



Australian Government

Department of Health and Aged Care
Office of the Gene Technology Regulator

Risk Assessment and Risk Management Plan for

DIR 200

Fermentation and processing of recombinant
proteins using genetically modified *Pichia pastoris*

Applicant: Cauldron Molecules Pty Ltd

07 February 2024

PAGE INTENTIONALLY LEFT BLANK

Summary of the Risk Assessment and Risk Management Plan for Licence Application DIR 200

Decision

The Gene Technology Regulator (the Regulator) has decided to issue a licence application to use genetically modified (GM) *Pichia pastoris* (yeast) for precision fermentation to produce bovine milk, chicken egg and spider silk fibre proteins. It qualifies as Dealings involving the Intentional Release (DIR) of genetically modified organisms (GMOs) into the Australian environment under the *Gene Technology Act 2000* (the Act).

The applicant, Cauldron Molecules Pty Ltd (Cauldron) proposes to produce a range of recombinant proteins using various strains of *Komagataella phaffii* (previously known as *Pichia pastoris*¹). *P. pastoris* is a non-pathogenic yeast that is widely used in the biotechnology industry to produce recombinant proteins for pharmaceutical or food enzyme use. The proposed application is to optimise the large-scale fermentation process and characterise GM yeast used to produce recombinant animal proteins from a non-animal source. The GM yeast will incorporate a protein expression cassette to produce a recombinant protein. The incorporated genes will encode for proteins in their native form. The production process will involve fermentation of GM yeast cultures in large volumes (approximately 12,500 L per tank) at Cauldron's purpose-built protein production facility in Borenore, New South Wales. The recombinant proteins will be purified and will not contain any GM yeast.

The Regulator has prepared a Risk Assessment and Risk Management Plan (RARMP) for this application in accordance with the Act and corresponding state and territory legislation, and finalised following consultation with a wide range of experts, agencies and authorities, and the public. The RARMP concludes that the proposed trial poses negligible risks to human health and safety and the environment and that any risks posed by the dealings can be managed by imposing conditions on the release.

¹ Host name *Pichia pastoris* will be used for this application.

The application

Project Title	Fermentation and processing of recombinant proteins using genetically modified <i>Pichia pastoris</i> ²
Parent organism	<i>Pichia pastoris</i>
Genetic modifications	<p>Insertion of expression cassette for producing bovine milk, chicken egg and spider silk fibre proteins. The expression cassette may also contain:</p> <ul style="list-style-type: none"> • antibiotic selectable marker gene that confers resistance to a specific antibiotic to enable selection for the GM yeast • secretion signal peptide to facilitate secretion of proteins • constitutive or inducible promoter to facilitate expression of introduced sequences • tags such as epitope or polyhistidine to detect and purify the recombinant proteins
Principal purpose	To optimise the fermentation process and characterise GM yeast during production of animal proteins.
Previous releases	There has been no previous application in Australia for these GMOs.
Proposed limits and controls	
Proposed duration	5 years
Proposed location	Cauldron facility in Borenore
Proposed controls	<ul style="list-style-type: none"> • Laboratory strains of yeast will be used in the production which require specific media and growth conditions. • Molecular characterisation for multiple generations will be undertaken to assess genetic stability and copy number. • GM yeast will be fully transformed, i.e. vector plasmids will not be present. • Fermentation will take place in closed systems and transfer of fluids will be aseptic. • Transport of viable GM yeast will follow the Regulator's <i>Guidelines for the Transport, Storage and Disposal of GMOs</i>. • Viable GM yeast will be decontaminated via steam sterilisation or chemical disinfection. • Staff handling the GM yeast will undergo licence specific training. • Stringent manufacturing practices and quality control procedures will be followed to ensure that GM yeast does not remain in the environment. • The production facility is located in a region where there are no oak or chestnut trees in the proximity, which are the sources of wild-type <i>P. pastoris</i>. • Only decontaminated and denatured (where antibiotic genes are present) GM yeast slurry may be used for animal feed preparations or as soil conditioner.

² The title of the project as supplied by the applicant is 'Precision fermentation of alternative proteins'.

Risk assessment

The risk assessment process considers how the genetic modifications and proposed activities conducted with the GMO might lead to harm to people or the environment. Risks are characterised in relation to both the seriousness and likelihood of harm, taking into account information in the application (including proposed controls), relevant previous approvals and current scientific/technical knowledge. Both the short- and long-term impacts are considered.

Credible pathways to potential harm that were considered included exposure of people or other organisms to the GM yeast, and the potential for persistence or dispersal of the GM yeast. Potential harms associated with these pathways included allergenicity to people, and environmental harms due to the potential for the GM yeast to spread in the environment.

The risk assessment concludes that the proposed dealings pose negligible risks to human health and safety and the environment. No specific risk treatment measures are required to manage these negligible risks.

The principal reasons for the conclusion of negligible risks are that the viable GM yeast will not be used for human food or animal feed, and that the proposed limits and controls will effectively minimise exposure to and dispersal of the GM yeast.

Risk management

The risk management plan describes measures to protect the health and safety of people and to protect the environment by controlling or mitigating risk. The risk management plan is given effect through licence conditions.

As the level of risk is considered negligible, specific risk treatment is not required. However, since this is a limited and controlled release, the licence includes limits on the location and duration of the release. Controls are included to prohibit the use of the viable GM yeast in human food and animal feed, to minimise dispersal of the GM yeast from the production facility, to transport GM yeast in accordance with the Regulator's guidelines, and to destroy GM yeast at the end of the protein production process.

In addition, there are several general conditions relating to ongoing licence holder suitability, auditing and monitoring, and reporting requirements which include an obligation to report any unintended effects.

Table of contents

SUMMARY OF THE RISK ASSESSMENT AND RISK MANAGEMENT PLAN	I
DECISION	I
THE APPLICATION	II
RISK ASSESSMENT	III
RISK MANAGEMENT	III
TABLE OF CONTENTS	IV
ABBREVIATIONS	VI
CHAPTER 1 RISK ASSESSMENT CONTEXT	1
SECTION 1 BACKGROUND	1
1.1 Interface with other regulatory schemes	2
SECTION 2 THE PROPOSED DEALINGS	2
2.1 Proposed limits of the dealings (duration, location, scale, people)	2
2.2 Proposed controls to restrict spread and persistence of the GMO in the environment	3
2.3 Details of the proposed dealings	3
SECTION 3 PARENT ORGANISM	7
3.1 Classification	7
3.2 Characteristics	7
3.3 Genome	8
3.4 Life cycle	8
3.5 Pathogenicity and toxigenicity.....	8
3.6 Reservoir	8
3.7 Environmental stability and decontamination	8
3.8 Risk Group and containment	9
3.9 Properties of <i>P. pastoris</i> expression system	9
SECTION 4 THE GMO - NATURE AND EFFECT OF THE GENETIC MODIFICATION	10
4.1 Development of GM yeast.....	10
4.2 Expected properties of the GM yeast.....	17
SECTION 5 THE RECEIVING ENVIRONMENT	17
5.1 Presence of related fungal species in the receiving environment	17
5.2 Presence of similar genes or products in the environment	17
SECTION 6 PREVIOUS AUTHORISATIONS.....	18
CHAPTER 2 RISK ASSESSMENT	19
SECTION 1 INTRODUCTION	19
SECTION 2 RISK IDENTIFICATION	20
2.1 Risk source	20
2.2 Causal pathway.....	21
2.3 Potential harms.....	22
2.4 Postulated risk scenarios	22
2.5 Risk scenario 1	25
2.6 Risk scenario 2	27
SECTION 3 UNCERTAINTY	28
SECTION 4 RISK EVALUATION	29
CHAPTER 3 RISK MANAGEMENT PLAN	31
SECTION 1 BACKGROUND.....	31
SECTION 2 RISK TREATMENT MEASURES FOR SUBSTANTIVE RISKS	31
SECTION 3 GENERAL RISK MANAGEMENT	31

Limits and controls on the release31

Other risk management considerations33

SECTION 4 ISSUES TO BE ADDRESSED FOR FUTURE RELEASES.....35

SECTION 5 CONCLUSIONS OF THE CONSULTATION RARMP.....35

REFERENCES.....36

APPENDIX A: SUMMARY OF SUBMISSIONS FROM PRESCRIBED AGENCIES ON THE CONSULTATION RARMP.....41

APPENDIX B: SUMMARY OF SUBMISSIONS FROM THE PUBLIC ON THE CONSULTATION RARMP44

Abbreviations

CMP	Cow milk protein
CMPA	Cow's milk protein allergy
DAFF	Department of Agriculture, Fisheries and Forestry
DIR	Dealings involving Intentional Release
DNA	Deoxyribonucleic acid
EFSA	The European Food Safety Authority
EWP	Egg white protein
FDA	Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
GM	Genetically modified
GM yeast	GM <i>P. pastoris</i>
GMO	Genetically modified organism
GRAS	Generally Recognised as Safe
GTTAC	Gene Technology Technical Advisory Committee
HGT	Horizontal gene transfer
IATA	International Air Transport Association
IBC	Institutional Biosafety Committee
kDa	Kilodalton
kL	Kilolitre
L	Litre
OGTR	Office of the Gene Technology Regulator
ppm	Part per million
Production facility	Non OGTR certified area of the Cauldron's purpose-built protein facility
RAF	Risk Assessment Framework
RARMP	Risk Assessment and Risk Management Plan
the Act	The <i>Gene Technology Act 2000</i>
the Regulations	The Gene Technology Regulations 2001
the Regulator	The Gene Technology Regulator
TSD guidelines	Regulator's <i>Guidelines for Transport, Storage and Disposal of GMOs</i>
US	The United States

Chapter 1 Risk assessment context

Section 1 Background

1. An application has been made under the *Gene Technology Act 2000* (the Act) for Dealings involving the Intentional Release (DIR) of genetically modified organisms (GMOs) into the Australian environment.
2. The Act and the Gene Technology Regulations 2001 (the Regulations), together with corresponding State and Territory legislation, comprise Australia's national regulatory system for gene technology. Its objective is to protect the health and safety of people, and to protect the environment, by identifying risks posed by or as a result of gene technology, and by managing those risks through regulating certain dealings with GMOs.
3. Section 50 of the Act requires that the Gene Technology Regulator (the Regulator) must prepare a Risk Assessment and Risk Management Plan (RARMP) in response to an application for release of GMOs into the Australian environment. Sections 50, 50A and 51 of the Act and sections 9 and 10 of the Regulations outline the matters which the Regulator must take into account and who must be consulted when preparing the RARMP.
4. The *Risk Analysis Framework* (RAF) (OGTR, 2013) explains the Regulator's approach to the preparation of RARMPs in accordance with the Act and the Regulations. The Regulator has also developed operational policies and guidelines that are relevant to DIR licences. These documents are available from the Office of the Gene Technology Regulator ([OGTR website](#)).
5. Figure 1 shows the information that is considered, within the regulatory framework, in establishing the risk assessment context. This information is specific for each application. Risks to the health and safety of people or the environment posed by the proposed dealings are assessed within this context. Chapter 1 describes the risk assessment context for this application.

RISK ASSESSMENT CONTEXT	
The GMO Modified genes Novel traits	Proposed GMO dealings Activities Limits Controls
Parent organism (comparator) Origin and taxonomy Cultivation and use Biology	Previous releases Australian approvals International approvals
Receiving environment Environmental conditions: abiotic and biotic factors Production practices Related organisms Similar genes and proteins	

Figure 1 Summary of parameters used to establish the risk assessment context, within the legislative requirements, operational policies and guidelines of the OGTR and the RAF.

6. In accordance with Section 50A of the Act, this application is considered to be a limited and controlled release application, as the Regulator was satisfied that it meets the criteria prescribed by the Act. Therefore, the Regulator was not required to consult with prescribed experts, agencies and authorities before preparation of the RARMP.

7. Section 52 of the Act requires the Regulator to seek comment on the consultation RARMP from agencies - the Gene Technology Technical Advisory Committee, State and Territory Governments, Australian Government authorities or agencies prescribed in the Regulations, Australian local councils and the Minister for the Environment - and from the public. The advice from the prescribed experts, agencies and authorities and how it was taken into account is summarised in Appendix A. Ten public submissions were received and their consideration is summarised in Appendix B.

1.1 Interface with other regulatory schemes

8. Gene technology legislation operates in conjunction with other regulatory schemes in Australia. The GMOs and any proposed dealings conducted under a licence issued by the Regulator may also be subject to regulation by other Australian government agencies that regulate GMOs or GM products, including Food Standards Australia New Zealand (FSANZ), the Australian Pesticides and Veterinary Medicines Authority, the Therapeutic Goods Administration, the Australian Industrial Chemicals Introduction Scheme and the Department of Agriculture, Fisheries and Forestry (DAFF).

9. The DAFF regulates products imported into Australia to protect Australia from biosecurity risks. Under the *Biosecurity Act 2015*, the importation of biological material such as live GMOs requires a permit from the DAFF.

Section 2 The proposed dealings

10. Cauldron Molecules Pty Ltd (Cauldron) is seeking authorisation to carry out precision fermentation using genetically modified (GM) *Pichia pastoris* (referred to as GM yeast from hereon) for production of animal proteins. The overall aim of the project is to establish culture conditions and the fermentation process using GM yeast to produce three different classes of proteins at volumes greater than 100 L. For this, the applicant will:

- establish culture conditions to produce various proteins (bovine milk, chicken egg and spider silk fibre) either in a batch or continuous culture; and
- characterise the GM yeast during culture.

11. The dealings involved in the proposed protein production process are to:

- (a) grow, raise or culture the GMO;
- (b) use the GMO in the course of manufacture of a thing that is not the GMO:
 - i. to produce recombinant proteins for analyses;
- (c) conduct the following experiments with the GMO:
 - i. to optimise the scale-up fermentation process; and
 - ii. to characterise GM yeast;
- (d) transport the GMO;
- (e) dispose of the GMO;

and the possession (including storage), supply and use of the GMO for the purposes of, or in the course of, any of these dealings.

2.1 Proposed limits of the dealings (duration, location, scale, people)

12. The protein production process is proposed to take place over a five-year period from the date of issue of the licence.

13. The protein production process will involve fermentation of GM yeast cultures in large volumes (approximately 6,500 L to 12,500 L per tank) at Cauldron's purpose-built protein production facility ('production facility' will be used from hereon) in Borenore, New South Wales. The applicant is

proposing to run eight single batch cultures and eight continuous flow cultures. A total volume of approximately 504 kL will be cultured per annum.

14. Only trained personnel will conduct dealings with the GM yeast.

2.2 Proposed controls to restrict spread and persistence of the GMO in the environment

15. The applicant has proposed a number of controls to restrict the spread and persistence of the GMO in the environment. These include:

- Laboratory strains of yeast will be used in the production which require specific media and growth conditions.
- Molecular characterisation for multiple generations will be undertaken to assess genetic stability and copy number.
- GM yeast will be fully transformed, i.e. vector plasmids will not be present.
- Fermentation will take place in closed systems and transfer of fluids will be aseptic.
- Transport of viable GM yeast will follow the Regulator's *Guidelines for the Transport, Storage and Disposal of GMOs*.
- Viable GM yeast will be decontaminated via steam sterilisation or chemical disinfection.
- Staff handling the GM yeast will undergo licence specific training and wear appropriate personal protective equipment (PPE) including coveralls, P2 mask and gloves, when required.
- Stringent manufacturing practices and quality control procedures will be followed to ensure that GM yeast does not remain in the environment.
- The production facility is located in a region where there are no oak or chestnut trees in proximity, which are the sources of wild-type *P. pastoris*.
- Only decontaminated³ and, where antibiotic genes are present, denatured⁴ GM yeast slurry may be used for animal feed preparations.

2.3 Details of the proposed dealings

16. The protein production process involving culture of GM yeast in volumes of no more than 25 L per vessel is classified as an exempt dealing (Schedule 2, Item 4 of the Regulations). Culture of GM yeast in volumes of more than 25 L per vessel in a OGTR certified PC2 Large Scale Facility (PC2 LS facility) will be covered under a notifiable low risk dealing (NLRD) authorisation (Schedule 3, Part 2, 2.1(f) of the Regulations). This DIR application is for a protein production process where more than 25 L per vessel of GM culture will be produced in a non-OGTR certified facility ("the production facility").

17. The GM yeast will be either manufactured overseas or within Australia by applicant's clients and will be supplied or imported in a purified form as a dry powder or in the form of a stab culture. This work will be regulated as an exempt dealing or a NLRD authorisation. Under these authorisations, frozen cells will be recovered from the purified powder or stab culture by culturing the GM yeast in media followed by seeding into 500 ml flasks. These cultures will be subsequently expanded to a 90 L culture in a 150 L vessel. During the culture expansion, the GM yeast will be

³ Decontaminate means a physical and/or chemical process which removes, kills or renders non-viable the GMOs used in the facility but does not necessarily result in sterility.

⁴ Denature means, a physical and/or chemical process which renders, in the context of genes, DNA and its proteins non-functional.

verified for integration of the gene expression cassettes and for the absence of plasmid sequences. The GM yeast will be characterised by repeat sequencing of approximately 12% of the genome covering the integration site at regular intervals to assess genetic stability over 50 generations. Finally, the production strains will be sequenced to characterise the exact copy number and the insertional location.

18. The GM yeast will be stored at the PC2 LS facility in liquid nitrogen. The cultures would be handled/transported in accordance with the Regulator's *Guidelines for Transport, Storage and Disposal of GMOs*.

2.3.1 Protein production process

19. The proposed protein production process will be conducted in three sequential parts:

1. Validation of the GM yeast during production;
2. Protein expression in fermentation culture tanks (fermenters); and
3. Purification of the expressed protein.

20. Once, the GM yeast is characterised (paragraph 17), the 90 L culture volume with a desired cell density and purity will be inoculated into a 6.5 kL of culture in a 10 kL fermenter tank outside the PC2 LS facility and then to 12.5 kL maturation vessels (Figure 2).

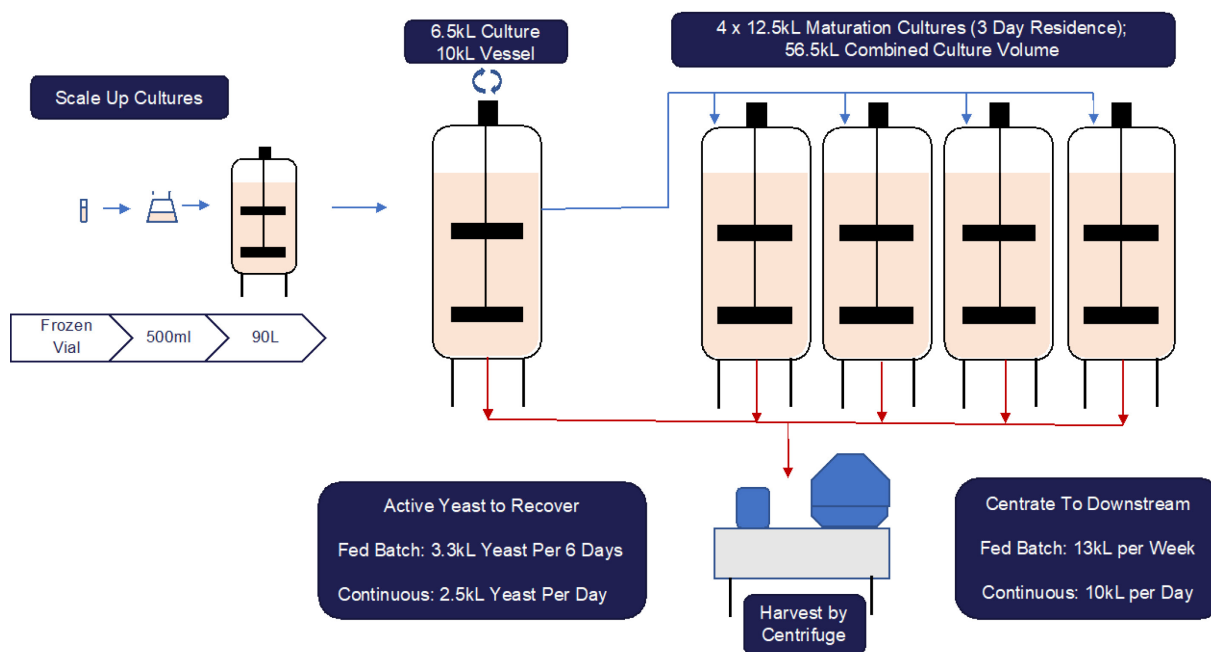


Figure 2 Process flow diagram for batch and continuous flow protein production (Source: Cauldron)

21. The final step involves downstream purification of the expressed protein which involves separation of cells by centrifugation, microfiltration, ultra-filtration and a drying step (Figure 3).

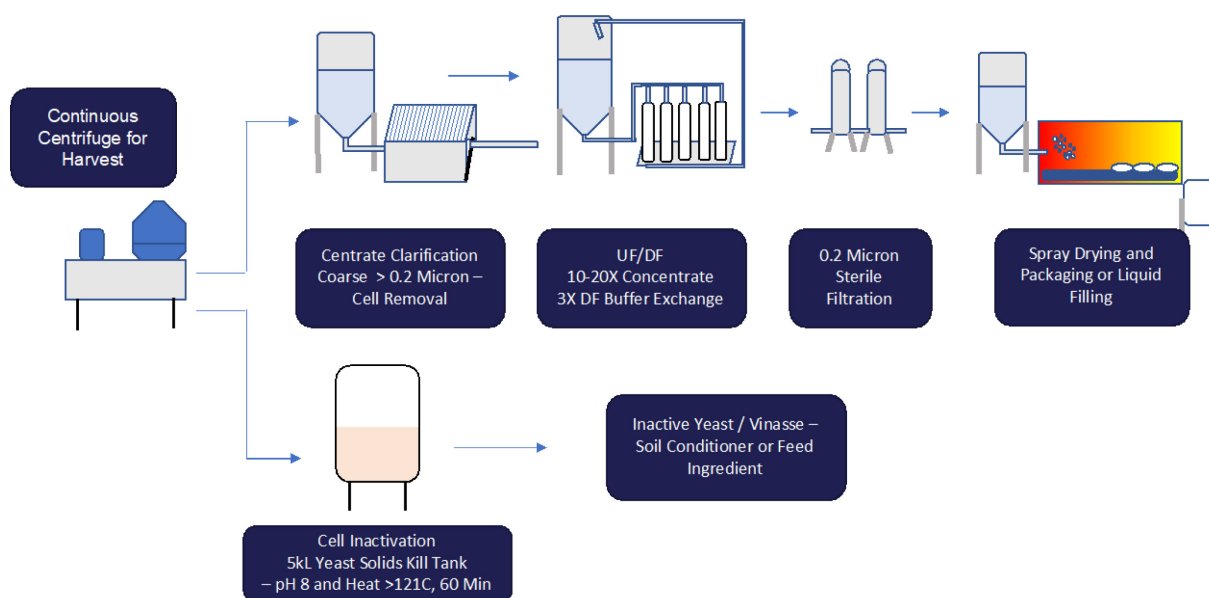


Figure 3 Downstream processing of the expressed proteins produced via fermentation (Source: Cauldron).

22. The final formulation will be tested by negative colony formation for absence of viable GM yeast, as well as testing for chemical and microbiological parameters.

23. The GM yeast and any ingredients used during the manufacturing process will be food grade ingredients which have a long history of safe use.

24. Fermentation cultures that do not meet the defined physico-chemical and microbiological testing criteria will be decontaminated and discarded. The fermenters and their contents will be heat sterilised or decontaminated chemically and/or the contents will be transferred to holding tanks for chemical decontamination.

25. The applicant has in place manufacturing systems such as Hazard Analysis Critical Control Point and Quality Management System to ensure compliance. There is a primary focus on food safety and the final product undergoes rigorous scrutiny to meet high quality standards without any impurities including viable GM yeast.

26. Purified proteins will be supplied to the applicant's clients in dry powder form and GM yeast will not enter human food. The decontaminated GM yeast slurry may be used for animal feed preparations or as a soil conditioner. If antibiotic resistance genes have been used, the GM slurry must also be denatured before use. Insertion of antibiotic resistance genes serve the purpose of GM yeast selection during the initial stages of development in the laboratory. The final intended uses of the purified proteins are in nutritional products, egg substitutes and fibre industry and therefore it may be used for human consumption following approval by FSANZ. For this the applicant will work with their clients to meet regulatory requirements with FSANZ.

2.3.2 Properties of the fermenters

27. The fermenters proposed to be used for the protein production will be constructed from 316 grade stainless steel. Therefore, these tanks will be highly resistant to corrosive acidic and alkaline environments and will allow for chemical disinfection and steam sterilisation.

28. The fermenters will form part of a closed system (this is a system for growth, processing and/or storage of large scale cultures of GM yeast within an enclosed vessel or vessels and transfer lines). The tanks will have ports for inoculation, media input, sampling, and air vents. All inputs will be sterilised either by filtration through a 0.2 μ m membrane or by subjecting to heat treatment at 121°C

for 15 minutes. Additionally, there will be multiple retractable and fixed probes for monitoring culture parameters that can be operated aseptically, and steam sterilised after use.

2.3.3 Training of staff

29. All staff will undergo core procedural and safety training regarding fermentation operations, section specific training and the individual job specific training. The training protocols will be documented in a training record form.

30. Staff will receive a licence specific training package which will contain relevant information on GM yeast. Signed statements will be recorded indicating that they have read, understood and agree to be bound by the licence conditions.

2.3.4 Transport and storage of the GMO

31. The applicant has stated that transfer of GM yeast culture between the PC2 LS facility and the outdoor production facility will be either by transfer vessel or stainless-steel piping. During transit, the transfer vessel will be maintained under pressure which is then connected to fermenters for aseptic transfer of culture medium. Alternatively, the GM yeast will be transferred through stainless-steel pipelines. Before, the transfer, both the pipeline and the fermenter will be steam sterilised. The pipelines are fitted with flanges across the PC2 LS facility which serve as a facility containment barrier that can be closed.

32. The GM yeast will be stored in fermenters in the production facility or in a PC2 LS facility.

2.3.5 Disposal of the GMO

33. Decontamination of GM yeast and waste containing GM yeast will be achieved either by autoclaving at $>121^{\circ}\text{C}$ for 60 minutes or chemical disinfection by 1% (w/v) Virkon for 10 minutes.

34. The fermenters and the stainless-steel pipelines will be steam sterilised before and after culture runs. Validation of effective decontamination will be confirmed by culturing samples on nutrient agar culture plates for seven days and by adding methylene blue indicator solution to observe yeast colonies under an optical microscope.

35. The applicant expects 25% of the culture volume to be wet yeast biomass slurry waste after the centrifugation and filtration step. The GM yeast slurry will be passed through a heat exchanger run at $>65^{\circ}\text{C}$ and held for 60 minutes at $>65^{\circ}\text{C}$ in a holding tank. This is known to effectively kill yeast.

36. Where the GM yeast has antibiotic resistance genes (used as a selectable marker), the applicant has proposed to use a combination of heat and pH to effectively denature the antibiotic resistance gene proteins and DNA. Studies have shown that DNA integrity is significantly compromised when subjected to alkaline pH. A correlation was observed between increase in pH (pH 7 to pH >13) and a reduction in PCR amplification of target DNA (Bivehed et al., 2023). Where protein production is induced, the GM yeast slurry will be collected in a separate jacketed vessel. The pH will be raised to >10 and held for 60 minutes at $>70^{\circ}\text{C}$. The pH will be readjusted to between 7 and 8 before disposal. Alternatively, where protein production is not induced, the pH will be raised to >10 and passed through a heat exchanger where the temperature is raised to 121°C for a minimum of 60 seconds. The pH will be readjusted to between 7 and 8 before disposal. Successful decontamination of GM yeast will be verified during the initial culture cycles by plating samples on agar plates for seven days as mentioned above followed by a viability test. The GM yeast will remain in the vessels until test results are negative for viable GMOs. This decontaminated and denatured (where antibiotic resistance genes are present) GM yeast waste would either be composted and used as a soil conditioner, it may be dried on site and used in animal feed preparations or disposed of by a waste contractor.

2.3.6 Accountability and Monitoring

37. The applicant has in place a documentation system that ensures that testing data and certificate of analyses is maintained for all batches of cultures. The testing parameters include physico-chemical parameters during culture and microbiological specifications including absence of GM yeast in purified protein.

2.3.7 Contingency plans

38. Dedicated spill kits will be co-located next to each fermenter which will contain absorbent material, PPE and decontamination solution. In the event of a small spill or leak, the spill will be treated with a chemical disinfectant such as 1% available chlorine for 20 minutes. In case of large spills, the drains lead to a small sump with level sensors which activate pumps to transfer the liquids to a concrete tank or 3 x 22 kL holding tanks for chemical inactivation.

39. If any material is found outside the production facility, the material will be placed in a double sealed container and transported to the Cauldron facility for destruction. The location's Global Positioning System (GPS) reading will be recorded, and the surrounding area will be inspected for any GM material. The storage and destruction location of the GM material will be documented.

40. The project supervisor will consult the compliance team and inform the IBC and the Regulator of the incident such as a spill or exposure to the GM yeast.

Section 3 Parent organism

41. The parent organism of the GM yeast is *P. pastoris*, also known as *Komagataella phaffi*. All the strains proposed to be used by the applicant are classified as *K. phaffi* (Barone et al., 2023). The characteristics of the parent organism provide a baseline for comparing the potential for harm from dealings with the GM yeast. The relevant biological properties of *P. pastoris* will be discussed here.

3.1 Classification

42. *P. pastoris* belongs to the genus *Pichia* within the family Saccharomycetaceae (Suh et al., 2006). *P. pastoris* was reclassified as genus *Komagataella* following phylogenetic analysis of the genome sequence (Yamada et al., 1995; Kurtzman, 2005), yet it is still commonly referred to as *Pichia*.

3.2 Characteristics

43. *P. pastoris* is a non-pathogenic single-celled yeast that occurs in decaying wood of trees (Enache-Angoulvant and Hennequin, 2005; Suh et al., 2006). Its unique feature is that it can metabolise methanol as a carbon source in addition to other sources, such as glucose, glycerol and ethanol, but cannot use lactose (Rebnegger et al., 2016). The ability to utilise methanol is mainly through the tightly regulated methanol inducible promoter alcohol oxidase (*AOX1*) and the methanol utilisation pathway genes (*MUT* genes). Glucose, glycerol and ethanol, when used in the culture medium, act as strong repressors of *AOX1* which gets derepressed when these alternate carbon sources are depleted and subsequently induced when methanol is introduced (Ahmad et al., 2014; Ata et al., 2021).

44. *P. pastoris* is used in laboratory research and biotechnology industries as a popular choice for expressing heterologous proteins and as an ingredient in stock feed. It has a long history of safe use. It is also used to produce human food ingredients such as enzyme processing aids, sweeteners, and nutritive substances (FSANZ, 2020).

45. *P. pastoris* is an obligate aerobe with a strong preference for respiratory growth (Walsh and Martin, 1977; Bernauer et al., 2020). Optimum growth occurs at 28.9°C and pH 6.9 (Chang et al., 2006).

3.3 Genome

46. The genome of *P. pastoris* has been sequenced and annotated. It is approximately 9.43 Mbp and organised into four chromosomes encoding 5,313 genes (De Schutter et al., 2009).

3.4 Life cycle

47. Cells of *P. pastoris* exist in both haploid and diploid states. The yeast normally exists in vegetative haploid form and reproduces by budding. However, under limited nitrogen availability, mating-type (*MAT*) genes are expressed which transform the haploid cells into mating forms which results in diploid cells. These diploid cells undergo meiosis to form haploid ascospores which in turn give rise to the haploid vegetative forms. *P. pastoris* is a homothallic fungus, wherein a single haploid cell is able to produce cells with both a-type and α -type genotypes.

48. Prolonged nitrogen limited conditions are required for induction of sporulation which is unlikely to occur in a cell culture environment (Ata et al., 2021). Under optimum culture conditions, *P. pastoris* cells double approximately every 60 - 120 minutes (Karbalaei et al., 2020).

49. Under adverse environmental conditions such as carbon source limitations, and pH variation, some cells may undergo a morphological switch to filamentous multicellular forms. These different morphological phenotypes may be able to adapt to environmental changes for better survival (Ata et al., 2021).

3.5 Pathogenicity and toxigenicity

50. *P. pastoris* is a non-pathogenic and non-toxic yeast.

51. Since *P. pastoris* is widely used in vaccine development, a study was conducted to profile its safety. Intravenous injection of *P. pastoris* in mice led to dissemination of *P. pastoris* to various tissues but did not induce a cell-mediated response or increase in the level of circulating antibodies. This indicates its safe profile in the development of vaccines (Becerril-García et al., 2022).

52. *P. pastoris* has been tested for toxigenicity in animal models as feed preparations. In broiler chicks fed with additional yeast supplement, it was found to have a beneficial probiotic influence as compared to control chicks fed with non-supplemented diet (Gil de los Santos et al., 2012; Gil de Los Santos et al., 2018). In another study, similar beneficial probiotic effects were noted in mice administered with *P. pastoris* through an intragastric route (Birmann et al., 2021). However, it is not clear from these studies whether the yeast was able to colonise the gut of these animals.

53. FSANZ has approved several processing aids, additives and nutritional products that have been produced using *P. pastoris*. One such example is the soy leghemoglobin produced via *P. pastoris* fermentation to be used as a meat analogue product (FSANZ, 2020).

3.6 Reservoir

54. In nature, *P. pastoris* is commonly associated with the decaying wood of trees and soft tissue of succulent plants (Mortimer, 2000; Chang et al., 2006).

3.7 Environmental stability and decontamination

55. Information relating to environmental stability and decontamination of *P. pastoris* is scarce. Hence, information relating to its well-studied distant relative *Saccharomyces cerevisiae* is used as a guide. Both *P. pastoris* and *S. cerevisiae* are single celled yeast with chitinous cell wall. They belong to the same family: Saccharomycetaceae.

56. *S. cerevisiae* is an anhydrobiote which is able to survive desiccation for at least six months (Tapia and Koshland, 2014). *S. cerevisiae* is effectively decontaminated by direct application of isopropyl alcohol or 0.3% peracetic acid (Korukluoglu et al., 2006). Quaternary amine compounds and aldehydes may be effective, depending on concentration and incubation time of the compounds.

57. The effect of hypochlorite on *S. cerevisiae* depends on solution pH, concentration and incubation time (Hays et al., 1967). *S. cerevisiae* was destroyed after 30 seconds exposure to 25 parts per million (ppm) (0.0025% v/v) hypochlorite in solutions buffered at pH 5 or 8.5 (Hays et al., 1967). Growth of *S. cerevisiae* on solid medium was completely inhibited at 3000 ppm sodium hypochlorite, while viability was lost in over 80% of cells when incubated for 15 minutes in 50 ppm sodium hypochlorite solution buffered at pH 6 (Kwolek-Mirek et al., 2011). In contrast, Korukluoglu et al. (2006) reported that a chlorine-based commercial disinfectant used concentrations of 0.5–2% did not effectively decontaminate *S. cerevisiae* within 14 days.

58. Likewise, Korukluoglu et al. (2006) found that iodophor (iodine) concentrations of 0.5–1% did not effectively decontaminate *S. cerevisiae*, while Hays et al. (1967) observed that *S. cerevisiae* was non-viable after 15 seconds at 6 ppm (0.0006%) iodophor.

59. The minimum inhibitory concentration of ethanol towards *S. cerevisiae* is approximately 13% (Kampf, 2018). The maximum concentration of ethanol reported to be tolerated by *S. cerevisiae* is 25%. Effective fungicidal activity against food-borne yeasts was reported following exposure to 70% ethanol for 5 minutes (Kampf, 2018).

60. Based on decontamination methods effective for *S. cerevisiae* and that single celled yeasts have cell wall made of chitin, it is expected that *P. pastoris* would also be decontaminated using these methods.

3.8 Risk Group and containment

61. The Australian Standard 2243.3:2022 *Safety in Laboratories Part 3: Microbiological safety and containment* (Standards Australia New Zealand, 2022) does not provide a classification for *P. pastoris*. However, according to the criteria listed in the Australian Standard, *P. pastoris* can be classified as a Risk Group 1 organism.

62. The American Type Culture Collection (ATCC) classifies the methylotrophic yeast *P. pastoris* as Biosafety Level 1, for organisms that are not known to cause disease in healthy adults (American Type Culture Collection, 2023). *P. pastoris* dried yeast is permitted to be used as an additive to the feed formulation of broiler chickens as a source of protein (US FDA, 2023a).

63. The Public Health Agency of Canada also classifies *P. pastoris* as a Risk Group 1 organism (Public Health Agency of Canada, 2023). Given this, PC1 containment and work practices are considered appropriate when working with the wild-type organism.

64. *P. pastoris* has been given the status as Generally Recognised as Safe (GRAS) by the US FDA (US FDA, 2017) and Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA) for use in enzyme production (EFSA, 2023).

3.9 Properties of *P. pastoris* expression system

65. As mentioned above (Section 3.2), *P. pastoris* is a methylotrophic non-pathogenic yeast which can metabolise methanol as a carbon source. There are several reasons for *P. pastoris* being established as a popular tool for research and in expression of heterologous proteins (Vinayagamurthy et al., 2007; Karbalaee et al., 2020; Gao et al., 2021):

- It possesses a regulatable AOX1 promoter;
- Expression cassettes can be integrated into the yeast genome at specific sites;
- It can reach very high cell densities in simple media consisting of methanol;
- High heterologous protein yields can be achieved up to >20g/L;

- Minimal endogenous proteins are expressed and heterologous proteins can be secreted into the supernatant leading to high purity of heterologous protein;
- Being a eukaryote, heterologous proteins can be expressed close to their native forms comprising post-translational modifications;
- It is very similar to the well-studied *S. cerevisiae*, a well known research and biotechnology industry model, the techniques of which are translatable to *P. pastoris*; and
- The cultures are easy to maintain, easy to scale-up and the growth requirements are inexpensive.

66. Despite these advantages, like with any other expression systems, *P. pastoris* has some limitations (Karbalaee et al., 2020):

- Transformation efficiencies are relatively poor requiring large quantities of plasmids for successful transformation; and
- Heterologous protein production using methanol as sole carbon source could result in methanol toxicity to the yeast.

Section 4 The GMO - nature and effect of the genetic modification

67. The applicant proposes to conduct precision fermentation to produce three classes of proteins (bovine milk proteins, chicken egg proteins and spider silk fibre) using GM yeast.

68. The purpose of this application is to optimise the fermentation process and characterise the GM yeast during fermentation to produce different animal proteins. The GM yeast will be constructed by the applicant's clients from commercially available *P. pastoris* strains and vector plasmids. It has been observed that the optimal strain for production of a recombinant protein differs based on the target protein (Brady et al., 2020). Hence, the proposal to use different strains and plasmids will allow for optimisation of expression of the three classes of recombinant animal proteins.

4.1 Development of GM yeast

69. To generate GM yeast, the expression cassettes carrying genes of interest will first be inserted into commercially available expression vector plasmids which will be multiplied in a suitable host. The expression cassette will then be excised from the plasmid and the linearised cassette will be introduced into the yeast. *P. pastoris* will be stably transformed with the linearised cassette by homologous recombination using standard methods such as electroporation. Integration into the yeast genome will be via homologous recombination. These GM yeast will be fully transformed and validated for integration of the expression cassette and for the absence of any unintegrated plasmid vector by the applicant's clients prior to their supply to the applicant.

70. In addition to the transgenes for expression of bovine milk, egg white and spider silk fibre proteins, the expression cassette may also contain:

- constitutive or inducible promoter (e.g., methanol inducible AOX1) to facilitate expression of introduced sequences;
- antibiotic selectable marker genes that confer resistance to specific antibiotics limited to zeocin, hygromycin, ampicillin, or kanamycin to enable positive selection for the GMO;
- secretion signal peptide (e.g., alpha factor) to facilitate extracellular secretion of proteins into the supernatant; and
- tags such as epitope or polyhistidine to detect and purify the recombinant proteins.

71. The applicant has provided an example of an expression cassette coding for bovine β -lactoglobulin protein of 275 amino acids with an approximate molecular weight of 30.4 kDa (Figure 4). The expression cassette may contain the following genetic elements:

Genetic element	Function
Methanol regulated <i>P. pastoris</i> Alcohol Oxidase 1 (AOX1) inducible promoter	Expression of recombinant protein
Alpha factor signal peptide	Facilitates extracellular secretion of the expressed protein into the culture medium
Bovine β -lactoglobulin	Expression of recombinant protein
c-Myc epitope	Detection of the recombinant protein
Poly-histidine tag	Purification of the recombinant protein
Antibiotic selection marker	Positive selection of the GM yeast
Termination sequence	Termination signal of transcription

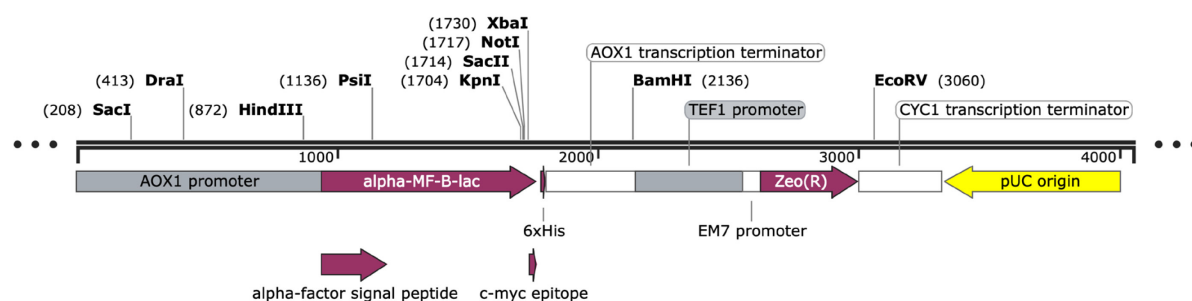


Figure 4 Example vector map for the expression of the β -lactoglobulin protein (Source: Cauldron)

72. The antibiotic resistance gene may not be removed from the GM yeast prior to scale-up fermentation.

4.1.1 Features of introduced genes

73. Though the proteins proposed to be expressed in their native form have a long history of safe use as food and fibre, some of them are known to be dietary allergens. Milk and egg proteins are listed among the nine major food allergens by US FDA (US FDA, 2023b) and among the top ten major food allergens in Australia (AIFS, 2023; FSANZ, 2023). The introduced genes, their structure and function are summarised below.

4.1.1.1 Bovine milk proteins

74. Milk is an essential nutrient source for new-born calves before they are gradually weaned to a solid diet. It is considered a complete diet as it provides all nine essential amino acids. Milk also provides immune protection and immunomodulatory effects during development of the calf's immune system. Broadly, there are two types of proteins present in the bovine milk: casein which comprises approximately 80% of the proteins and whey which forms approximately 20%. Casein proteins are relatively insoluble in water while the whey proteins form the soluble component (Davoodi et al., 2016). The source and properties of the bovine milk proteins expressed by the GM yeast are listed in Table 1.

4.1.1.2 Chicken egg proteins

75. Egg is mainly composed of eggshell (9-12%), egg white (60%) and yolk (30-33%). Egg white comprises of 90% water and 10% protein and is a major nutrient source for developing embryos. Egg white protein (EWP) comprises mainly of ovalbumin (54%), ovotransferrin (12%), lysozyme (3.5%) and ovomucin (11%). The source and properties of the chicken egg proteins expressed by the GM yeast are listed in Table 1.

4.1.1.3 Spider silk proteins

76. Spiders generate seven different types of silk from seven different glands. They have diverse functions, mainly to capture prey, escape from predators and forming a protective egg sac. The glands and the type of silk they produce are listed below:

- Major ampulate gland – dragline silk – Dragline silk is highly tensile comparable to steel. It is used to make the main frame of the web and to drop in order to escape.
- Minor ampulate gland – auxiliary spiral thread – it is elastic
- Flagelliform gland – core capture spiral
- Aggregated gland – sticky aqueous coating
- Pyriform gland – cement
- Aciniform gland – wrapping prey
- Cylindrical gland – egg sack

77. Spider silk proteins are not considered allergenic (Römer and Scheibel, 2008). The source and properties of the spider silk fibre proteins expressed by the GM yeast are listed in Table 1.

Table 1 Proteins proposed to be expressed in the GM yeast

Class 1: Bovine Proteins - Source organism Bovine (<i>Bos taurus</i>)			
Protein	Molecular weight	Properties and Function	Allergen
Whey proteins			
β-lactoglobulin	18.4 kDa	β-lactoglobulin is a small protein of 162 residues. It exists mainly in dimeric form but can form multimeric aggregates under various conditions. It is the major whey protein found in ruminant milk, accounting for 65% of whey protein. It is absent in human milk. It plays a role in transport of small molecules and micronutrients (Jensen et al., 2022). It is a known major food allergen when not in a bound state with a micronutrient.	Yes
α-lactalbumin	14.1 kDa	α-lactalbumin consists of a single peptide chain of 123 amino acids joined by 4 disulphide bonds. It accounts for 25% of whey protein. Its main role is in the regulation of lactose biosynthesis (Jensen et al., 2022) and its essential amino acid composition is crucial for new-born nutrition. It is a calcium binding protein and shares similar structure to lysozyme indicating an antimicrobial function. It is a known major milk allergen in some people.	
Bovine lactoferrin (bLf)	80kDa	Bovine lactoferrin is an iron-binding glycoprotein which belongs to the class of transferrin proteins. Its main function is to transfer iron to cells and stabilise iron levels in blood. FSANZ allergenicity assessment of bovine lactoferrin as a fortifying ingredient in infant formula, stated that it may be allergenic on the basis that some individuals have IgE antibodies to bovine lactoferrin (FSANZ, 2023).	
Casein Proteins			
α- Casein (α _{s1} and α _{s2})	20–25 kDa	Casein proteins belong to the phosphoprotein family. The casein proteins aggregate to form micelles of approximately 100 μm. The outermost layer of the micelles is composed of κ-caseins which are negatively charged and hence repel each other to form a colloidal suspension in an aqueous medium. β-Casein is the major casein protein found in ruminant milk and occurs in two genetic variant forms A1 and A2.	Yes
β-Casein (A1 and A2)			
κ casein			
Class 2: Chicken Egg Proteins - Source organism chicken (<i>Gallus gallus domesticus</i>)			
Egg white proteins			

Ovalbumin (Gal d 2)	45 kDa	Ovalbumin is a glycoprotein which consists of 386 amino acids (Abeyrathne et al., 2013). Ovalbumin mainly functions as a nutritional source for the developing embryo (Li et al., 2022). Other than being an excellent nutrient source, it is a known major allergen (Li et al., 2022).	Yes
Ovomucoid (Gal d 1)	23 kDa	Ovomucoid is a large glycoprotein and has antimicrobial properties (Caubet and Wang, 2011). Ovomucoid is known as a trypsin inhibitor thus causing indigestion; and the most significant allergen among EWP (Caubet and Wang, 2011).	
Ovotransferrin (Gal d 3)	76 kDa	Ovotransferrin is a metal binding monomeric glycoprotein belonging to the transferrin family. It is a single 686 amino acid peptide (Abeyrathne et al., 2013) and forms two homologous lobes (as a result of a gene duplication event) each joining a single iron atom. It comprises 13% of total EWP. The main function of ovotransferrin is homeostasis and transport of iron levels (Legros et al., 2021). It is the least allergenic protein among EWP.	
Egg shell membrane proteins			
Type 1 Egg collagen protein Col1A1 and Col1A2; collagen X (COL10A1)	NA	These proteins collectively crosslink to form eggshell membrane proteins. They form a foundation for the formation of the calcareous eggshell and an effective barrier that prevent bacterial infection (Du et al., 2015; Han et al., 2023). Eggshell membrane, though unutilised has been shown to improve skin health due to its collagen content (Yoo et al., 2015). A water soluble hydrolysate food product derived from eggshell membrane proteins was evaluated as safe for consumption (EFSA et al., 2018). The elastic and semipermeable properties of eggshell membrane proteins could have potential uses in industrial, nutraceutical, and biomedical applications (Yoo et al., 2015). Since they are not utilised, allergenicity data is not available for these proteins.	Not known
Fibrillin-1 (FBN1)	~320 kDa		
Cysteine rich eggshell membrane protein (CREMP)	NA		
Class 3: Silk Fibre - Source organism Orb weaver Spider (Nephila calvipes)			
Major ampullate spidroin 1 (MaSp1 or MaSp2)	250 kDa	Dragline silk is composed of two types of proteins: MaSp1 and MaSp2, with a molecular weight of 250 kDa each (Römer and Scheibel, 2008). They are large proteins with a highly repetitive core region bordered by non-repetitive N and C-terminal domains. This arrangement renders formation of β-sheets thus giving highly tensile properties comparable to steel (Ramezaniaghdam et al., 2022). It is used to make the main frame of the web and to drop in order to escape.	No

Minor ampullate spidroin1 and 2 (MiSp1 and 2)	250 kDa	MiSp1 and 2 have elastic properties and form the core spiral of the web which stabilises the main frame. They have similar biomechanical properties as MaSp (Ramezaniaghdam et al., 2022).	
Flagelliform protein (Flag)	360 kDa	Flag silk is highly elastic which can withstand the impact of a flying insect. The proteins are composed of repetitive and spacer motifs which form a spring like helix that give elastic properties (Ramezaniaghdam et al., 2022).	

4.1.1.4 Allergenicity of the proteins associated with the introduced genes

78. As shown in Table1, the bovine milk and chicken egg proteins expressed by the GM yeast are known allergens and can result in allergic reactions. Allergic reactions are a type of adverse effect, resulting from sensitization to a chemical, followed by an allergic response upon subsequent exposure. Allergenicity is the potential for a chemical to be recognised by the body as a foreign substance and to elicit a (disproportionate) immunological reaction.

79. The severity of allergic reactions depends on the allergen quantity, the exposure route, and the duration of treatment (Robles and Motheral, 2014). Clinical symptoms generally start within minutes and could result in different symptoms such as skin rashes, vomiting, and difficulty breathing. Food allergies are generally triggered by ingestion but can also be triggered by skin/dermal contact and inhalation. These route of exposure and their role in allergic reactions in response to proposed proteins have been described below.

Exposure via ingestion

80. Cow milk proteins (CMP) can elicit adverse immune reaction known as cow's milk protein allergy (CMPA) (Solinas et al., 2010). CMPA is characterised by allergic reactions such as anaphylaxis, asthma, rhinitis, atopic dermatitis, urticaria and gastrointestinal disorders (Docena et al., 1996). Both whey (β -lactoglobulin and α -lactalbumin) and casein proteins (β -casein) are considered major food allergens (Lam et al., 2008).

81. Infants get sensitised to CMPs by developing antibodies specific to individual proteins with prevalence ranging from 2 to 3% (Bar-On et al., 2022; Jensen et al., 2022). Tolerance usually develops as children mature and consequently is less prevalent occurring in less than 0.5% of adults (Bar-On et al., 2022; Jensen et al., 2022). As cited in a review, the allergy eliciting threshold to provoke a response in allergic children was 30 mL milk (Allen et al., 2007).

82. Allergy to eggs arises due to hypersensitivity to egg proteins, predominantly EWP and is especially common among children. It is the second most common allergy after CMPA. As in CMPA, children acquire tolerance to EWP allergies as they age. Prevalence ranges between 0.5 to 2.5% of young children (Rona et al., 2007). Like milk allergies, egg allergies decrease with age, although there have been a small number of cases of adults developing egg allergies (Unsel et al., 2007; Cremonte et al., 2021). As cited in a review, the allergy eliciting threshold to provoke a response in 50% of allergic children was 2000 mg of egg white (Allen et al., 2007).

83. Among the CMP, casein proteins are known to be major allergens. In an observational study involving 80 milk-atopic patients (3 months to 25 years, mean age: 6.19 years), casein, β -lactoglobulin and α -lactalbumin antigen specific antibodies were tested. All patients were tested positive for casein; 10/80 tested positive for β -lactoglobulin and 5/80 were positive for α -lactalbumin (Docena et al., 1996).

Exposure via dermal contact

84. Upon direct exposure to milk proteins via dermal contact, allergic reaction such as contact dermatitis (i.e., itchy rash) and contact urticaria (i.e., immediate swelling and redness of the skin) could occur.

85. In an observational study with children known to have cow's milk allergy, milk was applied to children's cheeks or exposed to milk via skin prick test. The study showed that exposure to milk in some children resulted in contact urticaria (Schichter-Konfino et al., 2015). In another study, contact dermatitis was observed in response to casein protein and was found to often be associated with impaired skin (e.g., suffering from eczema) (Nakonechna et al., 2019; Jensen et al., 2022).

86. A 16 year old boy experienced allergic reaction (such urticaria-angioedema, cough, bronchial obstruction) after accidental skin contact with cow milk (Liccardi et al., 2004). There are also other

studies where skin contact with cow's milk in infants, children and adults have resulted in severe systemic symptoms (Jarmoc and Primack, 1987; Tan et al., 2001; Ramirez and Bahna, 2009).

87. Due to being in the vicinity of uncooked fish or egg, a 6 year old boy with multiple food allergies experienced contact urticaria (Ramirez and Bahna, 2009). Similar, other studies have indicated that contact with egg could result in an allergic reaction (Yamada et al., 1995; Caubet and Wang, 2011).

Exposure via Inhalation

88. Different studies have demonstrated that oral inhalation of milk proteins could result in an allergic reaction (Robles and Motheral, 2014; Bar-On et al., 2022). In one study, 9-year-old boy with asthma and milk protein allergy experienced shortness of breath due to the presence of lactose in an asthma inhaler (Robles and Motheral, 2014). Similar observations to the previous study (such as contact urticaria around lips, angioedema, shortness of breath) were also observed in children and adults with asthma and CMPA in response to oral inhalation of lactose (Sa et al., 2011; Bar-On et al., 2022).

89. Similar to CMPA, EWP allergies can be induced via inhalation. Upon exposure to egg aerosols or egg proteins, bakery workers experienced allergic respiratory symptoms and inflammation of eyes and their skin prick tests showed positive results for EWP (Leser et al., 2001; Escudero et al., 2003).

4.2 Expected properties of the GM yeast

90. The applicant has proposed to use commercially available strains of *P. pastoris* and protein expression vectors for stable integration of expression cassettes into the *P. pastoris* genome (GM yeast). As these strains will be tested for stability under exempt or NLRD dealings, the GM yeast used for large scale fermentation is expected to stably express desired animal proteins. Some of these proteins produced by the GM yeast are known food allergens.

91. The GM yeast with constitutive promoters are expected to express proteins under all suitable culture conditions. However, the GM yeast with inducible promoters are expected to express proteins in the presence of an inducer.

92. Due to the presence of antibiotic resistance genes in the GM yeast, the yeast may have a selective advantage in the presence of the antibiotic.

Section 5 The receiving environment

93. The receiving environment forms part of the context in which the risks associated with dealings with the GMOs are assessed. Relevant information about the receiving environment includes abiotic and biotic interactions of the organism with the environment where the release would occur; production practices for the organism; presence of organisms that are sexually compatible with the GMO; and background presence of the gene(s) used in the genetic modification (OGTR, 2013).

94. The GM yeast to be used for the protein production process will be developed from laboratory *P. pastoris* strains which only grow under conducive culture conditions. In the event of a spill or leak, they are unlikely to survive in the environment and form a self-sustaining population.

5.1 Presence of related fungal species in the receiving environment

95. *P. pastoris* is commonly found in decaying wood and succulent plants (Mortimer, 2000; Chang et al., 2006). The natural hosts such as oak or chestnut trees are not present in the vicinity of the production facility. Hence, it is unlikely that the GM yeast will interact with wild-type yeast.

5.2 Presence of similar genes or products in the environment

96. The genes and their proteins for bovine milk, chicken egg and spider silk are ubiquitous in the environment. Also, the antibiotic resistance genes proposed in the application are naturally present in soil bacteria.

Section 6 Previous authorisations

97. The GM yeast has not been previously authorised for fermentation trials in Australia. However, several similar dealings have received Generally Recognised as Safe (GRAS) status in the United States. Most recent of these are GRAS No. 1056 for production of β -lactoglobulin and GRAS No. 967 for production of egg-white using *P. pastoris*.

Chapter 2 Risk assessment

Section 1 Introduction

98. The risk assessment identifies and characterises risks to the health and safety of people or to the environment from dealings with GMOs, posed by or as the result of gene technology (Figure 5). Risks are identified within the established risk assessment context (Chapter 1), taking into account current scientific and technical knowledge. A consideration of uncertainty, in particular knowledge gaps, occurs throughout the risk assessment process.

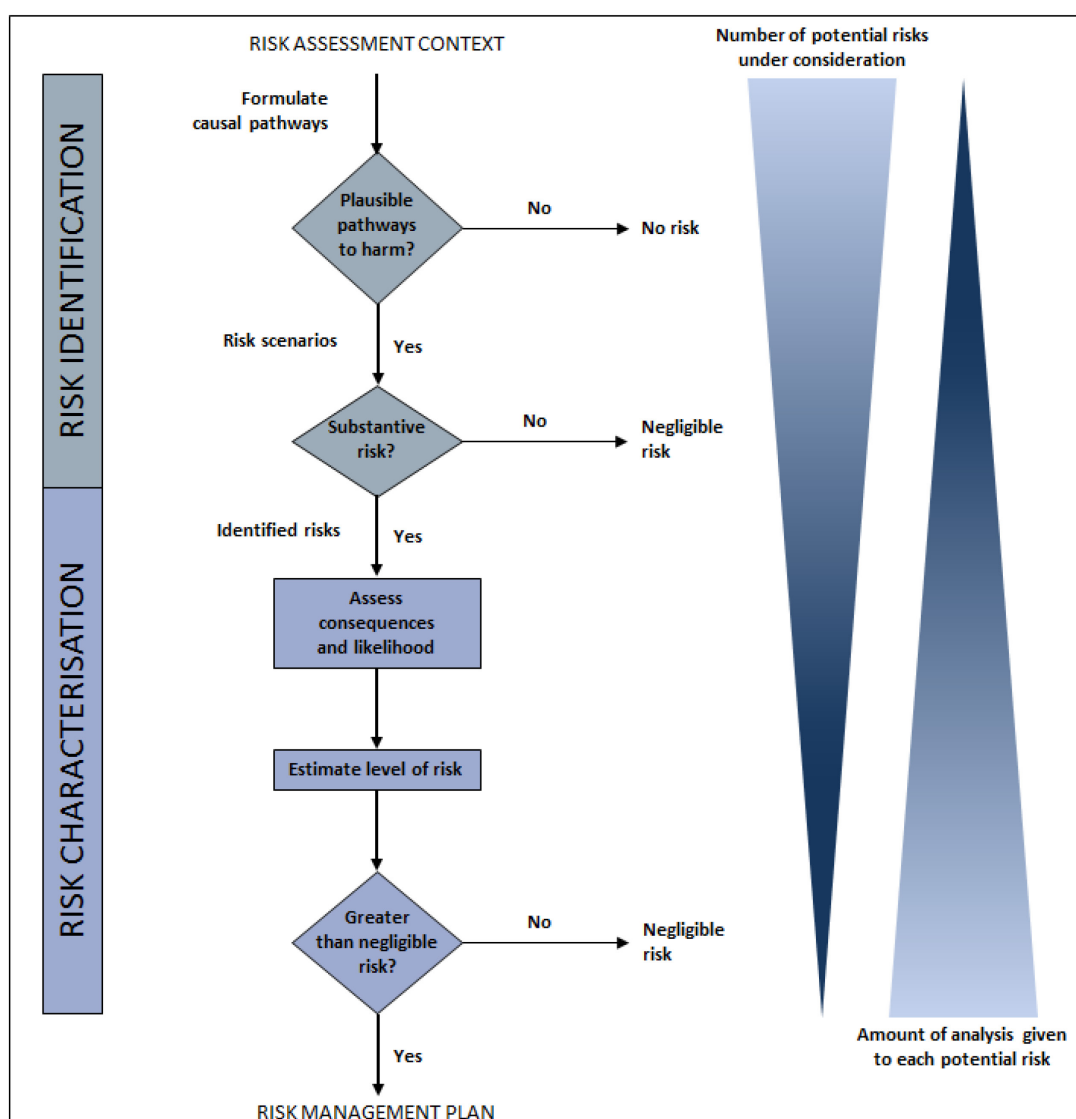


Figure 5 The risk assessment process

99. The Regulator uses a number of techniques to identify risks, including checklists, brainstorming, previous agency experience, reported international experience and consultation (OGTR, 2013).

100. Risk identification first considers a wide range of circumstances in which the GMO, or the introduced genetic material, could come into contact with people or the environment. This leads to postulating plausible causal pathways that may give rise to harm for people or the environment from dealings with a GMO. These are risk scenarios.

101. Risk scenarios are screened to identify substantive risks, which are risk scenarios that are considered to have some reasonable chance of causing harm. Risk scenarios that could not plausibly

occur, or do not lead to harm in the short and long term, do not advance in the risk assessment process (Figure 5), i.e., the risk is considered no greater than negligible.

102. Risk scenarios identified as substantive risks are further characterised in terms of the potential seriousness of harm (Consequence assessment) and the likelihood of harm (Likelihood assessment). The consequence and likelihood assessments are combined to estimate the level of risk and determine whether risk treatment measures are required. The potential for interactions between risks is also considered.

Section 2 Risk identification

103. Postulated risk scenarios are comprised of three components (Figure 6):

- i. the source of potential harm (risk source)
- ii. a plausible causal linkage to potential harm (causal pathway)
- iii. potential harm to people or the environment.

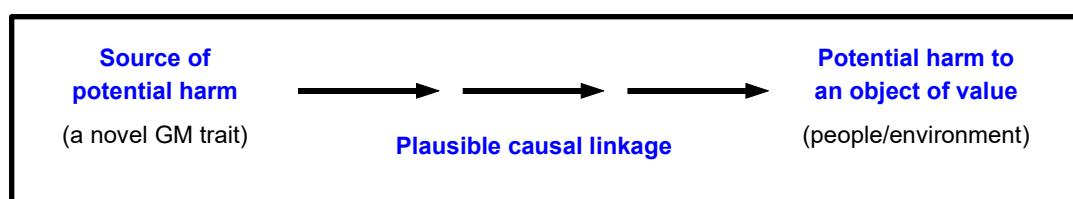


Figure 6 Components of a risk scenario

104. When postulating relevant risk scenarios, the risk context is taken into account, including the following factors detailed in Chapter 1:

- the proposed dealings;
- the proposed limits including the extent and scale of the proposed dealings;
- the proposed controls to limit the spread and persistence of the GMO; and
- the characteristics of the parent organism(s).

2.1 Risk source

105. The sources of potential harms can be intended novel GM traits associated with one or more introduced genetic elements, or unintended effects/traits arising from the use of gene technology.

106. As discussed in Chapter 1 Section 4, the GM yeast will be modified by inserting bovine milk, chicken egg or spider silk genes. The intended effect of insertion of these genes is to express them to produce animal proteins from a non-animal source. These introduced genes are considered further as a potential source of risk.

107. The introduced tags such as poly-histidine and epitope tags, and selectable antibiotic markers, are routinely used in the protein production process to identify the GM yeast (i.e., selection of the GM yeast) and to identify and purify the expressed proteins. The antibiotic selection markers used in the laboratory are not usually used as therapeutics. They are not considered as a direct source of risk in the proposed dealings.

108. It is noted that a large volume of GM yeast slurry is generated that may carry antibiotic resistance genes. If released into the environment, this could potentially result in horizontal gene transfer (HGT) resulting in antibiotic resistant organisms. Though HGT is considered a rare event in eukaryotes (Gonçalves and Gonçalves, 2022) such as yeast, as a cautionary approach, the GM yeast where antibiotic resistance genes are present is proposed to be denatured as well as decontaminated before it could be used in the preparation of animal feed, as soil conditioner or disposed as waste.

109. The introduced genes are controlled by introduced regulatory sequences such as promoters and termination sequences, and secretion signal peptides. These sequences are DNA that is not expressed as a protein, so exposure is to the DNA only and dietary DNA has no toxicity (Society of Toxicology, 2003). Hence, the introduced regulatory sequences will not be further considered as sources of potential harm for this application.

Unintended effects due to the process of genetic modification

110. Genetic modifications have the potential to cause unintended effects in several ways. These include insertional effects, where the introduced genetic elements are randomly integrated into the host's genome. These effects, such as interruptions, deletions, duplications or rearrangements of the genome, can lead to altered expression of endogenous genes as reviewed in plants (Schnell et al., 2015). As mentioned in paragraph 17, prior to conducting dealings in the production facility, the strains will be assessed and characterised in small volumes within the PC2 LS facility for more than 50 generations to demonstrate stable integration of the expression cassette and genetic stability. Each protein production strain of the GM yeast will be fully sequenced before selecting the desired GM yeast for the proposed protein production process. This would ensure that there are no unintended disruptions to other endogenous genes. Hence the potential for the processes of genetic modification to result in unintended effects will not be considered further.

111. The Act does not regulate activities with GM products, which are defined as a thing (other than a GMO) derived or produced from a GMO. Examples include purified recombinant proteins. Therefore, risks posed during handling and use of the purified proteins after separating them from the GM yeast will not be considered further in this risk analysis. However, risks posed by exposure to the proteins in the course of conducting dealings with the GM yeast are considered.

112. Risks arising from cross-contamination of production equipment, leading to unintended expansion of the GM yeast and protein expression while culturing other organisms, or product contamination at downstream processing stages, will not be considered further. Fermenters and processing equipment undergo cleaning processes after each use and are sterilised before initiating a new culture.

2.2 Causal pathway

113. The following factors are taken into account when postulating plausible causal pathways to potential harm:

- the proposed dealings with the GMO;
- proposed limits, including the extent and scale of the proposed dealings;
- characteristics of the parent organism;
- potential effects of introduced or deleted gene(s) on the properties of the organism;
- routes of exposure to the GMOs, the introduced gene(s) and gene product(s);
- potential exposure to the introduced gene(s) and gene product(s) from other sources in the environment;
- the receiving environment;
- spread and persistence of the GMOs (e.g. dispersal pathways and establishment potential);
- potential for gene transfer to sexually compatible organisms
- gene transfer by horizontal gene transfer (HGT); and
- unauthorised activities.

114. Although all of these factors were considered, some are not included in the risk scenarios below as a plausible pathway to harm could not be identified.

115. As discussed in Chapter 1 Section 3, under extreme conditions such as nutrient depletion, *P. pastoris* resorts to formation of mating-types which produce diploid cells. These diploid cells have the ability to sporulate. Similarly, *P. pastoris* under extreme conditions has been observed to form invasive morphological phenotypes, possibly to adapt to environmental changes for better survival (Ata et al., 2021). However, culture conditions proposed by the applicant are not conducive for sporulation or for modified phenotypes. Hence, the potential for spread through spore formation or through morphological adaptations will not be considered further.

116. The commercially available strains proposed to be used in the application are laboratory adapted *P. pastoris* strains that can only grow under optimal conditions. As a result, these strains are unlikely to grow in the environment in an event of escape. Moreover, the wild-type *P. pastoris*, are typically found in trees such as chestnut and oak, which are not found in the vicinity of the production facility. Hence, the potential of gene transfer to sexually compatible organisms or homologous recombination will not be considered further.

117. As discussed in Chapter 1 Section 3, *P. pastoris* does not produce any toxins and is not considered pathogenic. Hence, harms relating to toxicity and pathogenicity will not be considered further. Similarly, the potential for reversion of the GM yeast to the parental phenotype is not a plausible pathway to harm because the parent organism is not pathogenic or harmful (Chapter 1 Section 3). Therefore, reversion will not be considered further.

118. Oral ingestion of the GM yeast as a causal pathway will not be considered as the staff will be wearing a face mask when handling the cultures which will prevent accidental ingestion and the trained staff will not consider the culture contents as food which will preclude intentional ingestion. Moreover, as discussed in (Chapter 1, Section 4.1.1.4), the quantities of allergen required to be consumed to elicit an allergic response is higher than that which would be consumed accidentally.

119. The Act provides for substantial penalties for unauthorised dealings with GMOs or non-compliance with licence conditions, and also requires the Regulator to have regard to the suitability of an applicant to hold a licence prior to the issuing of the licence. These legislative provisions are considered sufficient to minimise risks from unauthorised activities. Therefore, unauthorised activities by the applicant will not be considered further.

2.3 Potential harms

120. The introduced genes encode for animal proteins. Therefore, the potential harms that will be considered are:

- risks to people undertaking this dealing from exposure to the GM yeast; and
- risks to people and the environment from an unintentional release of GM yeast.

2.4 Postulated risk scenarios

121. Two risk scenarios were postulated and screened to identify any substantive risks. These scenarios are summarised in Table 2 and discussed in depth in Sections 2.5 – 2.6.

122. In the context of the activities proposed by the applicant and considering both the short and long term, neither of the two risk scenarios gave rise to any substantive risks.

Table 2 Summary of risk scenarios from the proposed dealings with GM yeast

Risk scenario	Risk source	Possible causal pathway	Potential harm	Substantive risk	Reasons
1	GM yeast secreting	Fermentation of GM yeast expressing the introduced genes	Allergenicity in people	No	<ul style="list-style-type: none"> • The limits and controls of the proposed protein production process would minimise

Risk scenario	Risk source	Possible causal pathway	Potential harm	Substantive risk	Reasons
	animal proteins	<p>↓</p> <p>Exposure of people at the production facility via inhalation of aerosols or contact</p> <p>↓</p> <p>Expression of proteins</p>			<p>exposure of people to the GM yeast.</p> <ul style="list-style-type: none"> • The production facility will be limited to one location. • The facility will have restricted access. • Closed system equipment include probes for monitoring culture parameters; ports for inputs and sample collection limit exposure to GM yeast. • Transfer of fluids via stainless steel piping or through aseptic transfer vessels, will prevent people from getting in direct contact with GM yeast. • The GM yeast is not expected to colonise human gut and form sustainable population, so any effect will be transitory. • The introduced proteins are ubiquitously present in the environment and many in the human diet.
2	GM yeast secreting animal proteins	<p>Culture of GM yeast expressing animal proteins</p> <p>↓</p> <p>Escape of GM yeast into the environment via:</p> <ul style="list-style-type: none"> • Leakage from culture tanks or pipes • During sample collection • Transport of slurry or GMO waste 	Adverse effects in people or animals	No	<ul style="list-style-type: none"> • The proposed limits and controls would minimise the likelihood of dispersal of the GM yeast. • Closed systems will prevent animals getting into contact with GM yeast. • The culture tanks and pipes will be regularly inspected for any leakage. • The GM yeast are developed from

Risk scenario	Risk source	Possible causal pathway	Potential harm	Substantive risk	Reasons
		<p>↓</p> <p>Presence of GM yeast outside the production facility</p> <p>↓</p> <p>Establishment and persistence of GM yeast in the environment</p>			<p>laboratory strains which require a conducive culture environment and hence, are not likely to persist in the environment.</p> <ul style="list-style-type: none"> • It is not expected that the introduced genes would confer any selective advantage to the GM yeast to establish and persist in the environment. • Decontaminated and denatured (where antibiotic resistance genes are used) slurry will be tested for the absence of GMOs and antibiotic resistance genes, if relevant. • All GMO containing waste will be autoclaved or chemically disinfected before being disposed of. • Transport according to TSD guidelines • There are no flowing waterways nearby the facility that would facilitate the GM yeast from escaping and spreading in the environment in case of any extreme weather event.

2.5 Risk scenario 1

Risk source	GM yeast secreting animal proteins
Causal pathway	Fermentation of GM yeast expressing the introduced gene ↓ Exposure of people at the production facility via inhalation of aerosols or contact ↓ Expression of proteins
Potential harm	Allergenicity in people

2.5.1 Risk source

123. The source of potential harm for this postulated risk scenario is the GM yeast, which secretes animal proteins.

2.5.2 Causal Pathway

124. The GM yeast will be cultured at the production facility during which time animal proteins will be expressed.

125. People working at the production facility may be exposed to GM yeast and the expressed protein via direct contact with the GM yeast culture solution or by inhalation of aerosolised GM yeast resulting in exposure to expressed proteins causing allergenicity. This would be most likely to occur when people are working with GM yeast, e.g., while monitoring culture conditions, during separation of the GM yeast and the supernatant or during failure of closed systems, e.g., over pressurisation resulting in bursting of a fermenter and leakage or spill during transfer of GM yeast to a fermenter.

126. As mentioned in Chapter 1, Section 2.3.1, culture medium can be aerosolised by bubbles bursting in foams or in aerated fermenters. This could lead to inhalation of aerosolised GM yeast. Spills or leaks during culturing, harvest or post-harvest cleaning may lead to dermal contact of people with GM yeast.

127. As mentioned in Chapter 1, fermentation will be carried out using a closed system which will have probes for monitoring culture parameters; filtered gas exchange vents, ports for inputs and sample collection, thus preventing the need to open the system. Further, transfer of fluids will be via stainless steel piping or through aseptic transfer vessels. Moreover, staff working with the GM yeast will be trained and will wear appropriate PPE, which includes face masks and gloves. All these measures will prevent people from aerosol exposure or from direct contact with GM yeast cultures.

128. Allergy to milk and egg proteins is most common in young children and decreases rapidly with age (Allen et al., 2007; Jensen et al., 2022). Young children will not be present in the production facility. The most common route of exposure to these allergenic proteins is via the diet. As stated in paragraph 128, ingestion of the GM yeast expressing the milk and egg proteins is highly unlikely to occur as GM yeast will be cultured in closed systems and appropriate PPE will be worn.

129. The production process will be restricted to one location and for five years from the date of issue of the licence. Access to the production facility would be only given to authorised and appropriately trained people. This means that a limited number of people would be in the production facility or handling the GM yeast.

130. As mentioned in Chapter 1, GM yeast from this protein production process would not be used for human food and only decontaminated yeast slurry would be used as a soil conditioner or in animal feed preparation. The GM yeast will be decontaminated either by exposure to heat or chemicals. Decontamination methods would be tested for their efficiency in the initial stages. This would limit the exposure of people to viable GM yeast.

131. In the event of a spill or leakage, spill kits placed adjacent to the fermenters would be used. The spill kits contain absorbent material, PPE and decontamination solution. This could be deployed quickly to soak up and kill the GM yeast. In case of large spills, the drains lead to a small sump with level sensors which activate pumps to transfer the liquids to a concrete tank or 3 x 22 kL holding tanks for chemical disinfection.

132. These measures are expected to minimise exposure of people to GM yeast during a spill or leakage.

133. Overall, the proposed limits and controls minimise the potential for exposure of GM yeast to people working at the production facility.

2.5.3 Potential harm

134. In the case of exposure to GM yeast expressing bovine milk and chicken egg proteins, a range of allergic symptoms could occur (Chapter 1, Section 4.1.1.4). The most common symptoms reported in allergic individuals as a result of bovine milk and chicken egg consumption are anaphylaxis, asthma, rhinitis, atopic dermatitis, urticaria and gastrointestinal disorders. An allergic reaction is expected to occur rapidly, over the space of minutes to hours. Treatment ranges from antihistamines, epinephrine, glucocorticoids, and inhaled beta-agonists. Anaphylaxis is a systemic, rapid onset hypersensitivity reaction (Cardona et al., 2020). While anaphylaxis is a serious condition that may require hospitalisation, fatal and near-fatal events are rare (Umasunthar et al., 2013; Turner et al., 2017). An analysis of data from 1970 European children in 10 countries with anaphylaxis to food, there were a total of 5 fatal anaphylactic reactions, with 2 attributed to cow's milk and none to eggs (Grabenhenrich et al., 2016). In fact, out of a total of 134 children reported to have egg allergy, none experienced life-threatening anaphylaxis.

135. The standard of care for anaphylaxis is an intramuscular injection of adrenaline (epinephrine) to reduce the risk of death (Prince et al., 2018).

136. As discussed in Chapter 1, Section 4.1.1.3, spider silk fibre proteins are not known to be allergens and *P. pastoris* is not known to be toxic or pathogenic, therefore, exposure of people to GM yeast expressing spider silk protein is unlikely to result in harm to people.

137. People are generally exposed to bovine milk and chicken egg proteins as they form part of their daily diet, and a very small proportion of adults have allergic reactions to them. However, people who have allergies to the bovine milk and chicken egg proteins could have an allergic reaction through direct contact or from the inhalation of aerosolised GM yeast (Chapter 1, Section 4.1.1.4).

138. Given the widespread consumption of bovine milk and chicken egg proteins, it is highly unlikely that a person would not be aware of their allergy and would protect themselves against exposure to these proteins. The applicant has further advised that no current staff have any allergies or food intolerances to proteins proposed to be used under this licence application.

2.5.4 Conclusion

139. Risk scenario 1 is not identified as a substantive risk due to limited potential for exposure to the GM yeast, their introduced genes, and their encoded proteins and the low prevalence of contact or inhalation allergies to these proteins (Chapter 1, Section 4.1.1.4). Therefore, this risk could not be considered greater than negligible and does not warrant further detailed assessment.

2.6 Risk scenario 2

Risk source	GM yeast secreting animal protein
Causal pathway	<p>Culture of GM yeast expressing animal proteins</p> <p style="text-align: center;">↓</p> <p>Escape of GM yeast into the environment via:</p> <ul style="list-style-type: none"> • Leakage from fermenters or pipes • During sample collection • Transport of slurry or GM yeast waste <p style="text-align: center;">↓</p> <p>Presence of GM yeast outside the production facility</p> <p style="text-align: center;">↓</p> <p>Establishment and persistence of GM yeast in the environment</p>
Potential harm	Adverse effects in people or animals

2.6.1 Risk source

140. The source of potential harm for this postulated risk scenario is the GM yeast, which secretes animal proteins.

2.6.2 Causal Pathway

141. The GM yeast could escape from the production facility during events such as leakage from fermenters and pipelines, sample collection or transport of partially decontaminated GM yeast. The GM yeast could establish and persist in the environment. This could result in exposure of people, other desirable organisms, or the environment to the introduced genes and proteins.

Potential for persistence at the production facility

142. The GM yeast could persist at the production facility:

- in fermenters between batches of GM yeast culture
- in other fermenters, in which non-GM *P. pastoris* are grown while the GM yeast is present at the production facility
- in equipment other than fermenters used in connection with the GM yeast or
- in spills of culture solution suitable to grow yeast.

143. The applicant has proposed that GM yeast cultures will be grown at the site in closed systems; all equipment used in connection with the GM yeast will be cleaned/sterilised after use and before use for a new batch; and to regularly inspect equipment for any leakages. These controls would minimise the chances of GM yeast persisting in the production facility.

144. As mentioned above in risk scenario 1, if there is any leak or spill, area or cultures will be decontaminated using chemicals. Alternatively, drains lead to a sump from where the liquid is transferred to a concrete or holding tanks for chemical decontamination.

145. In addition, the GM yeast could persist at the production facility due to ineffective decontamination of GM yeast. The applicant has proposed to inactivate the GM yeast by either heat or chemical inactivation. The efficiency of these decontamination methods will be tested during initial stages.

Dispersal through human activity

146. Dispersal through human activity could occur if staff did not adhere to protocols or by accident. For example, GM yeast could be transported on clothing or footwear following a spill of GM yeast at the production facility.

147. Access to the site would be restricted to authorised, trained staff. The site will be checked for spills of GM yeast culture solution and action will be taken to decontaminate any such spills. All GM yeast would be transported in accordance with the Regulator's Guidelines for the Transport, Storage and Disposal of GMOs, which would minimise the opportunity for dispersal of GM yeast.

Dispersal by animals

148. The fermentation process will be conducted in closed systems. This will prevent access by wild or domestic animals to the facility and being exposed to the GM yeast. However, in case of any leak or spill, there is a chance that birds or rodents could be exposed to the GM yeast.

2.6.3 Potential harm

149. The potential harms for this risk scenario are that the GM yeast could spread and persist in the environment. This could lead to exposure of animals and people potentially causing adverse effects. The potential harms for this risk scenario for people are the same as the potential harms described in detail in Risk Scenario 1.

150. As discussed in Chapter 1, Section 3.5, chickens and mice fed with yeast had beneficial probiotic effects. The yeast itself and proteins expressed in GM yeast are commonly present in the environment and there are no reports of toxicity in animals.

151. The GM yeast could spread and persist in the environment as an invasive species and out compete native yeast. However, as the strains used for development of GM yeast are laboratory strains that require a conducive environment for growth, they are not expected to survive in the environment.

152. The production facility is located where the natural hosts of *P. pastoris* such as oak or chestnut trees do not exist. This will minimise the GM yeast from finding a suitable environment to spread and persist.

153. Further, there are no flowing waterways nearby the facility that would facilitate the GM yeast from escaping and spreading in the environment in case of any extreme weather event.

2.6.4 Conclusion

154. Risk scenario 2 is not identified as a substantive risk due to the limited ability of the GM yeast to establish and persist outside of culture conditions at a density that would have an appreciable adverse effect on the biotic environment or desirable organisms. Therefore, this risk could not be considered greater than negligible and does not warrant further detailed assessment.

Section 3 Uncertainty

155. Uncertainty is an intrinsic part of risk analysis and is present in all aspects of risk analysis. This is discussed in detail in the Regulator's Risk Analysis Framework document.

156. Uncertainty is addressed by approaches such as balance of evidence, conservative assumptions, and applying risk management measures that reduce the potential for risk scenarios involving uncertainty to lead to harm. If there is residual uncertainty that is important to estimating the level of risk, the Regulator will take this uncertainty into account in making decisions.

157. For DIR 200, uncertainty is noted particularly in relation to:

- Molecular characterisation of the GM yeast including gene sequences and amino acid sequences of the expressed proteins;
- potential for allergenicity of GM yeast expressing the introduced milk, egg and spider silk proteins to people;
- potential for GM yeast to sporulate or form adaptable structures under culture conditions;
- validity of decontamination of GMOs in large volumes; and denaturation methods (where antibiotic resistance genes are present).

158. Additional data, including information to address these uncertainties, may be required to assess possible future applications with reduced limits and controls, such as the commercial release of these GM yeast.

159. Chapter 3, Section 4, discusses information that may be required for future release.

Section 4 Risk evaluation

160. Risk is evaluated against the objective of protecting the health and safety of people and the environment to determine the level of concern and, subsequently, the need for controls to mitigate or reduce risk. Risk evaluation may also aid consideration of whether the proposed dealings should be authorised, need further assessment, or require collection of additional information.

161. Factors used to determine which risks need treatment may include:

- risk criteria,
- level of risk,
- uncertainty associated with risk characterisation, and
- interactions between substantive risks.

162. Two risk scenarios were postulated whereby the proposed dealing might give risk to harm to people or the environment.

163. A risk is substantive only when the risk scenario may, because of gene technology, have some chance of causing harm. Risk scenarios that do not lead to harm, or could not reasonably occur, do not represent a substantive risk and do not advance in the risk assessment process.

164. Two risk scenarios were postulated whereby the proposed dealings might give rise to harm to people or the environment. In the context of the control measures proposed by the applicant, and considering both the short and long term, neither of these scenarios were identified as substantive risks. The principal reasons for these conclusions are summarised in Table 2 and include:

- fermentation will occur in closed systems.
- no GM yeast would enter human food;
- limits on the duration of the proposed release;
- people conducting dealings will wear appropriate PPE;
- GM yeast will be decontaminated and denatured (where antibiotic resistance genes are present) before being used in animal feed preparation or disposed of as soil conditioner;
- waste containing GM yeast will be autoclaved before its disposal; and
- suitability of proposed controls to restrict the spread and persistence of the GM yeast and its genetic material.

165. Therefore, risks to the health and safety of people, or the environment, from the proposed release of the GM yeast into the environment are considered negligible. The *Risk Analysis Framework* (OGTR, 2013), which guides the risk assessment and risk management process, defines negligible risks as risks of no discernible concern with no present need to invoke actions for mitigation.

Therefore, no controls are required to treat these negligible risks. Hence, the Regulator considers that the dealings involved in this proposed release do not pose a significant risk to either people or the environment⁵.

⁵ As none of the proposed dealings are considered to pose a significant risk to people or the environment, Section 52(2)(d)(ii) of the Act mandates a minimum period of 30 days for consultation on the RARMP.

Chapter 3 Risk management plan

Section 1 Background

166. Risk management is used to protect the health and safety of people and to protect the environment by controlling or mitigating risk. The risk management plan addresses risks evaluated as requiring treatment and considers limits and controls proposed by the applicant, as well as general risk management measures. The risk management plan informs the Regulator's decision-making process and is given effect through proposed licence conditions.

167. Under section 56 of the Act, the Regulator must not issue a licence unless satisfied that any risks posed by the dealings proposed to be authorised by the licence are able to be managed in a way that protects the health and safety of people and the environment.

168. All licences are subject to three conditions prescribed in the Act. Section 63 of the Act requires that each licence holder inform relevant people of their obligations under the licence. The other statutory conditions allow the Regulator to maintain oversight of licensed dealings: Section 64 requires the licence holder to provide access to premises to OGTR inspectors and Section 65 requires the licence holder to report any information about risks or unintended effects of the dealing to the Regulator on becoming aware of them. Matters related to the ongoing suitability of the licence holder are also required to be reported to the Regulator.

169. The licence is also subject to any conditions imposed by the Regulator. Examples of the matters to which conditions may relate are listed in Section 62 of the Act. Licence conditions have been imposed to limit and control the scope of the dealings. In addition, the Regulator has extensive powers to monitor compliance with licence conditions under Section 152 of the Act.

Section 2 Risk treatment measures for substantive risks

170. The risk assessment of risk scenarios listed in Chapter 2 concluded that there are negligible risks to people and the environment from the proposed trial of GM yeast. The risk scenarios were considered in the context of the proposed release (Chapter 1 Section 2), the proposed control measures (Chapter 1, Section 2.2) and the receiving environment (Chapter 1 Section 5), and considering both the short and the long term. The risk evaluation concluded that no specific risk treatment measures are required to treat these negligible risks. Limits and controls proposed by the applicant and other general risk management measures are discussed below.

Section 3 General risk management

171. The limits and controls proposed in the application were important in establishing the context for the risk assessment and in reaching the conclusion that the risks posed to people and the environment are negligible to low. Therefore, to maintain the risk context, licence conditions have been imposed to limit the number of production trial sites and duration of the trial, as well as a range of controls to restrict the spread and persistence of the GMOs and their genetic material in the environment. The conditions are discussed and summarised in this Chapter and listed in detail in the licence.

Limits and controls on the release

172. Section 2.1 and Section 2.2 in Chapter 1 list the limits and controls proposed by the applicant in their application. Many of these are discussed in the two risk scenarios considered in Chapter 2. The appropriateness of the limits and controls is considered further in the following sections.

3.1.1 Consideration of limits proposed by Cauldron

173. The proposed work would take place at a single location at Cauldron's purpose-built protein production facility in NSW, which is owned and managed by Cauldron. The production trials would be run over eight single batches per annum and eight continuous batches per annum where GM yeast would be cultured in volumes of up to 12.5 kL. The proposal is to run these production trials for five years from the issue of licence. The limited size and duration of the trial would restrict the potential exposure of people and desirable animals to the GM yeast (Risk scenario 1). Considering a possibility that some culture runs might fail, a restriction on the number of runs per annum has not been imposed in the licence. Taking into consideration the limits proposed, a licence condition is included to restrict the location to one production facility and licence period to five years from the issue of the licence and to restrict the trial to one site.

174. The applicant proposes that only trained and authorised staff would be permitted to deal with the GM yeast. Standard conditions included in the licence state that only people authorised by the licence holder are covered by the licence and that the licence holder must inform all people dealing with the GM yeast of applicable licence conditions. These measures would limit the exposure of people to potential harm from the GM yeast.

3.1.2 Consideration of proposed controls to manage exposure to the GMOs

175. Access to the GM yeast is restricted to authorised people and this is included as a licence condition. This condition restricts the exposure of people not authorised under the licence and animals to the GM yeast.

176. The applicant has proposed that staff would be wearing appropriate PPE when required. A licence condition is included that requires staff to wear coveralls, P2 mask and gloves where there is possibility of exposure to GM yeast.

177. The applicant has indicated that all waste from the GM yeast protein production process would be disposed of in a manner that would not disperse GM yeast. A licence condition is included that allows only decontaminated and denatured (where antibiotic resistance genes are present) GM yeast to be used as a soil conditioner or in animal feed preparation.

178. The Applicant has proposed that transport of GMOs will follow Regulator's *Guidelines for Transport, Storage and Disposal of GMOs* (TSD guidelines). Licence conditions require that transport complies with minimum requirements for packaging and labelling the GMO for risk group 1 organisms according to the TSD guidelines.

179. A licence condition requires that a record of each GM yeast including its genetic modification is kept and made available to the Regulator, if requested.

3.1.3 Consideration of proposed controls to manage dispersal and persistence of the GMOs

180. The applicant has proposed control measures to limit the dispersal of GM yeast during the fermentation process. The applicant proposes that fermentation will be carried out in closed fermenters to minimise the likelihood of aerosol release, direct contact and ingestion. The closed system will have several inlet and outlet ports to allow inoculation of cultures, insertion of fixed and retractable probes, and supply and release of gases. The applicant also states that liquids will be transferred through stainless steel piping or transfer vessels. The closed fermenters, stainless-steel pipes and transfer vessels would be decontaminated before and after contact with the GM yeast. These controls are considered appropriate to minimise the likelihood of dispersal of the GM yeast and are covered in several licence conditions.

181. The applicant has proposed physical and behavioural control measures to limit the dispersal of GM yeast during harvest, by minimising aerosol formation, direct contact and ingestion. GM yeast are separated by pumping the culture solution from the fermenters directly to the centrifuge. The

residual slurry will be transferred to a vessel where GM yeast will be decontaminated and the supernatant transferred for further filtration through a stainless-steel pipe network. All the vessels and piping network will be inspected regularly for any leakage. These controls are considered appropriate and are included in the licence.

182. The methodology for decontamination and denaturation (where antibiotic resistance genes are present) of the GMOs will be tested during the initial stages of batch culture for effectiveness of killing and denaturing the GM yeast. A licence condition is included that stipulates that both methods of heat and chemical inactivation be tested and validated for their effectiveness in killing the GM yeast after the first run and these results must be provided to the Regulator before proceeding with further runs. In addition, where antibiotic resistance genes are present in the GM yeast, the GM yeast is also required to be denatured using validated methods and these results must be provided to the Regulator before proceeding with further runs.

183. The applicant proposed that all other waste such as filter residue and filter material containing GM yeast would be disposed of via autoclaving. This is an acceptable means of disposing of the GM yeast and is included in the licence.

184. The applicant has proposed a contingency plan detailing several steps to deal with any inadvertent small spills. As an additional back-up, for example in case of a large spill volume, the drain in the culture area could be blocked and the GM yeast culture pumped to a holding tank where it could be chemically inactivated. These steps are considered appropriate and are imposed as licence conditions.

185. A standard condition is included in the licence requiring the licence holder to ensure that dealings are conducted to not compromise the health and safety of people and minimise unintentional exposure to the GM yeast.

3.1.4 *Summary of licence conditions to be implemented to limit and control the dealings*

186. A number of licence conditions have been drafted to limit and control the proposed dealings, based on the above considerations. These include requirements to:

- limit the duration of the release to a maximum of five years from issue of the licence;
- limit the release to a single location in NSW: Cauldron's protein production facility at Borenore, NSW;
- the fermenters be of closed systems;
- inspect the culture area for spilled media regularly during culture of the GM yeast;
- culture and harvest GM yeast in a manner that avoids dispersal of the GM yeast;
- clean areas and equipment exposed to GM yeast after use;
- clean any equipment used with the GM yeast before use for any other purpose;
- destroy all GM yeast not required for further experiments or future trials;
- transport and store the GM yeast in accordance with the Regulator's guidelines;
- not allow the GM yeast to be used for human food;
- only decontaminated and denatured (where antibiotic resistance genes are present) GM yeast may be used as soil conditioner or in animal feed preparation;
- not allow the GM yeast to be released into the environment; and
- dispose of GM yeast via autoclave or effective chemical decontamination methods.

Other risk management considerations

187. All DIR licences issued by the Regulator contain a number of conditions that relate to general risk management. These include conditions relating to:

- applicant suitability

- contingency plans
- identification of the persons or classes of persons covered by the licence
- reporting requirements
- access for the purpose of monitoring for compliance.

3.1.5 *Applicant suitability*

188. In making a decision whether or not to issue a licence, the Regulator must have regard to the suitability of the applicant to hold a licence. Under Section 58 of the Act, matters that the Regulator must take into account include:

- any relevant convictions of the applicant
- any revocation or suspension of a relevant licence or permit held by the applicant under a law of the Commonwealth, a State or a foreign country
- the capacity of the applicant to meet the conditions of the licence.

189. A licence condition requires the licence holder to inform the Regulator of any information that would affect their suitability.

190. In addition, the applicant organisation must have access to an IBC and be an accredited organisation under the Act.

3.1.6 *Contingency plans*

191. The applicant has submitted a contingency which details measures to be undertaken in the event of any unintended presence of the GM yeast outside permitted areas.

192. Before culturing the GM yeast, the applicant would also be required to provide the Regulator with a method to detect the GM yeast reliably and uniquely.

3.1.7 *Identification of the persons or classes of persons covered by the licence*

193. The persons covered by the licence will be the licence holder and employees, agents or contractors of the licence holder and other persons who are, or have been, engaged or otherwise authorised by the licence holder to undertake any activity in connection with the dealings authorised by the licence.

194. Prior to culture of the GM yeast, the applicant would be required to provide the role and contact details for key persons responsible for the management of the protein production process at the site; and the persons or class of persons working with the GMOs.

3.1.8 *Reporting requirements*

195. The licence requires the licence holder to immediately report any of the following to the Regulator:

- any additional information regarding risks to the health and safety of people or the environment associated with the dealings
- any contraventions of the licence by persons covered by the licence
- any unintended effects of the dealings.

3.1.9 *Monitoring for compliance*

196. The Act stipulates, as a condition of every licence, that a person who is authorised by the licence to deal with a GMO, and who is required to comply with a condition of the licence, must allow inspectors and other persons authorised by the Regulator to enter premises where a dealing is being undertaken for the purpose of monitoring or auditing the dealing.

197. If monitoring activities identify changes in the risks associated with the authorised dealings, the Regulator may also vary licence conditions, or if necessary, suspend or cancel the licence.

198. In cases of non-compliance with licence conditions, the Regulator may instigate an investigation to determine the nature and extent of non-compliance. The Act provides for criminal sanctions of large fines and/or imprisonment for failing to abide by the legislation, conditions of the licence or directions from the Regulator, especially where significant damage to the health and safety of people or the environment could result.

Section 4 Issues to be addressed for future releases

199. Additional information has been identified that may be required to assess an application for a commercial release of these GM yeast, or to justify a reduction in limits and controls. This includes:

- additional molecular and biochemical characterisation of the GM yeast, including data on allergenicity; and
- molecular characterisation of the recombinant proteins, including amino acid sequence, to demonstrate equivalence to native proteins.

Section 5 Conclusions of the consultation RARMP

200. The RARMP concludes that the proposed limited and controlled release of GM yeast poses negligible risks to the health and safety of people or the environment as a result of gene technology, and that these negligible risks do not require specific risk treatment measures.

201. Conditions are imposed on the location and duration of the release, and to restrict the spread and persistence of the GM yeast and their genetic material in the environment, as these were important considerations in establishing the context for assessing the risks.

References

- Abeyrathne, E.D.N.S., Lee, H.Y., and Ahn, D.U. (2013). Egg white proteins and their potential use in food processing or as nutraceutical and pharmaceutical agents—A review. *Poultry Science* 92, 3292-3299.
- Ahmad, M., Hirz, M., Pichler, H., and Schwab, H. (2014). Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Appl Microbiol Biotechnol* 98, 5301-5317.
- AIFS (2023). 10 Most common food allergens in Australia. (Australian Institute of Food Safety).
- Allen, C.W., Campbell, D.E., and Kemp, A.S. (2007). Egg allergy: are all childhood food allergies the same? *J Paediatr Child Health* 43, 214-218.
- American Type Culture Collection (2023). *Komagataella pastoris*.
- Ata, Ö., Ergün, B.G., Fickers, P., Heistering, L., Mattanovich, D., Rebnegger, C., and Gasser, B. (2021). What makes *Komagataella phaffii* non-conventional? *FEMS Yeast Res* 21.
- Bar-On, O., Levine, H., Stafler, P., Shmueli, E., Jacobi, E., Goldberg, O., Steuer, G., *et al.* (2022). Lactose-Containing Dry-Powder Inhalers for Patients with Cow's Milk Protein Allergy-The Conundrum; A National Survey of Pediatric Pulmonologists and Allergologists. *J Clin Med* 11.
- Barone, G.D., Emmerstorfer-Augustin, A., Biundo, A., Pisano, I., Coccetti, P., Mapelli, V., and Camattari, A. (2023). Industrial Production of Proteins with *Pichia pastoris*-*Komagataella phaffii*. *Biomolecules* 13.
- Becerril-García, M.Á., Flores-Maldonado, O.E., González, G.M., García-González, G., Hernández-Bello, R., and Palma-Nicolás, J.P. (2022). Safety profile of intravenous administration of live *Pichia pastoris* cells in mice. *FEMS Yeast Research* 22.
- Bernauer, L., Radkohl, A., Lehmayr, L.G.K., and Emmerstorfer-Augustin, A. (2020). *Komagataella phaffii* as Emerging Model Organism in Fundamental Research. *Front Microbiol* 11, 607028.
- Birmann, P.T., Casaril, A.M., Pesarico, A.P., Caballero, P.S., Smaniotto, T.Â., Rodrigues, R.R., Moreira, Â.N., *et al.* (2021). *Komagataella pastoris* KM71H modulates neuroimmune and oxidative stress parameters in animal models of depression: A proposal for a new probiotic with antidepressant-like effect. *Pharmacological Research* 171, 105740.
- Bivehed, E., Hellman, B., Fan, Y., Haglöf, J., and Buratovic, S. (2023). DNA integrity under alkaline conditions: An investigation of factors affecting the comet assay. *Mutat Res Genet Toxicol Environ Mutagen* 891, 503680.
- Brady, J.R., Whittaker, C.A., Tan, M.C., Kristensen, D.L., 2nd, Ma, D., Dalvie, N.C., Love, K.R., *et al.* (2020). Comparative genome-scale analysis of *Pichia pastoris* variants informs selection of an optimal base strain. *Biotechnol Bioeng* 117, 543-555.
- Cardona, V., Ansotegui, I.J., Ebisawa, M., El-Gamal, Y., Fernandez Rivas, M., Fineman, S., Geller, M., *et al.* (2020). World allergy organization anaphylaxis guidance 2020. *World Allergy Organization Journal* 13, 100472.

- Caubet, J.C., and Wang, J. (2011). Current understanding of egg allergy. *Pediatr Clin North Am* 58, 427-443, xi.
- Chang, S.W., Shieh, C.J., Lee, G.C., Akoh, C.C., and Shaw, J.F. (2006). Optimized growth kinetics of *Pichia pastoris* and recombinant *Candida rugosa* LIP1 production by RSM. *J Mol Microbiol Biotechnol* 11, 28-40.
- Cremonte, E.M., Galdi, E., Roncallo, C., Boni, E., and Cremonte, L.G. (2021). Adult onset egg allergy: a case report. *Clinical and Molecular Allergy* 19, 17.
- Davoodi, S.H., Shahbazi, R., Esmaeili, S., Sohrabvandi, S., Mortazavian, A., Jazayeri, S., and Taslimi, A. (2016). Health-Related Aspects of Milk Proteins. *Iran J Pharm Res* 15, 573-591.
- De Schutter, K., Lin, Y.C., Tiels, P., Van Hecke, A., Glinka, S., Weber-Lehmann, J., Rouzé, P., *et al.* (2009). Genome sequence of the recombinant protein production host *Pichia pastoris*. *Nat Biotechnol* 27, 561-566.
- Docena, G.H., Fernandez, R., Chirido, F.G., and Fossati, C.A. (1996). Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy* 51, 412-416.
- Du, J., Hincke, M.T., Rose-Martel, M., Hennequet-Antier, C., Brionne, A., Cogburn, L.A., Nys, Y., *et al.* (2015). Identifying specific proteins involved in eggshell membrane formation using gene expression analysis and bioinformatics. *BMC Genomics* 16, 792.
- EFSA (2023). Updated list of QPS-recommended microorganisms for safety risk assessments carried out by EFSA.
- EFSA, Panel o.D.P.N., Allergies, Turck, D., Bresson, J.-L., Burlingame, B., Dean, T., Fairweather-Tait, S., *et al.* (2018). Safety of egg membrane hydrolysate as a novel food pursuant to Regulation (EU) 2015/2283. *EFSA Journal* 16, e05363.
- Enache-Angoulvant, A., and Hennequin, C. (2005). Invasive *Saccharomyces* infection: a comprehensive review. *Clinical Infectious Diseases* 41, 1559-1568.
- Escudero, C., Quirce, S., Fernández-Nieto, M., Miguel, J., Cuesta, J., and Sastre, J. (2003). Egg white proteins as inhalant allergens associated with baker's asthma. *Allergy* 58, 616-620.
- FSANZ (2020). Risk and technical assessment report – Application A1186 Soy leghemoglobin in meat analogue products, FSANZ, ed.
- FSANZ (2023). Food allergies.
- Gao, J., Jiang, L., and Lian, J. (2021). Development of synthetic biology tools to engineer *Pichia pastoris* as a chassis for the production of natural products. *Synth Syst Biotechnol* 6, 110-119.
- Gil de Los Santos, D., Gil de Los Santos, J.R., Gil-Turnes, C., Gaboardi, G., Fernandes Silva, L., França, R., Gevehr Fernandes, C., *et al.* (2018). Probiotic effect of *Pichia pastoris* X-33 produced in parboiled rice effluent and YPD medium on broiler chickens. *PLoS One* 13, e0192904.
- Gil de los Santos, J.R., Storch, O.B., Fernandes, C.G., and Gil-Turnes, C. (2012). Evaluation in broilers of the probiotic properties of *Pichia pastoris* and a recombinant *P. pastoris* containing the *Clostridium perfringens* alpha toxin gene. *Veterinary Microbiology* 156, 448-451.

- Gonçalves, P., and Gonçalves, C. (2022). Horizontal gene transfer in yeasts. *Curr Opin Genet Dev* 76, 101950.
- Grabenhenrich, L.B., Dölle, S., Moneret-Vautrin, A., Köhli, A., Lange, L., Spindler, T., Ruëff, F., *et al.* (2016). Anaphylaxis in children and adolescents: The European Anaphylaxis Registry. *J Allergy Clin Immunol* 137, 1128-1137.e1121.
- Han, C., Chen, Y., Shi, L., Chen, H., Li, L., Ning, Z., Zeng, D., *et al.* (2023). Advances in eggshell membrane separation and solubilization technologies. *Frontiers in Veterinary Science* 10.
- Hays, H., Elliker, P.R., and Sandine, W.E. (1967). Microbial destruction by low concentrations of hypochlorite and iodophor germicides in alkaline and acidified water. *Applied Microbiology* 15, 575-581.
- Jarmoc, L.M., and Primack, W.A. (1987). Anaphylaxis to cutaneous exposure to milk protein in a diaper rash ointment. *Clin Pediatr (Phila)* 26, 154-155.
- Jensen, S.A., Fiocchi, A., Baars, T., Jordakieva, G., Nowak-Wegrzyn, A., Pali-Schöll, I., Passanisi, S., *et al.* (2022). Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines update - III - Cow's milk allergens and mechanisms triggering immune activation. *World Allergy Organ J* 15, 100668.
- Kampf, G. (2018). Ethanol. In *Antiseptic Stewardship: Biocide Resistance and Clinical Implications*, G. Kampf, ed. (Cham: Springer International Publishing), pp. 9-35.
- Karbalaei, M., Rezaee, S.A., and Farsiani, H. (2020). *Pichia pastoris*: A highly successful expression system for optimal synthesis of heterologous proteins. *J Cell Physiol* 235, 5867-5881.
- Korukluoglu, M., Sahan, Y., and Yigit, A. (2006). The fungicidal efficacy of various commercial disinfectants used in the food industry. *Annals of Microbiology* 56, 325-330.
- Kurtzman, C.P. (2005). Description of *Komagataella phaffii* sp. nov. and the transfer of *Pichia pseudopastoris* to the methylotrophic yeast genus *Komagataella*. *Int J Syst Evol Microbiol* 55, 973-976.
- Kwolek-Mirek, M., Bartosz, G., and Spickett, C.M. (2011). Sensitivity of antioxidant-deficient yeast to hypochlorite and chlorite. *Yeast* 28, 595-609.
- Lam, H.-Y., Van Hoffen, E., Michelsen, A., Guikers, K., Van Der Tas, C.H.W., Bruijnzeel-Koomen, C.A.F.M., and Knulst, A.C. (2008). Cow's milk allergy in adults is rare but severe: both casein and whey proteins are involved. *Clinical & Experimental Allergy* 38, 995-1002.
- Legros, J., Jan, S., Bonnassie, S., Gautier, M., Croguennec, T., Pezennec, S., Cochet, M.F., *et al.* (2021). The Role of Ovotransferrin in Egg-White Antimicrobial Activity: A Review. *Foods* 10.
- Leser, C., Hartmann, A.L., Praml, G., and Wüthrich, B. (2001). The "egg-egg" syndrome: occupational respiratory allergy to airborne egg proteins with consecutive ingestive egg allergy in the bakery and confectionery industry. *J Investig Allergol Clin Immunol* 11, 89-93.
- Li, Z., Huang, X., Tang, Q., Ma, M., Jin, Y., and Sheng, L. (2022). Functional Properties and Extraction Techniques of Chicken Egg White Proteins. *Foods* 11, 2434.

- Liccardi, G., De Falco, F., Gilder, J.A., D'Amato, M., and D'Amato, G. (2004). Severe systemic allergic reaction induced by accidental skin contact with cow milk in a 16-year-old boy. A case report. *J Investig Allergol Clin Immunol* 14, 168-171.
- Mortimer, R.K. (2000). Evolution and variation of the yeast (*Saccharomyces*) genome. *Genome Research* 10, 403-409.
- Nakonechna, A., Matthews, D., and Sargur, R. (2019). Occupational asthma, rhinitis, contact dermatitis, and severe milk allergy caused by primary occupational exposure to casein. *Ann Allergy Asthma Immunol* 123, 224-225.
- OGTR (2013). Risk Analysis Framework 2013, 4th edn (Canberra: Office of the Gene Technology Regulator).
- Prince, B.T., Mikhail, I., and Stukus, D.R. (2018). Underuse of epinephrine for the treatment of anaphylaxis: missed opportunities. *Journal of Asthma and Allergy* 11, 143-151.
- Public Health Agency of Canada (2023). ePATHogen - Risk Group Database.
- Ramezaniaghdam, M., Nahdi, N.D., and Reski, R. (2022). Recombinant Spider Silk: Promises and Bottlenecks. *Frontiers in Bioengineering and Biotechnology* 10.
- Ramirez, D.A., Jr., and Bahna, S.L. (2009). Food hypersensitivity by inhalation. *Clin Mol Allergy* 7, 4.
- Rebnegger, C., Vos, T., Graf, A.B., Valli, M., Pronk, J.T., Daran-Lapujade, P., and Mattanovich, D. (2016). *Pichia pastoris* Exhibits High Viability and a Low Maintenance Energy Requirement at Near-Zero Specific Growth Rates. *Appl Environ Microbiol* 82, 4570-4583.
- Robles, J., and Motheral, L. (2014). Hypersensitivity reaction after inhalation of a lactose-containing dry powder inhaler. *J Pediatr Pharmacol Ther* 19, 206-211.
- Römer, L., and Scheibel, T. (2008). The elaborate structure of spider silk: structure and function of a natural high performance fiber. *Prion* 2, 154-161.
- Rona, R.J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., Sigurdardottir, S.T., *et al.* (2007). The prevalence of food allergy: a meta-analysis. *J Allergy Clin Immunol* 120, 638-646.
- Sa, A.B., Oliveira, L.C.L., Miyagi, K.V.M., Mello, Y.A.M.F., Cabral, E.C., Carvalho, A.P.E., Komaroff, F.F., *et al.* (2011). Reaction Due To Milk Proteins Contaminating Lactose Added To Inhaled Corticosteroid. *Journal of Allergy and Clinical Immunology* 127, AB241.
- Schichter-Konfino, V., Almog, M., Bamberger, E., Berkowitz, D., and Kessel, A. (2015). The significance of allergic contact urticaria to milk in children with cow's milk allergy. *Pediatric Allergy and Immunology* 26, 218-222.
- Schnell, J., Steele, M., Bean, J., Neuspiel, M., Girard, C., Dormann, N., Pearson, C., *et al.* (2015). A comparative analysis of insertional effects in genetically engineered plants: considerations for pre-market assessments. *Transgenic Research* 24, 1-17.
- Society of Toxicology (2003). Society of Toxicology position paper: The safety of genetically modified foods produced through biotechnology. *Toxicological Sciences* 71, 2-8.

Solinas, C., Corpino, M., Maccioni, R., and Pelosi, U. (2010). Cow's milk protein allergy. *The Journal of Maternal-Fetal & Neonatal Medicine* 23, 76-79.

Standards Australia New Zealand (2022). The Australian Standard 2243.3:2022 *Safety in Laboratories Part 3: Microbiological safety and containment*.

Suh, S.-O., Blackwell, M., Kurtzman, C.P., and Lachance, M.-A. (2006). Phylogenetics of Saccharomycetales, the ascomycete yeasts. *Mycologia* 98, 1006-1017.

Tan, B.M., Sher, M.R., Good, R.A., and Bahna, S.L. (2001). Severe food allergies by skin contact. *Ann Allergy Asthma Immunol* 86, 583-586.

Tapia, H., and Koshland, Douglas E. (2014). Trehalose is a versatile and long-lived chaperone for desiccation tolerance. *Current Biology* 24, 2758-2766.

Turner, P.J., Jerschow, E., Umasunthar, T., Lin, R., Campbell, D.E., and Boyle, R.J. (2017). Fatal anaphylaxis: mortality rate and risk factors. *The Journal of Allergy and Clinical Immunology: In Practice* 5, 1169-1178.

Umasunthar, T., Leonardi-Bee, J., Hodes, M., Turner, P.J., Gore, C., Habibi, P., Warner, J.O., *et al.* (2013). Incidence of fatal food anaphylaxis in people with food allergy: a systematic review and meta-analysis. *Clinical & Experimental Allergy* 43, 1333-1341.

Unsel, M., Sin, A.Z., Ardeniz, O., Erdem, N., Ersoy, R., Gulbahar, O., Mete, N., *et al.* (2007). New onset egg allergy in an adult. *J Investig Allergol Clin Immunol* 17, 55-58.

US FDA (2017). GRAS NOTIFICATION FOR SOY LEGHEMOGLOBIN PROTEIN PREPARATION DERIVED FROM PICHIA PASTORIS.

US FDA (2023a). CFR - Code of Federal Regulations Title 21 Sec. 573.750 *Pichia pastoris* dried yeast.

US FDA (2023b). Food Allergies - Major food allergens.

Vinayagamurthy, B., Reddy, G.R., and Suryanarayana, V.V.S. (2007). *Pichia pastoris*: A notable heterologous expression system for the production of foreign proteins - Vaccines. *Indian Journal of Biotechnology* 6, 175-186.

Walsh, R.M., and Martin, P.A. (1977). Growth of *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in a temperature gradient incubator. *Journal of the Institute of Brewing* 83, 169-172.

Yamada, Y., Matsuda, M., Maeda, K., and Mikata, K. (1995). The phylogenetic relationships of methanol-assimilating yeasts based on the partial sequences of 18S and 26S ribosomal RNAs: the proposal of *Komagataella* gen. nov. (Saccharomycetaceae). *Biosci Biotechnol Biochem* 59, 439-444.

Yoo, J.H., Kim, J.K., Yang, H.J., and Park, K.M. (2015). Effects of Egg Shell Membrane Hydrolysates on UVB-radiation-induced Wrinkle Formation in SKH-1 Hairless Mice. *Korean J Food Sci Anim Resour* 35, 58-70.

Appendix A: Summary of submissions from prescribed agencies on the consultation RARMP

The Regulator received a number of submissions from prescribed experts, agencies and authorities on the consultation RARMP. All issues raised in submissions that related to risks to the health and safety of people and the environment were considered in the context of the currently available scientific evidence and were used in finalising the RARMP that formed the basis of the Regulator's decision to issue the licence. Advice received is summarised below.

Submitter	Summary of issues raised	Comment
1	<p>Agrees with the overall conclusion of the RARMP.</p> <p>The Regulator should seek further clarification from the applicant on the fate of DNA and DNA products and the efficacy of the decontamination and inactivation pathways and methods for these. In the absence of such data, consider segregating GM yeast containing antibiotic resistance genes from use for soil conditioning or in animal feed preparation.</p> <p>The Regulator should further clarify the definition of 'inactivation' in the licence.</p>	<p>Submission has been noted.</p> <p>Section 2.3.5 has been amended to include denaturation of nucleic acid and proteins in GM yeast where antibiotic resistance genes will be present. Further, licence condition 23 has been modified to include that only decontaminated and, where antibiotic resistance genes are present, denatured GM yeast may only be used for the purpose of animal feed, as a soil conditioner or otherwise disposed of as waste. Further, licence condition 35 requires testing and validation for effectiveness in killing and denaturation of GM yeast.</p> <p>The word 'inactivation' has been replaced with 'decontamination' for consistency and definitions of both 'decontamination' and denaturation have been added to the RARMP and the licence.</p>
2	Noted that the licence will prohibit the use of GM yeast in human food or commercial animal feed. Do not have any further comments on the licence application at this stage.	Submission has been noted.
3	Do not have any advice or comments on the RARMP.	Submission has been noted.
4	Agrees with the approach and considers that the risk posed by the GMO to the health of humans and the environment is minimal, providing that there is strict adherence to the proposed control measures to mitigate the uncontrolled release of the GM yeast into the wider environment.	Submission has been noted.
5	Do not have any concerns.	Submission has been noted.

Submitter	Summary of issues raised	Comment
6	<p>Suggests amending the parent organism name from <i>Pichia pastoris</i> to its correct binomial name <i>Komagataella pastoris</i>.</p> <p>Sought clarification on whether only inactivated GM yeast slurry may be used for animal feed preparations or as soil conditioner.</p> <p>Sought clarification on how GM waste is decontaminated, whether by autoclaving or heat exchanger process, and on efficiency and validation of the decontamination methods. Further, how the material is to be kept during the 7 day culture.</p> <p>Sought clarification on how a major spill of 3 x 22 kL be chemically decontaminated and whether this decontamination agent would be present on site. Further clarification sought on what is meant by “any material outside the production facility” in paragraph 38.</p>	<p>After cross checking the list of strains proposed to be used for the trial, <i>Komagataella phaffi</i> or <i>Pichia pastoris</i> is the correct parent organism.</p> <p>Yes, only decontaminated (inactivated) GM yeast may be used for animal feed preparations or as a soil conditioner. No viable GM yeast will be used as human food or animal feed.</p> <p>As discussed in Section 2.3.5, general decontamination of GMO and associated waste would be by either >121°C for 60 minutes by autoclaving or chemical disinfection by 1% (w/v) Virkon™ for 10 minutes. The efficacy of these methods has been validated by the applicant by conducting swab cultures and will also be tested for large volumes as part of the work under this licence.</p> <p>As discussed in Section 2.3.5, the GM yeast slurry will be subjected to a heat exchanger run at >65°C and held for 60 minutes at >65°C in a holding tank. This is known to effectively kill yeast. Alternatively, where protein production is induced, the GM yeast slurry will be collected in a separate jacketed vessel. The pH will be raised to >10 and held for 60 minutes at >70°C. The pH will be readjusted to between 7 and 8 before disposal. The licence requires the licence holder to report the test results of these decontamination methods for the first run before proceeding with successive runs. The material will remain stored in the holding tank until the test results are negative for any viable GMOs.</p> <p>The 3 x 22 kL tanks are backup holding tanks which will be used for chemical disinfection in case of any spills from fermentation tanks. In the event of a spill, the effluent will be collected into a collection sump which will be diverted to the holding tanks. The decontamination agent will be co-located next to each fermenter.</p> <p>Reference to any material outside the production facility refers to any unlikely leak or spill leading to</p>

Submitter	Summary of issues raised	Comment
		presence of viable GM yeast outside of Cauldron's production facility.

Appendix B: Summary of submissions from the public on the consultation RARMP

The Regulator received ten submissions from the public on the consultation RARMP. The issue raised in the submission is summarised in the table below. All issues that related to risks to the health and safety of people and the environment were considered in the context of currently available scientific evidence in finalising the RARMP that formed the basis of the Regulator's decision to issue the licence.

Submitter	Summary of issues raised	Comment
1	Strongly objects to use of GMOs.	Submission has been noted. The Regulator makes licence decisions on the basis of consideration of risks associated with GMOs to people and the environment. The RARMP prepared for this application concluded that risks to people and the environment are negligible.
2	Endorses the risk assessment in protecting people and the environment	Submission has been noted.
3	Opposes alteration of food supply	Submission has been noted. The Regulator makes licence decisions on the basis of consideration of risks associated with GMOs to people and the environment. The RARMP prepared for this application concluded that risks to people and the environment are negligible. The licence prohibits the use of the GMOs in human food.
4	<p>Doubts the bonaifides of the applicant and their source of funding noting that the company is a start-up company.</p> <p>Concerned about the ability of the licence holder to report and monitor the work as they have no track record.</p> <p>The yeast contains chitin that could be a source of allergenicity and indigestion.</p>	<p>The Regulator has assessed the suitability of the applicant including their financial suitability as part of the application assessment process and found them to be suitable to hold a licence as an Australian legal entity.</p> <p>As discussed in Section 3.8, Chapter 1, yeast proposed to be used in this application has a long history of safe use in biotechnology industry and is approved for use of enzyme production and <i>P. pastoris</i> has been given the status as Generally Recognised as Safe (GRAS) by the US FDA and Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA) for use in enzyme production and food additives. No viable GM yeast will be sold for human food.</p>

Submitter	Summary of issues raised	Comment
	<p>Raises the issue that the GM yeast could escape (e.g., in case of an extreme weather event); and spread and persist in the environment.</p> <p>Points out that Virkon, proposed to be used as a disinfectant, is an environmental concern and could make its way into animal feed.</p>	<p>The risk assessment took into consideration the closed systems that are employed for the fermentation process and controls that are considered effective in operating in a contained environment. As mentioned in Section 2.3.7 of Chapter 1 and Risk Scenario 2, Chapter 2, the contingency plan in the RARMP refers to the drains that lead to a sump with level sensors that activate pumping to holding tanks for inactivation. There are no flowing waterways nearby the facility. Noting that the systems used are closed systems, flood events are unlikely to affect the operations.</p> <p>As mentioned in the RARMP, laboratory strains will be used in the fermentation process which require specific media and growth conditions and hence unlikely to survive in the wild noting that there are no natural host trees in the proximity of the facility.</p> <p>As mentioned in Section 2.3.5 in Chapter 1, Virkon is proposed to be used to disinfect GMOs <i>in vitro</i> and not to decontaminate yeast that will be composted or enter the animal feed chain.</p>
5	Endorses the risk assessment noting that the GM yeast poses negligible risks to the health and safety of people or the environment. The submitter states that the risk assessment has effectively covered risk identification, inherent uncertainty and evaluation; and developed a risk management plan to mitigate and control risk. Foresees that the licence, if issued will provide the opportunity to develop scale-up conditions and strain/protein characterisation data to support future applications for commercial production.	Submission has been noted.
6	Endorses the safety assessment and concurs with the identified risk scenarios that proposed controls effectively mitigate any potential harm. Further, acknowledges the extensive safety record of <i>P. pastoris</i> and its use in biotechnology for expression of proteins in their native form as food and fibre.	Submission has been noted.
7	Submitter is generally in favour of the trials. However, sees no reference to contingency plans in	Risk scenario 2 in Chapter 2 has been amended to include that there are no flowing waterways nearby the facility to cause flooding. As mentioned in Section 2.3.7

Submitter	Summary of issues raised	Comment
	case of flood noting that the region experiences unusual weather conditions.	of Chapter 1 and Risk Scenario 2, Chapter 2, the contingency plan in the RARMP refers to the drains that lead to a sump with level sensors that activate pumping to holding tanks for decontamination. Noting that the systems used are closed systems, flood events are unlikely to affect the operations.
8	Endorses the risk assessment and supports its conclusion. Submitter states that this will allow further pilot local scale-up trials and facilitate other regulatory approvals to drive investment and innovation in Australian precision food manufacturing sector.	Submission has been noted.
9	Endorses the risk assessment noting that the risk posed by the GMOs is negligible and the risk management plan effectively mitigates the minimal risk posed. The trial will be a valuable development for the agricultural sector.	Submission has been noted.
10	<p>The OGTR must clarify and discuss with the public whether the applicant proposes contained experimental or commercial protein production and the conditions and time frames.</p> <p>Must limit the batch size of any laboratory research or initial production of GMOs to no more than 25 litres.</p> <p>Before commencement of the trial, the OGTR must require tests exist to identify every strain.</p> <p>Must require the applicant to remove the antibiotic resistance marker genes from all the GM yeast strains that it produces for commercial scale-up.</p>	<p>In accordance with Section 50A of the Act, the trial is considered to be a limited and controlled release application as it meets the criteria prescribed by the Act. Its principal purpose is to conduct experiments with limits and control measures to restrict the dissemination or persistence of the GMOs in the environment.</p> <p>The protein production process involving culture of GM yeast in volumes of no more than 25 L per vessel are classified as an exempt dealing (Schedule 2, Item 4 of the Regulations). Culture of GM yeast in volumes of more than 25 L per vessel in a OGTR certified PC2 Large Scale Facility (PC2 LS facility) are covered under a notifiable low risk dealing (NLRD) authorisation (Schedule 3, Part 2, 2.1(f) of the Regulations). This DIR application is for a protein production process where more than 25 L per vessel of GM culture will be produced in a non-OGTR certified facility.</p> <p>A licence condition has been added that requires the licence holder to keep a record of GMOs and to have a method of detecting the GMO in the environment.</p> <p>This application is for a limited and controlled release, the principal purpose of which is to conduct experiments. Any future application for a commercial release will be assessed independently.</p> <p>Section 2.3.5 has been amended to include denaturation of nucleic acid and proteins in GM yeast where antibiotic resistance genes will be present. Further, licence condition 23 has been modified to include that where antibiotic resistance genes are present GM yeast must be denatured before being used</p>

Submitter	Summary of issues raised	Comment
	<p>Clarify that the recombinant proteins produced in Cauldron facilities will not be used for human food. The company makes clear that its intention is to produce food, feed and fibre.</p> <p>Require the applicant's contingency plans to include notice to the Cabonne Shire Council of any accidental leak or deliberate release of GMOs into the environment, so that full and timely information reaches residents.</p> <p>Require another round of public consultation on the revised plans.</p>	<p>for the purpose of animal feed, as a soil conditioner or otherwise disposed of as waste. Further licence condition 34 requires testing and validation for effectiveness of the processes used for denaturation of DNA and proteins introduced into the GM yeast.</p> <p>Though the future aim of the applicant is to produce recombinant proteins that could be used as food, feed and fibre, the intent of the proposed application is to optimise the fermentation process and characterise GM yeast during production of recombinant proteins.</p> <p>Licence conditions state that the GMOs must not be used, sold or otherwise disposed of for any purpose, which would involve or result in their use as food for humans.</p> <p>The Act does not regulate activities with GM products, which are defined as a thing (other than a GMO) derived or produced from a GMO. Examples include purified recombinant proteins. The recombinant proteins will be purified and will not contain any viable GM yeast. Therefore, risks posed during handling and use of the purified proteins after separating them from the GM yeast are not considered further in this RARMP.</p> <p>As part of the consultation process, Cabonne Shire Council was notified of the application and consulted on the risk assessment. The contingency plan includes a requirement that any incident such as a spill or exposure to the GM yeast be immediately reported to the Regulator. The Regulator will then make a decision who to notify based on the context of such incident.</p> <p>Section 52 of the Act requires the Regulator to seek comment on the consultation RARMP from agencies - the Gene Technology Technical Advisory Committee, State and Territory Governments, Australian Government authorities or agencies prescribed in the Regulations, Australian local councils and the Minister for the Environment - and from the public. The consultation was conducted accordingly.</p>