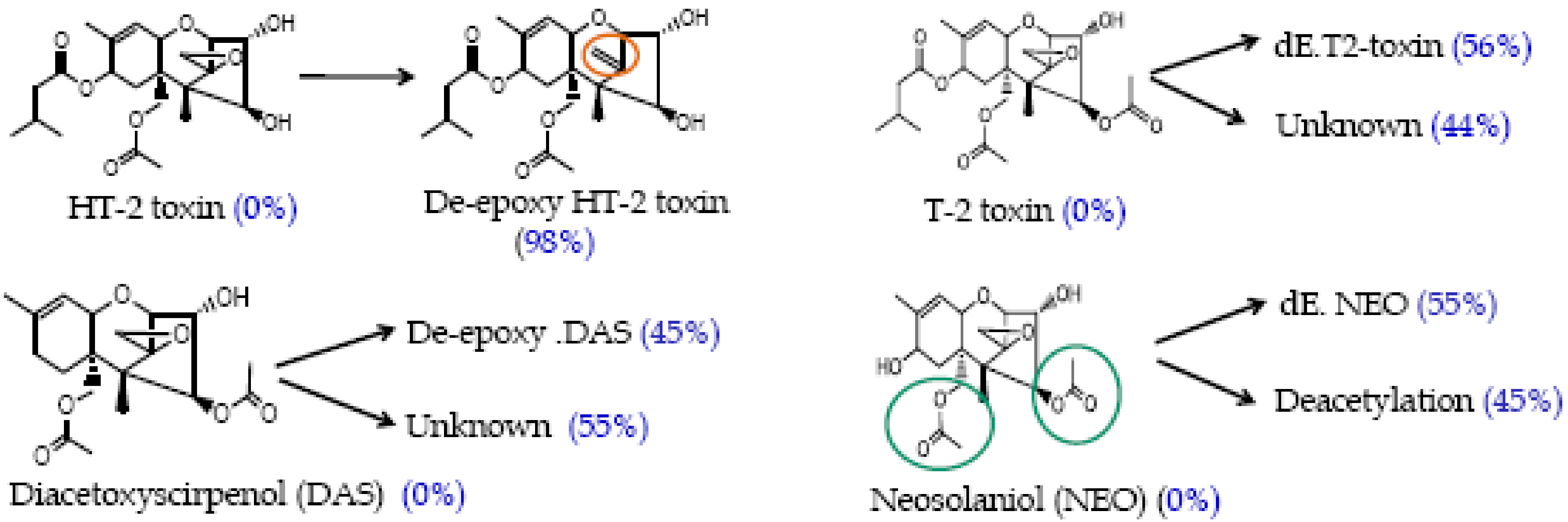
ADS47



Barpee

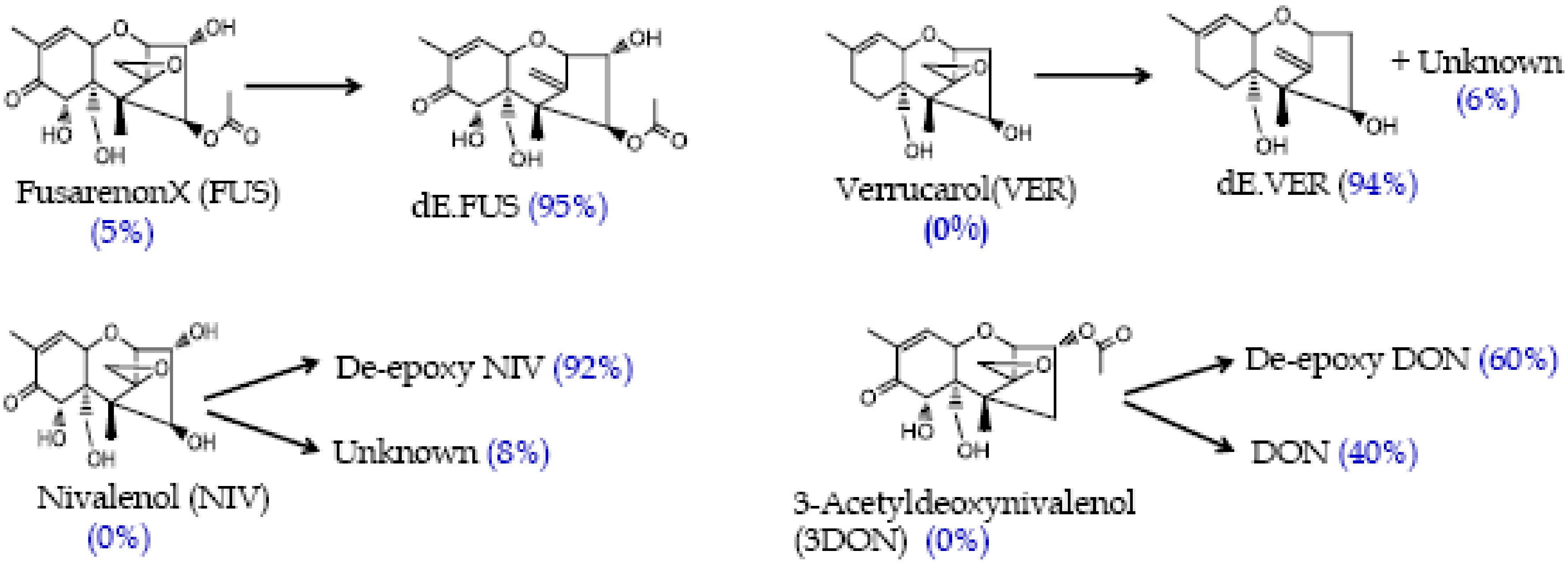
Detoxifying multiple mycotoxins

Detoxification of type-A trichothecene mycotoxins by ***Citrobacter* sp. ADS47**



Detoxifying multiple mycotoxins

Detoxification of type-B trichothecene mycotoxins by *Citrobacter* sp. ADS47



Applications of DON detoxifying microorganisms

Application of microbial detoxification in food chain

Expression of trichothecene-O-acetylase in plants

- Gene *Tri*101 encoding trichothecene-3-O-acetyltransferase from *F. graminearum* and *F. sporotrichioides* was cloned and the three-dimensional structures and kinetic properties of the enzyme have been studied in detail
- The gene has been expressed in several plant species and resulted in very limited success

Plant	Source of the gene	Results	Reference
Tobacco	*F. sporotrichioides*	Increased tolerance to trichothecenes	Muhitch et al. 2000
Wheat	*F. sporotrichioides*	Moderate tolerance to infection	Okubara et al. 2002
Arabidopsis	*F. sporotrichioides*	Resistance to the trichothecene diacetoxyscirpenol	Hohn et al. 2002
Rice	*F. graminearum*	Low expression, no tolerance to trichothecenes, infection not tested	Higa et al. 2003
Wheat	*F. sporotrichioides*	Field trial destroyed by opponents of GMO technology	Anonymous 2004
Barley	*F. sporotrichioides*	No effect on infection in field trial	Manoharan et al. 2006
Rice	*F. graminearum*	Increased tolerance to DON, tolerance to infection not tested	Ohsato et al. 2007

Karlovsky, P. Appl Microbiol Biotechnol (2011) 91:491–504

Expression of *Tri101* gene in maize

(trichothecenes 3-o-actetyltrasferease – from *Fusarium graminearum*)

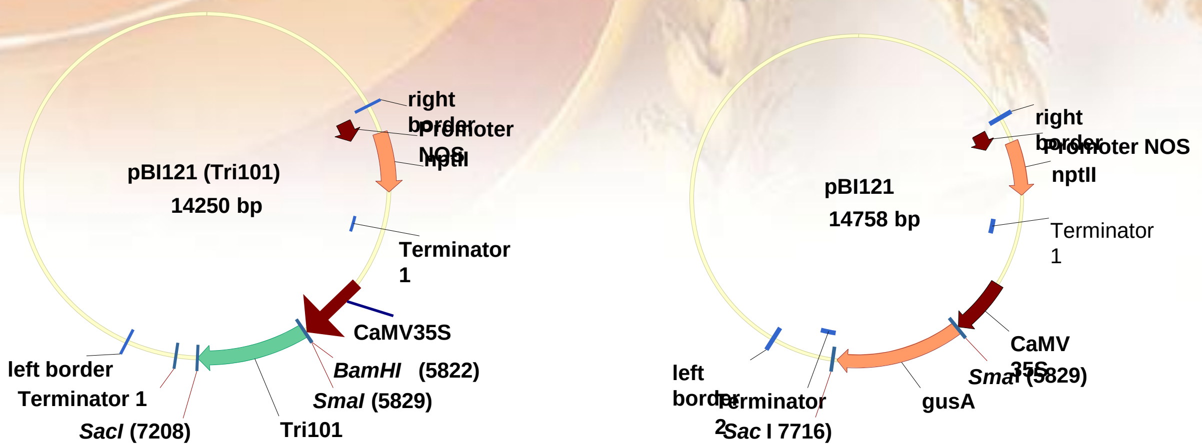
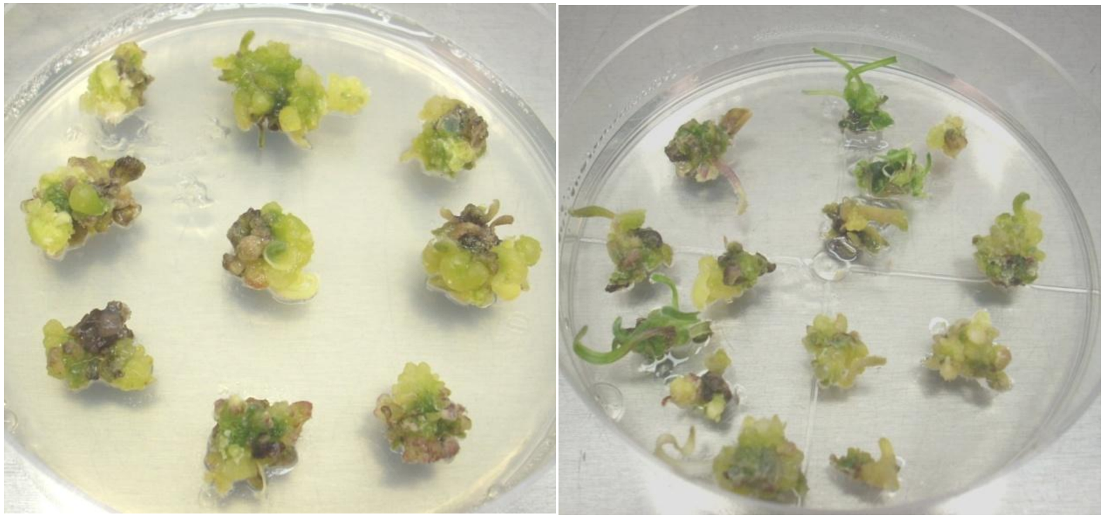


Figure 1. Diagram showing the plant expression vector PBI121 and PBI121-*Tri101*.



Selection medium Regeneration medium

Figure2. Maize multiple shoot clumps transformed with PBI121-Tri101 plasmid.

Pauls, P. and Zhou, T. Project report, 2010

Expression of *Tri101* gene in maize

(trichothecenes 3-o-actetyltrasferease – from *Fusarium graminearum*)

- All tests confirmed that maize transformation with the *F. graminearum Tri*101 gene was successful
- Effects on pathogen infection will be evaluated

T0 plants	Crossed with	PCR positive	southern positive	Western blot leaves	Western blot seeds
Tri101-1	CG65	+	+	+	+
Tri101-3	CG65	+	+	+	ND
Tri101-4	CG65	+	+	+	+
Tri101-5	CG65	+	+	+	ND
Tri101-8	CG65	+	+	+	+
Tri101-10	CG65	+	+	+	+

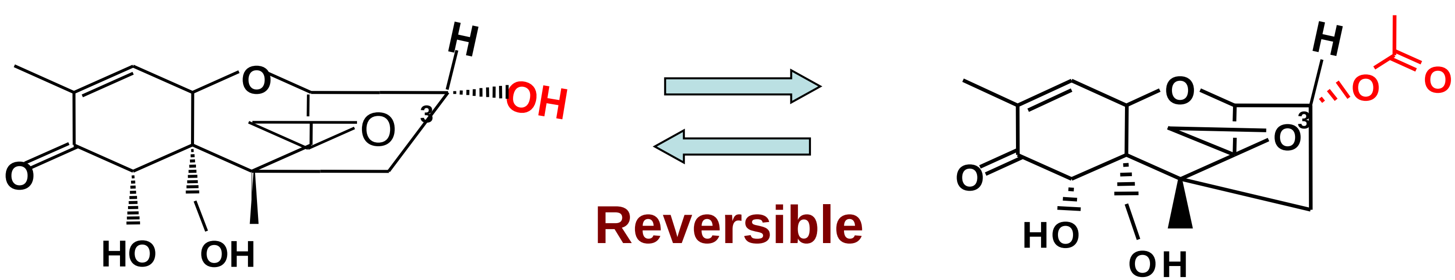
Pauls, P. and Zhou, T. Project report, 2010

Expression of *Tri101* gene in maize

(trichothecenes 3-o-actetyltrasferease – from *Fusarium graminearum*)

- Protein extracts from the transgenic maize tissues, leaves and seeds, transformed DON to 3-ADON, ranged from 24- 44%. No transformation by the WT protein

Protein extracts	DON to 3 ADON Transformation (%)	
	Leaf	**Seed**
Tri101-8-10	30.2	43.9
Tri101-8-2	37.9	42.7
Tri101-8-50	23.9	29.5
WT	0.0	0.0



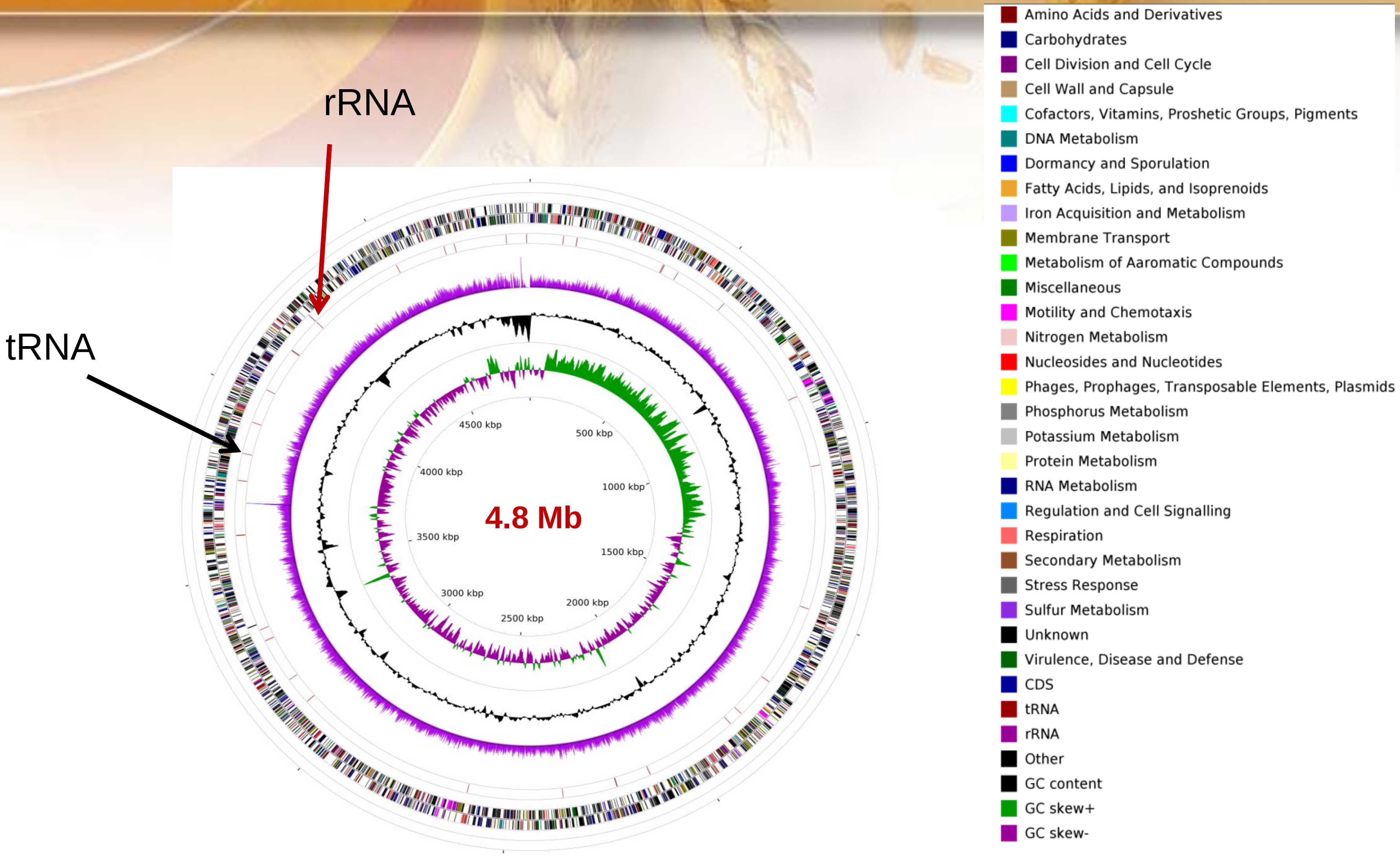
deoxynivalenol (DON) 3-acetyl DON (3ADON)

Isolation of the bacterial mycotoxin detoxifying gene(s)

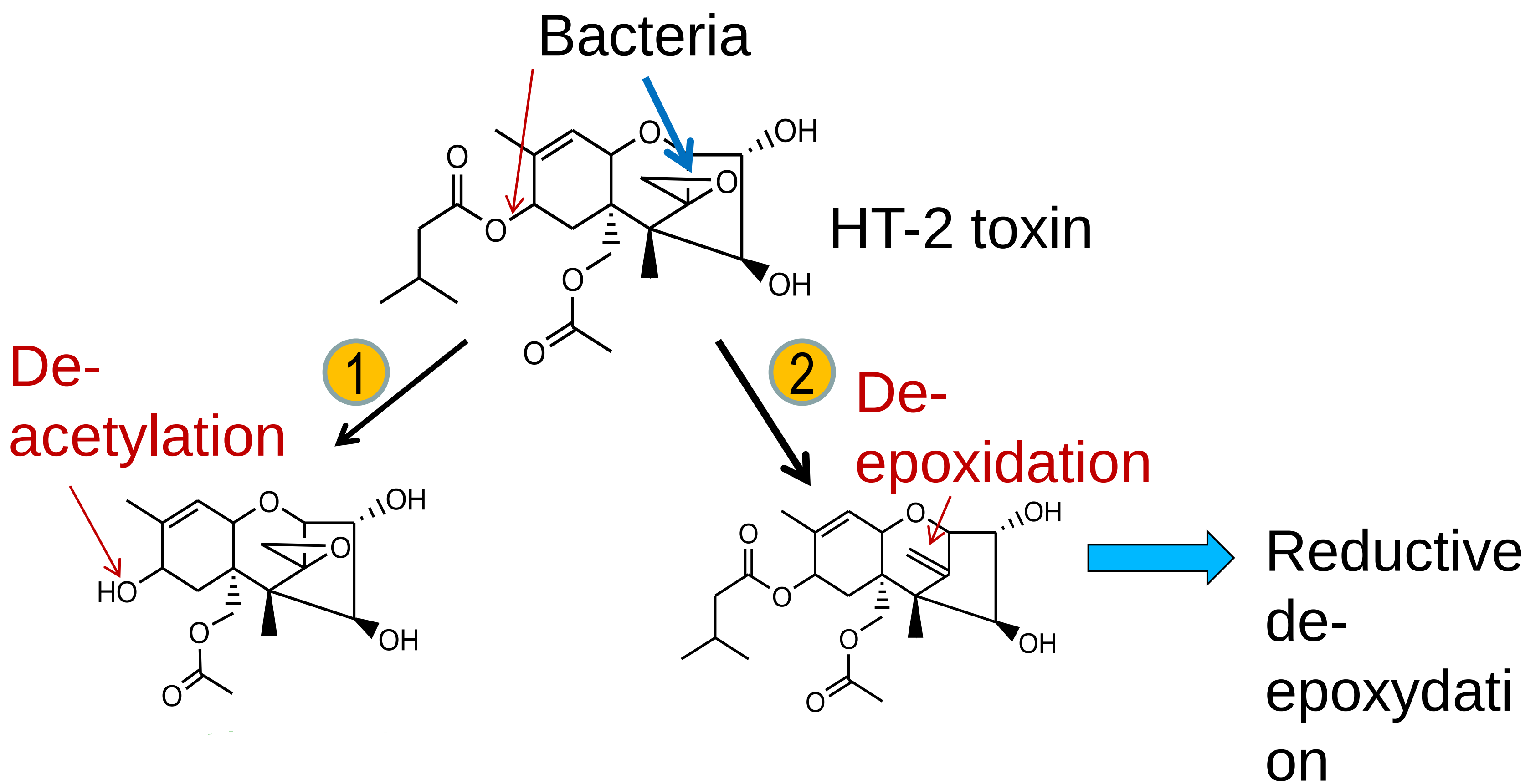
- Optimization of DON acetylases may improve the kinetic properties for the next generation of transgenic crops
- Genes encoding other DON detoxifying enzymes, particular for deepoxidations, should provide new opportunities for FHB resistance breeding and DON reduction
- Currently, we are actively working on identification of gene(s) responsible for DON deepoxidation.
- De-epoxidation results in greater toxicity reduction compared to acetylation, and the reaction is irreversible

- Method-1: Bacterial genomic DNA library preparation and functional screening of the library clones
- Method-2: Sequencing the whole-bacterial genome and identification of potential anti-toxin genes

ADS47 Genome Map developed by CGView software



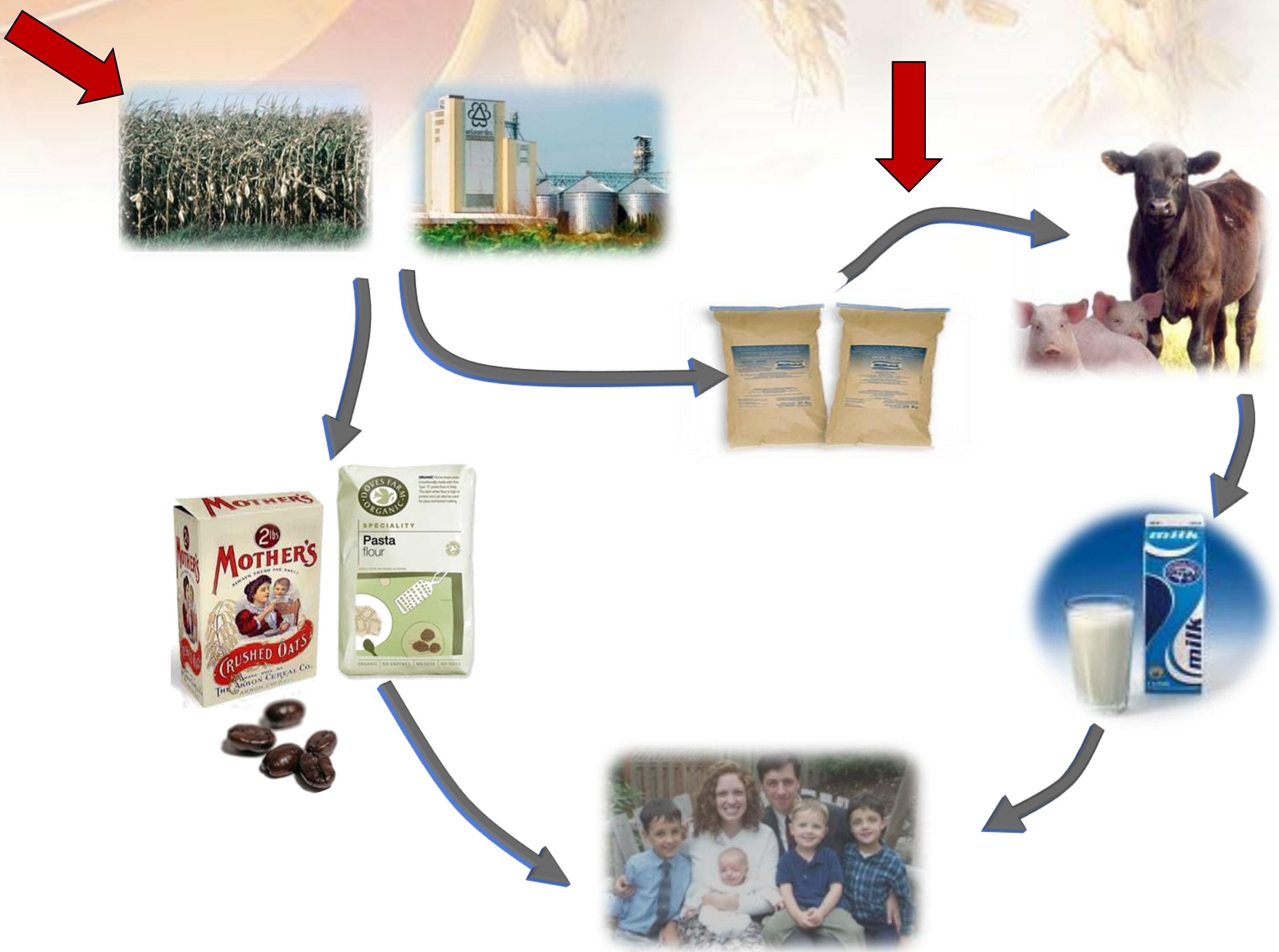
Bacterial mycotoxins detoxification reactions



Identified **unique genes** of ADS47 to predict mycotoxins degrading genes

Unique gene classes	Number
Total unique genes	270
1. Reductases /oxidoreductases	9
2. Deacetylase	1
3. Dehydratase	1
4. Hydrolase	2
5. Transferase	4
6. Sulfatase	3
7. Bacteriocin	2
8. Type VI secretion	2
9. Kinase	4
10. Transporter	9
11. Membrane/exported protein	8
12. Regulator	12
13. Integrase/recombinase	5
14. Phage/prophage/transposae	27
15. Miscellaneous	34
16. Hypothetical protein	147

Application of microbial detoxification in food chain



Application in feed / livestock

Animal feeding

Microorganisms capable of detoxifying mycotoxins

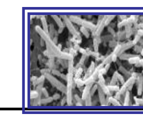
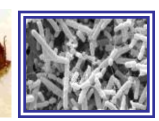
Feed ingredients

Applied as probiotics

Detoxification by fermentation

Detoxification in Pig Trial

Pig starter III (corn and soy) as the base diet

Ingredient	A	B	C	D
Corn				
Fermented Corn				 
Moldy Corn				
Fermented Moldy Corn			 	
DON	0	5 ppm	0	0
dE-DON (DOM-1)	0	0	equivalent 5 ppm	0

Detoxification of DON in Moldy Corn

- Single isolate LS-100 propagated in culture medium at 37°C in anaerobic chamber for 3 days to 10^9
- Moldy corn was autoclaved at 121°C for 15 min
- Inoculation of grounded moldy corn with LS-100 cell culture
- Incubation at 37°C, 72 h in anaerobic chamber (5%H_2 and 95% CO_2)
- Freeze dry the fermented moldy corn
- Analysis the product – DON was transformed to DOM 100%

Li, X-Z, Zhu, C., de Lange, C.F.W., Zhou, T., He, J., Yu, H., Gong, J. and Young, J.C. (2011). Food Additives & Contaminants: Part A, 28:7, 894-901

Demonstration of microbial detoxification in pig trial

Dietary treatments[a]	Calculated[b] DON ($mg\,kg^{-1}$)	Calculated DOM ($mg\,kg^{-1}$)	Analysed DON ($mg\,kg^{-1}$)	Analysed DOM ($mg\,kg^{-1}$)
Non-toxic Corn	0	0	n.d.	n.d.
Toxic Corn	5.0	0	7.6 ± 1.1	n.d.
LS100-De-toxic Corn	0	5.0	n.d.	4.3 ± 3.1
LS100-Non-toxic Corn	0	0	n.d.	n.d.

Growth Performance of Starter Pigs

	A	B	C	D
				
Toxin period (day 9 - 18)				
Adjusted Daily Weight Gain (g/d)	882^{b}	458^{a}	835^{b}	835^{b}
Adjusted Daily Feed Intake	1,337^{b}	943^{a}	1,367^{b}	1,347^{b}
Gain : Feed	0.66^{b}	0.47^{a}	0.62^{b}	0.61^{ab}

Pig Performance

- De-epoxidation by bacterial cells was able to eliminate the adverse effects of mycotoxin DON on swine performance

Microbial detoxification of other ingredients

- Contaminated wheat, barley ……
- By-products from grain processing e.g. Steep water, wheat bran ……
- By-products from Bio-energy industry

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