Classification and Spoilage Characteristics of *Chryseobacterium* Isolates from Fish

by

Lydia Gavu

Submitted in fulfilment of the requirements
for the degree of

MASTER OF SCIENCE
(FOOD MICROBIOLOGY)

In the

Department of Microbial, Biochemical and Food Biotechnology
Faculty of Natural and Agricultural Sciences
University of the Free State

Supervisor: **Prof. C.J. Hugo**
Co-supervisors: **Dr. A.C. Hitzeroth**
Dr. M. Gryzenhout

2019

DECLARATION

I Lydia Gavu, declare that the thesis hereby submitted by me for the M.Sc. degree in the Faculty of Natural and Agricultural Sciences at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I furthermore cede copyright of this thesis in favour of the University of the Free State.

L. Gavu

January 2020

TABLE OF CONTENTS

Chapter **Title** **Page**

AKNOWLEDGEMENTS

I would like to direct my genuine appreciation and gratitude to the following persons and institutions for their assistance in the accomplishment of this study:

Firstly, and foremost, God Almighty for giving me the strength, wisdom, persistence and ability to complete this study,

My Supervisor, Prof. C.J. Hugo, for her unlimited persistence and supervision throughout this study, for her guidance and encouragement. Her guidance, motivated and gave me strength to carry on, in all the time of the research and writing of the thesis.

Dr. A. Jansen, my co-supervisor for her willingness to travel from far to offer her guidance, assistance and thoughtful remarks.

Dr. M. Gryzenhout, my co-supervisor, for her introduction to MEGA and Genious lectures.

Prof. A. Hugo, for his outstanding assistance with statistical analysis.

Dr. C. Bothma together with Mrs. L. Van der Walt and Mrs. C. Hills, for their assistance with the sensory analysis.

Ms. A. LumNde for her incredible assistance with phylogenetic analysis, offering me her notes, her time, opinion, support and for always being there to provide appropriate and insightful comments.

Dr. W. Van der Westhuizen for his assistance with molecular techniques.

The Veterinary Biotechnology lab, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, for the use of their facilities and equipment.

Mr. S. Marais, for his assistance with SPME GC/MS analysis;

The National Research Foundation for financial support throughout the course of this study.

NRF National Research Foundation

The Post Graduate School (UFS) for financial assistance.

Lastly, my family, and friends for their motivation, advice, moral support, simplest words of comfort and for believing in me.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

^{o}C	Degrees Celcius
ANOVA	Analysis of Variance
C.	*Chryseobacterium*
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DDH	DNA-DNA hybridization
E.	*Elizabethkingia*
Ed(s)	Editor(s)
e.g.	For example
et al.	(*et alii*) and others
etc.	Et cetera
F.	*Flavobacterium*
Fig.	Figure
FJB	Fish juice broth
g	gram
GC/MS	Gas chromatography and/ Mass spectrometry
G+C	Guanine plus cytosine
GN	Gram-negative
h	Hour
H_2S	Hydrogen sulphide
kg	Kilogram
MCA	MacConkey agar
min	Minute
mg	Milligram

ml	Millilitre
mm	Millimetre
Mol	Mole
NaCl	Sodium chloride
Mol%	Mole percentage
NA	Nutrient Agar
NCTC	National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom
ND	Not detected
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pp	Page(s)
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal Ribonucleic Acid
sec	Second(s)
SOS	Specific Spoilage Organisms
sp.	Species or unknown/unidentified/unspecified species
SPCA	Standard plate count agar
TBC	Total bacteria count
T_m	Melting temperature
™	Trade mark
TSA	Trypticase soy agar
R2A	Reasoners2 agar
µl	Microlitre
w/v	Weight per volume

Abstract

The genus *Chryseobacterium* was initially reported to have only six species and the number has increased rapidly over the years. The genus is represented by a total of 113 species at present. Species belonging to the genus *Chryseobacterium* are widespread in nature. They have been reported from clinical, environmental, industrial and food sources. Their spoilage characteristics have been well defined in food products including poultry, meat, milk and milk products and fish. Development of unpleasant odours in food contaminated by *Chryseobacterium* species usually results from the activity of proteolytic enzymes.

The first aim of this study was to classify 11 potential *Chryseobacterium* fish isolates from a previous study. The methods that were used included 16S rRNA gene sequence analysis, conventional phenotypic methods and the BIOLOG^{TM} Omnilog identification system. *Chryseobacterium balustinum, C. gleum* and *C. piscium* were used as reference strains throughout the whole study. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the 11 *Chryseobacterium* isolates represented members of the genus *Chryseobacterium.* The highest similarity were obtained for *C. piscium* by strains SH 23-4 (98.89%), SH 28-3 (100%) and SH 30-1 (100%) and it was therefore concluded that these three strains could be other strains of *C. piscium*. This finding was confirmed by the phylogenetic treeing methods used in this study because the three strains clustered closely with *C. piscium*. Strains SH 11-3(a), SH 11-3(b), SH 20-4, SH 25-4, SH 40-3, SH 19-2(b) and SH 11-4(b) had sequence similarity values lower than 98.7%, it is therefore a possibility that these strains might be novel species of *Chryseobacterium*, however, further investigations need to be performed for confirmation. The BIOLOG^{TM} Omnilog Gen III identification system and conventional phenotypic methods were also useful in classification. The BIOLOG^{TM} Omnilog was also able to identify strains SH 23-4, SH 28-3 and SH 30-1 as possible strains of *C. piscium.* The other strains could not be identified because of its limited database.

The second aim of this study was to estimate the spoilage potential of the 11 *Chryseobacterium* isolates by determining their spoilage potential in terms of substrate oxidation by the BIOLOG^{TM} Omnilog system. The results indicated that all the isolates could be potential fish spoilers, however, strains SH 11-4(b) and SH 23-4 were able to oxidise the most of the carbon sources (25/31) and could be regarded as being able to cause the most spoilage.

Sensory analysis of inoculated FJB samples did not show significant differences in terms of odour production, however, odours such as smelly feet, cabbage-like, fruity, sour and putrid sewage were noted for the 11 *Chryseobacterium* isolates and their reference strains.

Chryseobacterium species were able to produce a total of 87 volatile compounds in fish juice broth (FJB) at 4 and 25 °C. The composition of the volatile compounds detected was, however, slightly higher for samples that were incubated at 25 °C than at 4 °C. The frequently detected compounds occured in FJB samples containing *C. gleum* and strain SH 30-1 and the least detected compounds occurred in FJB samples containing strains SH 19-2(b) and SH 11-4(b). The frequently produced compounds with more pronounced odours were identified as 2-ethyl-1-hexanol, indole, dimethyl disulphide and 2-phenylethanol. FJB samples inoculated with strains SH 30-1, SH 28-3, SH 25-4 had the majority of volatile compounds associated with unpleasant odours, such as fishy from trimethylamine; spoiled, putrid from dimethyl sulphide; and faecal, nauseating from indole.

It was concluded that 7/11 *Chryseobacterium* strains in this study could be novel *Chryseobacterium* species. Sensory analysis and Gas-Chromatrography/Mass-Spectrometry were useful in estimating the spoilage potential of the *Chryseobacterium* species through odour production. The BIOLOG$^{\text{TM}}$ system can be used as an effective screening method for identifying the carbon sources utilised by the *Chryseobacterium* species which could then be investigated further for the potential of these starins to cause food spoilage. *Chryseobacerium* species used in this study have the potential to spoil fish and/other fish products because of their psychrotolerant and proteolytic nature.

Keywords: *Chryseobacterium*, taxonomy, fish spoilage, sensory analysis, volatile production

CHAPTER 1

Introduction

Fish is a high-protein, low-fat food that provides extensive health benefits to human beings and therefore serves as a vital part of the human diet as human bodies are unable to produce significant amounts of essential nutrients (Engelbrecht 1992). Fish however, provides an outstanding growth medium for a majority of microorganisms as it contains nutrients required for their growth (Engelbrecht 1992). About 30% of fish are lost due to the activity of spoilage microorganisms globally (Ghaly et al. 2010; Kuley et al. 2017). Heading and gutting of fish directly after capture is the prime cause of the contamination with psychrotolerant spoilage bacteria (Engelbrecht 1992).

Psychrotolerant bacteria are bacterial species which are capable of growing below 7 °C regardless of their optimum growth temperature (Champagne et al. 1994). Psychrotolerant bacteria, specifically Gram-negative rods such as *Pseudomonas, Alteromonas, Flavobacterium/Chryseobacterium, Moraxella, Acinetobacter* and *Vibrio* are mainly responsible for limiting the quality of fish, however, not all bacteria that are found on fish can be regarded as active spoilers (Cousin 1982; Engelbrecht 1992). Active spoilers are those organisms that have proteolytic activity, are able to produce off-odours and flavours, hydrogen sulphide (H_2S) and/or can reduce trimethylamine oxide (TMAO) to trimethylamine (TMA) (Wekell and Barnett 1991; Engelbrecht 1992).

Chryseobacterium species are widely distributed in food and food-related sources, water, soil, clinical, environmental and other sources (Jooste and Hugo 1999; Bernardet and Nakagawa 2006). The genus *Chryseobacterium* comprises of cold-tolerant and proteolytic spoilage bacteria that are commonly isolated from food (Hugo et al. 2003; de Beer et al. 2005, 2006; Charimba et al. 2013). *Chryseobacterium balustinum* (Harrison 1929; Vandamme et al. 1994), *C. piscium* (de Beer et al. 2006), *C. piscicola* and *C. scophthalmum* (Mudarris et al. 1994; Vandamme et al. 1994) are examples of *Chryseobacterium* species isolated from fish. *Chryseobacterium balustinum* was isolated from fresh halibut (*Hippoglossus hippoglossus*) and produced a yellowish slime, and was consequently treated as a spoilage agent rather than a pathogen (Austin and Austin 1999).

When comparing *Chryseobacterium* species with other well-known spoilage organisms such as *Pseudomonas*, it was reported that *Chryseobacterium joostei* showed greater spoilage abilities than *Pseudomonas fluoresences* based on their lipolytic and proteolytic activity and sensory analysis in terms of odour (Bekker 2011; Bekker et al. 2015, 2016).

In a study of Engelbrecht (1992) at the University of the Free State, several yellow-pigmented bacteria were isolated from Cape marine fish. Many of these bacteria were identified as members of the *Flavobacteriaceae* family (Engelbrecht et al. 1996a) and some of these isolates produced H_2S and hydrolyzed several substrates such as casein and gelatine which are spoilage indicators (Engelbrecht et al. 1996b). Later, in 2006, some of these isolates were identified as a new species, *Chryseobacterium piscium,* which was proposed to be a spoilage organism because of the activities of the enzymes urease and phenylalanine deaminase (de Beer et al. 2006). Several of these flavobacterial isolates, however, remained unidentified and their role in food spoilage, specifically fish spoilage, were also unknown.

For any organism to be identified, the taxonomy of the organism needs to be studied first. Taxonomy is the concept and practice of classifying organisms (Prakash et al. 2007). The methods that have been used to classify and identify microorganisms include molecular-based analysis, conventional methods and automated biochemical analysis (Morgan et al. 2009). The modern identification methods are as a result of the development in molecular biology, unravelling the structure and function of microorganisms (Khatib et al. 2014). These methods are more productive and time effective for microbial identification in comparison to traditional methods of detection (Senthilraj et al. 2016).

Food spoilage is associated with modifications in the physical properties of food that are unpleasant and unacceptable to the consumer (Ayres et al. 1980). Food spoilage results from microbial, chemical or mechanical activity (Ayres et al. 1980). Microbial spoilage is, however, the major source of deterioration of delicate food, which manifests as growth, changes in texture or in the form of off-odours and off-flavours.

Appearance, texture, odour, and taste are the main quality parameters for fish freshness directly linked to the sensory characteristics observed by consumers (Hassoun and Karoui 2017). Freshness is a critical measure for quality of the seafood products, since factors, such as rigor mortis, autolysis processes, and microbiological spoilage after death have a negative impact on it (Cheng and Sun 2014). Sensory evaluation is required to differentiate between products with distinct flavours (Krüsemann et al. 2019). Sensory analysis and GC/MS are methods that are used to evaluate characterising flavours and odours in food products (Krüsemann et al. 2019). Sensory expert panels are usually used to measure sensory product characteristics while consumer panels are used to examine consumers’ perceptions and reactions to product characteristics (Rees et al. 2009).

Purpose and objectives of this study

Purpose:

Several bacterial isolates belonging to the genus *Chryseobacterium* were previously isolated from fish in a study in the Food Science section of the UFS (Engelbrecht 1992). However, they were not identified to species level and it is not known whether they can spoil fish. This study will therefore firstly classify and identify these bacterial isolates. Secondly, determine their spoilage characteristics by using the BIOLOG^{TM} system, sensory and GC analysis. This study will therefore offer essential information on the spoilage capability of these bacterial isolates and the effect they may have on the quality of food, specifically fish products. The results attained from this study will also expand the existing information available on the role of *Chryseobacterium* in the microbial ecology of food spoilage.

Objectives:

I. To classify the 11 *Chryseobacterium* fish isolates by using 16S rRNA gene sequence analysis, conventional phenotypic tests and the BIOLOG^{TM} Omnilog Gen III identification system.

II. Estimation of spoilage characteristics of the *Chryseobacterium* strains by determination of odour production by employing sensory analysis.

III. Estimation of spoilage characteristics by determination of the production of volatile compounds by the *Chryseobacterium* strains by using GC/MS analysis.

CHAPTER 2

Classification and Spoilage Characteristics of *Chryseobacterium* species – A Review

2.1 INTRODUCTION

Taxonomy plays a vital role in the nomenclature, identification and classification of microorganisms. Previously, microrganisms were classified based on phenotypic qualities such as form, colony colour, cell size, staining properties, motility, host range and pathogenicity (Prakash et al. 2007). Taxonomists later developed polyphasic methods for the description and classification of new taxa which entailed the use of conventional, phenotypic, chemotypic and genotypic methods. Genotypic methods focusing on DNA or RNA molecules dominate modern taxonomy (Vandamme et al. 1996).

Growth of microorganisms in foods cause spoilage by the production of unacceptable changes that have a negative influence on the overall sensory characteristics. The ability of *Chryseobacterium* species to produce proteolytic enzymes enhances their spoilage potential; however, methods such as sensory analysis and GC/MS analysis of the volatile compounds assist in investigating spoilage in terms of odour. Psychrotolerant bacteria, specifically Gram-negative rods such as *Pseudomonas, Alteromonas, Flavobacterium/Chryseobacterium, Moraxella, Acinetobacter* and *Vibrio* are mainly responsible for limiting the quality of fish, however not all bacteria that are found on fish can be regarded as active spoilers (Cousin 1982; Engelbrecht 1992). *Chryseobacterium* species that have been isolated from fish include *Chryseobacterium scophthalmum* (Mudarris et al. 1994), *Chryseobacterium shigense* (Zamora et al. 2012c), *Chryseobacterium piscium* (de Beer et al. 2006) and *Chryseobacterium piscicola* (Ilardi and Avendaño-Herrera 2008; Ilardi et al. 2009).

Sensory analysis and GC/MS are methods that could be used to assess characterising flavours and odours in food products (Krüsemann et al. 2019). The BIOLOG^{TM} system can be used to assess spoilage potential of microorganisms by evaluating the substrates that organisms can oxidise. These tests assist with estimating the spoilage potential of microorganisms, particularly, their ability to produce metabolites causing off-odours or off-flavours (Dalgaard 2000; Gram and Dalgaard 2002). Sensory analysis of seafood is a critical tool used by the Federal Drug Administration (FDA) to protect customers from seafood that has been contaminated due to putrefaction (ORA Laboratory Manual 2013), while gas chromatography (GC) and mass spectrometry (MS) can be used to estimate food spoilage in terms of odour, detecting volatile compounds likely to result in off-odour production (Bekker 2011).

The aims of this literature review will be to give a brief historical overview of the taxonomy of the genus *Chryseobacterium* and the family *Weeksellaceae*; then to give a taxonomic description of the genus *Chryseobacterium*; the ecology of this genus will be indicated and the focus will be on food sources, especially fish sources; taxonomic methods used to classify and identify bacterial organisms will be discussed; spoilage characteristics of *Chryseobacterium* will be detailed in terms of lipolytic and proteolytic activities and then sensory analysis and gas chromatography-mass spectrometry will be discussed as methods to determine spoilage characteristics of *Chryseobacterium* species. The results obtained from this study will therefore broaden the current knowledge available on the role of *Chryseobacterium* species in the microbial ecology of food spoilage.

2.2 THE GENUS *Chryseobacterium*

2.2.1 Historical Overview

The genus *Chryseobacterium* belongs to the family *Weeksellaceae*, the kingdom *Bacteria*, phylum *Bacteroidetes*, class *Flavobacteria* and order *Flavobacteriales* (García-López et al. 2019). However, since 1994 when this genus name was suggested, until 2019, the genus belonged to the *Flavobacteriaceae* family coined by Jooste (1985) and validated in 1992 (Reichenbach 1992).

The genus *Chryseobacterium* was originally recommended by Vandamme et al. (1994) to contain six species which were previously classified as *Flavobacterium*. *[Flavobacterium] balustinum, [F.] gleum, [F.] indologenes, [F.] indoltheticum, [F.] meningosepticum* and *[F.] scophthalmum* were the six original isolates that were transferred to the genus *Chryseobacterium. Chryseobacterium gleum* was selected as the type species as it was well characterised and its genotypic and phenotypic relationships were known (Vandamme et al. 1994).

[Chryseobacterium] meningosepticum, previously identified as *[Flavobacterium] meningosepticum,* is the species most commonly described as a human pathogen in the genus *Chryseobacterium*, and was originally defined for a case of neonatal meningitis by Elizabeth King in 1959. The 16S rRNA gene sequence similarity studies, however, revealed that *[C.] meningosepticum* can be readily differentiated from other *Chryseobacterium* species

and therefore resulted in its transfer to a new genus called *Elizabethkingia.* It is now referred to as *Elizabethkingia meningoseptica* (Kim et al. 2005b).

The genus *Chryseobacterium* currently consists of about 113 species with validly published names including the not validly published "*Chryseobacterium proteolyticum*" (Euzéby 2019). The number of species is expected to increase as several bacteria phylogenetically related to members of the genus *Chryseobacterium* will be reported from investigations of various environments.

2.2.2 The description of *Chryseobacterium* species

Chryseobacterium species are straight, single, Gram-staining-negative rods with rounded ends, which are typically about 0.5 µm in width and their length varies, often about 1–3 µm. The cells are filamentous and they do not form endospores (Shimomura et al. 2005). Their colonies are pale to bright yellow due to the production of flexirubin-type pigments (Vandamme et al. 1994). They are non-motile and non-gliding in nature. They are strictly aerobic. *Chryseobacterium* species are chemo-organotrophic, meaning they acquire energy from the metabolism of organic compounds.

Growth of environmental isolates is most often at 5 °C. Growth occurs for all strains at 15-30 °C, and some grow up to a temperature of 37 °C, however, clinical isolates do not grow at 5 °C, but grow at 15-37 °C and there are also a few that grow at up to 42 °C (Vandamme et al. 1994; Bernardet et al. 2002, 2006; Kämpfer et al. 2003). Growth occurs on the standard commercial media, and no growth factors are needed. Several species also grow in media containing 3–5% NaCl. They are catalase and oxidase positive and most strains are unaffected by a wide variety of antimicrobial agents (Vandamme et al. 1994; Bernardet et al. 2011; Hugo et al. 2019).

The members of the genus *Chryseobacterium* are not difficult to isolate and cultivate; they usually do not require growth-promoting supplements and they grow at ambient temperatures (Bernardet et al. 2011). The isolation of *Chryseobacterium* strains from freshwater fish, however, is best when tryptic soy or nutrient agars are used (Bernardet et al. 2005) and cultivation of marine fish isolates, is best on brain heart infusion, glucose-yeast extract, marine 2216E, or nutrient agars (de Beer et al. 2006).

An extended incubation period of some species in the genus results in the production of extracellular slimy constituents which may result in slimy colonies (Vandamme et al. 1994). The ability of some members of the genus *Chryseobacterium* to produce proteases (gelatin and casein degrading proteases) are considered a major virulence factor (Bernardet et al. 2006).

The production of acid from glucose, indole production and the hydrolysis of starch varies between the *Chryseobacterium* species (Bernardet et al. 2002; de Beer et al. 2005; Kim et al. 2005a). The G+C content varies between 28.8–49.3 mol% G+C (Hugo et al. 2019).

2.2.3 Ecology

Chryseobacterium species have been procured from clinical, environmental, industrial and food sources. These sources will now be discussed in more detail and emphasis will be on the food sources, especially the fish sources.

2.2.3.1 Clinical sources

Chryseobacterium species are widely distributed in hospital environments and may end up on the patients' outer and inner body surfaces. Human's normal microbiota, however, do not contain *Chryseobacterium* species (Holmes and Owen 1981).

Chryseobacterium species can cause infections of the respiratory tract and urinary tract in patients with a compromised immune system and have been found to be tolerant to a variety of antimicrobial agents (Bernardet and Nakagawa 2006; Bernardet et al. 2011). *Chryseobacterium indologenes* and *C. gleum* are species that have been linked with infections in humans (Bernardet et al. 2005). *Chryseobacterium indologenes* have been involved in infections of new-borns (Bernardet et al. 2005).

Chryseobacterium gleum has been isolated from several human clinical samples (Holmes et al. 1984). *Chryseobacterium indologenes* was originally isolated in the course of a post-mortem in a human trachea and ever since the organism has been isolated, multiple times, in burn wounds. *Chryseobacterium indologenes* have caused bacteraemia in a diabetic child (Cascio et al. 2005).

Eight strains of *C. anthropi* were isolated from various clinical origins, four isolates were recovered from the blood of a hospitalized patient, and the remaining four were isolated from a bite wound, finger abscess, lung biopsy and urethra, respectively (Kämpfer et al. 2009). *Chryseobacterium bernardetii*, *C. carnis* and *C. nakagawai* were among the 182 phenotypically similar isolates that were assessed by DNA–DNA reassociation and 16S rRNA gene sequence analysis originating from clinical samples (Holmes et al. 2013). *Chryseobacterium hominis* was recovered from a Belgian patient's blood in 1998 (Vaneechoutte et al. 2007) while *C. treverense* was isolated from human blood from Trier in Germany (Yassin et al. 2010).

2.2.3.2 Environmental sources

Several members of the genus *Chryseobacterium* have been isolated from soil and soil samples which includes "*C. proteolyticum*" (Yamaguchi and Yokoe 2000), *C. taichungense* (Shen et al. 2005), *C. taiwanense* (Tai et al. 2006), *C. wanjuense* (Weon et al. 2006), *C. flavum* (Zhou et al. 2007), *C. jejuense* , *C. soli* (Weon et al. 2008), *C. vietnamense* (Li and Zhu 2012), *C. solincola* (Benmalek et al. 2010), *C. shandongense* (Yang et al. 2015), *C. solani* (Du et al. 2015), *C. arachidis* (Kämpfer et al. 2014a), *C. artocarpi* (Venil et al. 2014) and *C. frigidum* (Kim et al. 2016). *Chryseobacterium ginsengisoli* was isolated from a soil sample that was recovered from the roots of ginseng (Nguyen et al. 2013) and *C. arachidiradicis* and *C. geocarposphaerae* from the soil around very immature peanuts (Kämpfer et al. 2014a, Kämpfer et al. 2015a).

Chryseobacterium species have also been isolated from marine, freshwater and lake sediments. *Chryseobacterium yonginense* was isolated from a mesotrophic lake (Joung and Joh 2011), *C. daecheongense* from a freshwater lake sediment (Kim et al. 2005a), *C. piperi* from a fresh water creek (Strahan et al. 2011), *C. taihuense* from an eutrophic lake (Wu et al. 2013), *C. rigui* from the estuarine wetland of the Han River (Park et al. 2013) and *C. aurantiacum* from fresh water of a diseased farmed Murray cod (Luo et al. 2018). Other species have been found in watery environments such as *C. indoltheticum* which has been isolated from marine mud, *C. aquaticum* from a water reservoir (Kim et al. 2008), *C. aquifrigidense* from a water-cooling system (Park et al. 2008), *C. hispanicum* from drinking water (Gallego et al. 2006) and *C. angstadtii* from a newt tank (Kirk et al. 2013).

Some species, such as *C. formosense* (Young et al. 2005), *C. luteum* (Behrendt et al. 2007), *C. soldanellicola* and *C. taeanense* (Park et al. 2006) have all been isolated around the roots of growing plants. *Chryseobacterium camelliae* was isolated from green tea (Kook et al. 2014), *C. elymi*, *C. hagamense, C. lathyri* and *C. rhizosphaerae* were all isolated from the rhizosphere of coastal sand dune plants (Cho et al. 2011). *Chryseobacterium gregarium* on the other hand was recovered from decaying plant matter (Behrendt et al. 2008) while *C. hispalense* is a plant-growth-promoting bacterium isolated from a rainwater pond in an olive plant nursery

(Montero-Calasanz et al. 2013). The impact of *Chryseobacterium* species in these environments has not been investigated (Hugo et al. 2019).

2.2.3.3 Industrial Sources

Chryseobacterium defluvii was isolated from a wastewater treatment plant (Kämpfer et al. 2003), while *C. caeni* was isolated from bioreactor sludge (Quan et al. 2007), *C. limigenitum* from dehydrated sludge (Kampfer et al. 2015d) and *C. daeguense* from waste water from textile dye works (Yoon et al. 2007). *Chryseobacterium humi* and *C. palustre* were both isolated from industrially polluted sediments (Pires et al. 2010). *Chryseobacterium taichugense* was isolated from soil contaminated with tar (Shen at al. 2005), while *C. nepalense* was isolated from oil contaminated soil (Chaudhary and Kim 2017). *Chryseobacterium hungaricum* (Szoboszlay et al. 2008) and *C. solincola* (Benmalek et al. 2010) were both isolated from hydrocarbon-contaminated soil. The significance of these species at these particular sites is, however, still unknown. Some strains of *Chryseobacterium* have been shown to be involved in the degradation of various toxic compounds such as pentachlorophenol (PCP) (Yu and Ward 1996).

2.2.3.4 Food and food-related sources

Chryseobacterium species are widely distributed in food sources such as milk, fish, meat and poultry (Hugo et al. 2003; de Beer et al. 2005, 2006; Charimba et al. 2013). Table 2.1 indicates the *Chryseobacterium* species that have been isolated from food and food-related sources. The fish sources will be discussed in the next section (2.2.3.5).

Table 2.1: *Chryseobacterium* species isolated from a variety of food except fish.

Type of food	Species	Source	Reference
Dairy sources	*C. bovis*	Cow's raw milk	Hantsis-Zacharov et al. 2008a
	C. haifense	Cow's raw milk	Hantsis-Zacharov et al. 2007a
	C. halperniae	Cow's raw milk	Hahnke et al. 2016
	C. gleum	Dairy environments	Holmes et al. 1984
	C. joostei	Raw milk	Hugo et al. 2003
	C. lactis	Milk	Holmes et al. 2013
	C. oranimense	Cow's raw milk	Hantsis-Zacharov et al. 2008b
	C. indologenes	Raw milk	Yabuuchi et al. 1983
Meat and Poultry	*C. vrystaatense*	Raw chicken	de Beer et al. 2006
	C. carnipullorum	Raw chicken portion	Charimba et al. 2013
	C. gallinarum	Chicken	Kämpfer et al. 2014b
Water	*C. aquaticum*	Water reservoir in Korea	Kim et al. 2008
	C. lineare	Fresh water stream	Zhao et al. 2017
	C. marinum	Sea water	Lee et al. 2007
	C. piperi	Fresh water creek	Strahan et al. 2011
	C. sediminis	River sediment	Kämpfer et al. 2015b
	C. taihuense	Eutrophic lake	Wu et al. 2013
	C. yonginense	Mesotrophic artificial lake	Joung and Joh 2011
	C. hispanicum	Drinking water distribution system	Gallego et al. 2006
Beer-bottling Plants	*C. gambrini*	Steel surface from a beer bottling plant	Hertzog et al. 2008
	C. pallidum	Steel surface from the plant	Hertzog et al. 2008
	C. ureilyticum	Steel surface from a beer bottling plant	Hertzog et al. 2008
	C. molle	Steel surface from a beer bottling plant	Hertzog et al. 2008
Soil and rhizophere of Edible	*C. formosense*	Rhizophere of garden lettuce	Young et al. 2005
	C. cameliae	Leaves of green tea	Kook et al. 2014

Plants	*C. oleae*	Olive tree	Montero-Calasanz et al. 2014
	“C. proteolyticum”	Soil of a rice field	Yamaguchi and Yokoe 2000
	C. rhizoplanae	Rhizoplane of sweet corn	Kämpfer et al. 2015c
	C. shandongense	Soil	Yang et al. 2015
	C. solani	Eggplant rhizosphere soil	Du et al. 2015
	C. soldanellicola	Roots of sand-dune plants	Park et al. 2006
	C. taeanense	Roots of sand-dune plants	Park et al. 2006
	C. taiwanense	Farmland soil in Taiwan	Tai et al. 2006
	C. takakiae	Takakia lepidozioides	Zhao et al. 2015
	C. wanjuense	Greenhouse soil	Weon et al. 2006
Other sources	*C. shigense*	Lactic acid beverage	Shimomura et al. 2005
	C. balustinum	Potatoes	Krechel et al. 2002
	C. cucumeris	Cucumber	Jeong et al. 2017
	C. endophyticum	Maize leaf	Lin et al. 2017
	C. kwanjueense	Pepper (*Capsicum annuum* L.) root	Sang et al. 2013
	C. indologenes	Sugar beet leaves	Beattie 2006

2.2.4.5 Fish sources

Chryseobacterium isolates are prevalent in water environments. They are most of the times recovered from the external or the interior parts of marine animals. Niswati et al. (2005) described strains from the bacterial communities on the surface of freshwater micro-crustaceans, they were also isolated in a study led by Winters and colleagues in 2015 of the benthic amphipod *Diporeia* in the Great Lakes and from the microflora of amoebae. Two ultra-small *Chryseobacterium* strains from Lake Baikal (Russia) colonized the surface of *Bacillus subtilis* cells (Suzina et al. 2011).

Chryseobacterium species form part of the microorganisms found on the skin of newts regardless of the significant amount of harmful substances in the mucus (Vences et al. 2015). Their presence at the surface of lake sturgeon (*Acipenser fulvescens*) eggs before sterilization was also reported (Chalupnicki et al. 2015). A study that was conducted by Kan et al. (2015)

on the shift of the gut microbial community after exposing goldfish (*Carassius auratus*) to the herbicide pentachlorophenol, showed that *Chryseobacterium* strains were amongst the bacterial isolates in the gut. It was also shown in a study conducted by Ringø et al. (2006) that *Chryseobacterium* species form a central part of bacteria in the gastrointestinal tract of fish served soybean meal.

Some *Chryseobacterium* species linked with fish include *C. arothri*, which was recovered from the kidneys of a pufferfish (Campbell et al. 2008). *Chryseobacterium balustinum* was isolated from marine fish (Harrison 1929; Bergey and Breedt 1948; Engelbrecht et al. 1996a, 1996b), and four strains of *C. piscium* were isolated from fish caught in the South Atlantic Ocean near South Africa (de Beer et al. 2006). *Chryseobacterium balustinum, C. gleum* and *C. indologenes* strains which were isolated from Cape marine fish of South Africa exhibited wide-ranging proteolytic activities and therefore may be responsible for proteolytic spoilage of fish products during initial cold storage (Engelbrecht et al. 1996b).

Chryseobacterium scopthalmum is associated with gill disease of fresh water and marine fish (Mudarris et al. 1994), while *C. joostei* has been isolated from both milk (Hugo et al. 2003) and fish (Bernardet et al. 2005). *Chryseobacterium joostei* was isolated from diseased fish, however, it is not known if it was the cause of the pathogenesis (Bernardet et al. 2005).

Chryseobacterium species which have been isolated from fish sources and which were linked to pathogenicity and/or spoilage of the fish, are indicated in Table 2.2.

2.3 Taxonomic techniques

Taxonomy is the principle and practice of classifying organisms (Prakash et al. 2007) and is based on the nomenclature, classification and identification of organisms (Bisen 2014). Nomenclature involves the naming of organisms based on the Linnaeus system developed by Carl von Linné in the 18th century (Bisen 2014). Classification is the systematic arrangement of bacteria into groups (taxa) while identification is the act and outcome of assigning unidentified organisms to a specific rank in a previously made classification system (Baron 1996, Bisen 2014).

The purpose of classification is to create homogeneous groups which comprise of progenies of the nearest common ancestor (Prakash et al. 2007). Phylogeny is the process in which lineages of organisms evolved by separation from a common ancestor and involves phylogenetic and evolutionary framework (Sandle et al. 2013).

Table 2.2: *Chryseobacterium* species isolated from fish.

Chryseobacterium **Species**	**Source**	**Spoilage agent or pathogenic**	**Reference**
C. aahli	Brown trout (*Salmo trutta*)	Facultatively pathogenic	Loch and Faisal 2014
C. arothri	Pufferfish (*Arothron hispidus)*	Pathogenic	Campbell et al. 2008
C. balustinum	Diseased freshwater fish	Pathogenic	Harrison, 1929; Vandamme et al. 1994
C. chaponense	Farmed Atlantic salmon (*Salmo salar*)	Spoilage agent	Kämpfer et al. 2011
C. indologenes	Diseased yellow perch	Pathogenic	Pridgeon et al. 2012
C. joostei	Diseased fish	Spoilage agent	Bernadet et al. 2005
C. oncorhynchi	Gills of rainbow trout (*Oncorhynchus mykiss*)	Pathogenic	Zamora et al. 2012a
C. piscicola	External lesion of salmonid fish	Pathogenic	Ilardi et al. 2010
C piscium	Healthy marine fish	Spoilage agent	de Beer et al. 2006
C. senegalense	Mouth of a lungfish	Pathogenicity unknown	Lo et al. 2016
C. scophthalmum	Gills of diseased turbot (*Scopthalmus maximus*)	Pathogenic	Mudarris et al. 1994, Vandamme et al. 1994
C. shigense	Farmed rainbow trout	Pathogenic	Zamora et al. 2012b
C. tructae	Liver of diseased rain bow trout	Pathogenicity unknown	Zamora et al. 2012c
C. viscerum	Gills of diseased rainbow trout	Pathogenic	Zamora et al. 2012d

A comprehensive range of taxonomic studies of bacteria have increasingly substituted the earlier dependence on morphological, biological, and biochemical classification (Vandamme et al.1994). However, any species characterized according to phylogenetic comparisons must demonstrate phenotypic uniformity (Wayne et al. 1987). Figure 2.1 shows the taxonomic resolution of some of the currently used taxonomic techniques. The important taxonomical techniques will now be discussed briefly:

2.3.1 Genotypic methods

2.3.1.1 16S rRNA Gene Sequencing

The 16S rRNA gene sequence analysis has offered a foundation for studies of the regular relationships between prokaryotes and its application-transformed classification (Kämpfer 2010).

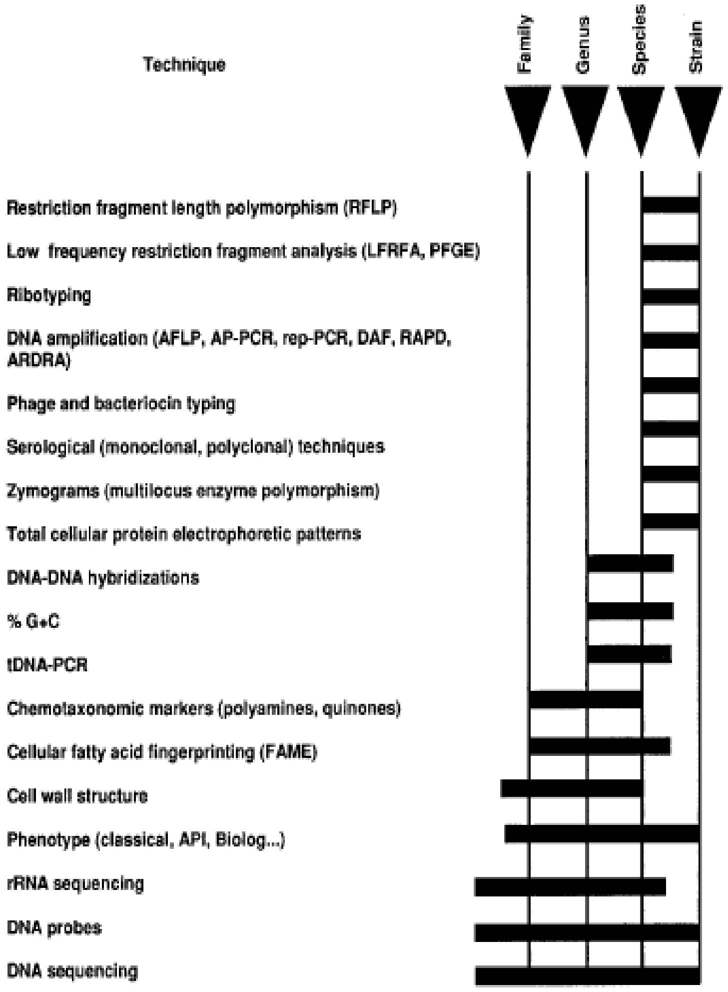

Fig. 2.1. Illustration of some of the currently used taxonomic techniques (Vandamme et al. 1996). AFLP, amplified fragment length polymorphism; AP-PCR, arbitrarily primed PCR; ARDRA, amplified rDNA restriction analysis; DAF, DNA amplification fingerprinting; FAMEs, fatty acid methyl esters; LFRFA, low frequency restriction fragment analysis; LMW, low molecular weight; PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; rep-PCR, repetitive element sequence-based PCR; RFLP, restriction fragment length polymorphism; tDNA-PCR, transfer DNA intergenic spacer PCR; 1D, 2D, one and two-dimensional, respectively.

The 16S rRNA gene sequence has the benefit that it is widely distributed, highly conserved, plays a fundamental role in protein synthesis, cannot be transferred horizontally and has an evolution rate which characterizes a suitable level of differences between organisms (Clarridge 2004). Organisms that have a 16S rRNA gene sequence similarity value of 97% or more are classified as members of the same species (Tindall et al. 2010), but it is necessary to compare several strains of a species because sequence similarities can differ up to 5% between strains of the same species (Bernardet et al. 2002). Higher 16S rRNA similarity values of 98.7–99.0% (Stackebrandt and Ebers 2006) and 98.2–99.0% (Meier-Kolthoff et al. 2013) have been recommended for species delineation. However, the 16S rRNA gene cannot be used alone for species delineation among groups such as the chryseobacteria and additional DNA-DNA reassociations are sometimes compulsory (Stackebrandt and Goebel 1994; Gillis et al. 2001).

After 16S rRNA sequencing, phylogenetic trees should be constructed. At least two methods should be used when constructing a phylogenetic tree (Bernardet et al. 2002). The following methods are available: maximum-likelihood, maximum-parsimony, unweighted pair group method (UPGMA) and the neighbour-joining method. Maximum-parsimony uses sites that contain at least two or more nucleotides or amino acids that are different while the UPGMA method accepts that the rate of nucleotide and amino substitutions of all evolutionary lineages are the same (Nei and Kumar 2000). A tree created through the maximum-likelihood method, is first built using e.g., the neighbour-joining method then the data likelihood is maximized by adjusting the branch lengths. The Nearest Neighbour Interchange (NNI) method is used to create variants. Maximum-likelihood branch lengths are computed and only variants that have the highest likelihood are retained (Nei and Kumar 2000).

The consistency of branching of the phylogenetic tree is proven through bootstrap analysis (Bernardet et al. 2002) and when the bootstrap value of the interior branch has a value of 95% or higher, only then can the topology of the branch be regarded as correct (Nei and Kumar 2000). The significance of the phylogenetic treeing method used will be enhanced if all the related organisms are included in the phylogenetic tree (Bernardet et al. 2002).

Sequences of the 16S rRNA gene that are new should be incorporated into a database that is well-known, e.g., GenBank, EMBL-EBI (European Bioinformatics Institute*)* and DDBJ (DNA Data Bank of Japan) (Tindall et al. 2010). The organism's description and accession number should be included in the database. Confusion should be avoided by depositing the new 16S rRNA gene sequences under the laboratory code or culture collection number rather than using the binomial name of the organism (Bernardet et al. 2002). Another database, EzBioCloud, is a new database that contains16S rRNA gene and genome sequences of *Bacteria* and *Archaea* (Yoon et al. 2017).

The 16S rRNA gene sequencing method, however, has some limitations when it comes to interpretation, incomplete databases in some cases and the inability to assign a species for organisms that have diverged recently. More comprehensive approaches e.g., whole-genome sequencing are becoming more important (Land et al. 2015).

2.3.1.2 Pulsed Field Gel Electrophoresis (PFGE)

Pulse Field Gel Electrophoresis (PFGE) is a fundamental genotyping method utilised for the separation of large DNA molecules after digestion with exclusive restriction enzymes (Sharma-Kuinkel et al. 2016). The method offers a good illustration of the whole bacterial chromosome in a single gel with a very high reproducible restriction profile (Sharma-Kuinkel et al. 2016). This technique can resolve huge DNA fragments which are visualised by staining (Prakash et al. 2007). Strains with at least 70% DNA binding values have a tendency of exhibiting similar protein fingerprints, with only slight variations (Pot et al. 1994).

2.3.1.3 Restriction Fragment Length Polymorphism (RFLP) and Plasmid DNA profiling

Restriction fragments are pieces of DNA that have been cut from larger molecules by restriction enzymes (Williams 1989). RFLP is a molecular method used to differentiate minor nucleotide sequence variations in homologous fragments of DNA (Balraj et al. 2011). Agarose or acrylamide gel electrophoresis separates DNA molecules, genomic, plasmid, or PCR products, incubated with restriction enzymes and the assimilated product by size (Balraj et al. 2011).

Even though RFLP is an important identification tool in molecular biology, it results in profiles that are challenging and difficult to compare while plasmid profiling generates profiles which may not be stable since it is difficult for bacteria to retain plasmids over several generations (Regnault et al. 1997).

Derivatives of the RFLP technique include methods like ribotyping, amplified ribosomal DNA restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD) and repetitive PCR (rep-PCR) (Prakash et al. 2007).

Ribotyping is a technique that is based on the total genomic DNA cleavage by restriction endonucleases, accompanied by electrophoretic separation, Southern blot transfer, and reassociation of transferred DNA fragments with a radiolabelled ribosomal operon probe (Bouchet et al. 2008).

ARDRA is mainly used to monitor great numbers of isolates at the same time. The method uses the digestion of amplified ribosomal DNA utilizing different restriction enzymes to yield patterns that are joined to get a profile (Maslow et al. 1993).

The RAPD technique is used for rapid epidemiologic typing of a wide-ranging microorganisms (Koeleman et al. 1998). A number of amplified fragments are formed which, when fixed on the gel, creates a strain-specific profile (Czekajlo et al. 2006). Even though the RAPD method offers the benefits of simplicity and rapidity, a lack of reproducibility has been described because of its high vulnerability to variation by primer and DNA concentration, DNA template quality, gel electrophoresis, and the type of DNA polymerase.

Repetitive-PCR (Rep-PCR) is a technique used for fingerprinting of bacterial genomes through the examination of strain or subtype-specific patterns attained from PCR of repetitive DNA elements existing within a bacterial genome (Versalovic et al. 1994).

2.3.1.4 DNA-DNA Hybridization

The DNA-DNA hybridization or DNA-DNA reassociation technique is a practice in classification for description of species. Wayne and collegues (1987) proposed that bacterial species in general would comprise of strains with 70% or greater DNA-DNA affiliation and with 5% or less ΔT_m values and both values must be taken into consideration. DNA affiliation is estimated by letting single-stranded DNA from one strain reassociate with single-stranded DNA from a second strain, resulting in a double-stranded DNA molecule (Baron 1996). The reaction is fully dependent on temperature. The optimal temperature for DNA reassociation is 25 to 30°C (ΔT_m) lower than the temperature at which integral double-stranded DNA denatures into single strands (Baron 1996).

DNA-DNA reassociation is a technique that offers more resolution than 16S rDNA sequencing, and the 70% standard has been the basis for describing a bacterial species (Cho and Tiedje 2001). However, the method is unpopular. Main disadvantages of DNA-DNA reassociation, are the lengthy nature of pairwise cross-hybridizations, the necessity for isotope utilization, and the impracticality of establishing a central database (Cho and Tiedje 2001). Another disadvantage is that the technique gives the relative percentage of similarity, but not the definite sequence identity (Prakash et al. 2007).

2.3.1.5 Guanine and Cytosine Ratio (G+C Ratio)

The G+C content in bacterial DNA ranges from 25–75%. This percentage is precise, however, not exclusive to some degree. Two isolates with the same G+C content might or might not belong to the same species (Baron 1996). If the G+C contents vary greatly, then it means the strains cannot be members of the same species. Analysis of DNA G+C ratio is one of the conventional genotypic methods in taxonomy (Prakash et al. 2007). In apparent species, the G+C content of strains generally vary by less than three mol%.

2.3.2 Conventional Phenotypic Tests

Conventional microbiological methods for bacterial identification have been utilised for many years and continue being an essential part of all microbiological laboratories at present (Morgan et al. 2009). These methods include basic growth parameters such as growth at different temperatures, growth under aerobic or anaerobic conditions, the need for specific nutrients, the ability to produce specific metabolites, and production of enzymes always gives essential information on not only the identity, but also the spoilage potential of an organism (Banwart 1989).

The description of the phenotypic characteristics of bacteria is an important taxonomic tool and should be used together with molecular-based techniques for bacterial identification (de Beer 2005). The classification of organisms has been performed by using traditional phenotypic methods for a long time as they form an integral part of classification, however, it has become clear that classification of microorganisms using these methods alone does not really correlate well with evolutionary relationships (Lane et al. 1985).

2.3.2.1 Morphological and Biochemical Methods

Phenotypic characteristics of bacteria include morphology of the bacterial cell and the bacterial colony as well as the biochemical reactions of the bacterium (Bisen et al. 2012). Morphological and biochemical characteristics of organisms can only be observed after cultivation and incubation. Morphological characteristics include, colony appearance in terms of shape, elevation, edge, optical characteristics, consistency, colony surface and pigmentation (Abiola and Oyetao 2016). Biochemical characteristics include enzyme production, metabolism and oxidation of a wide range of carbohydrates, aerobic or anaerobic reactions (Bisen et al. 2012).

It is essential to obtain a pure culture in order to identify an organism and the streak plate method is specifically used for this purpose (Bisen et al. 2012). After a pure culture is obtained, it can then undergo a series of cell treatment procedures such as Gram or Ziehl-Neelsen stains which assist in the microscopic determination of the morphology of the bacterial cells and the presence or absence of spores (Aguilera-Arreola 2015). Gram staining classifies bacterial strains into the Gram-positive and Gram-negative groups (Jesumirhewe et al. 2016). Proper atmospheric (e.g. aerobic or anaerobic) conditions are essential for the isolation and identification of bacteria (Bisen et al. 2012). Incubation temperature, pH, nutrients needed for growth, and antibiotic resistance are also essential characteristics that need to be determined (Bisen et al. 2012).

Biochemical methods of identification include the catalase; oxidase; motility; haemolytic; oxidation-fermentation and glucose fermentation tests (Prakash et al. 2007; Aguilera-Arreola 2015).

The majority of the genera in the family *Weeksellaceae* produce yellow to bright orange yellow-pigmented colonies on agar media and this primarily depends on the media and incubation temperature or the time (Hugo et al. 2019). Valuable information can also be obtained from microscopic examination with different staining techniques and biochemical reactions which will make it possible to differentiate between different genera and existing species. The majority of the species of *Chryseobacterium* produce lipolytic and proteolytic enzymes (Hantsis-Zacharov and Halpern 2007a, b; Yuan et al. 2018).

Identification of bacteria can be attained by utilizing a manual or a computerised system based on numerical classification (Sneath and Sokal 1973). This procedure involves the comparison of a large number of typically phenotypic characteristics of one organism with similar phenotypic characteristics of other organisms (Busse et al. 1996).

2.3.2.2 Automated BIOLOG^{TM} Omnilog System

The BIOLOG^{TM} Omnilog identification method is designed for the phenotypic identification of bacteria and fungi (filamentous and yeasts) (Sandle et al. 2013). The BIOLOG^{TM} method identifies microorganisms based on the exchange of electrons produced during respiration when microorganism cells oxidize a carbon source in the 96-well microplate, leading to tetrazolium-based colour changes that allows colorimetric determination of the increased respiration that takes place when microbial cells are oxidizing a carbon source (Miller et al. 1993). In a 96-well microtitre plate, all the wells are initially colourless, however, when a chemical in a well is oxidized, a burst of cellular respiration causes the reduction of the tetrazolium dye in the well, forming a purple colour which indicates that the tetrazolium-based reagent has been irreversibly reduced to a soluble purple compound called formazan (Miller et al. 1993, Sandle et al. 2013).

The BIOLOG^{TM} Omnilog system can be used to classify and differentiate a variety of microorganisms. Ecological and virulent microorganisms produce a distinctive pattern from discrete test reactions within the 96-well microplate (Biolog 2008). Resulting metabolic fingerprints are automatically read and verified in seconds. These patterns are evaluated with sophisticated interpretation software, the MicroLog^{TM} database software, and compared to extensive organism and pathogen databases. These fingerprint reaction patterns offer a massive amount of information appropriately contained in a single BIOLOG^{TM} Microplate (Biolog 2008; Sandle et al. 2013).

For identification purposes, the metabolic profiles attained are compared with a database (the GEN III database) containing characteristic profiles of a range of microorganisms (currently about 2500) (Sandle et al. 2013). Identification of the organism is obtained when the algorithm compares the properties of the test results for the unidentifiedn microorganism with a number of alternative microorganisms held in the reference well which does not have any carbon source (Miller et al. 1993; Sandle et al. 2013).

2.4 FOOD SPOILAGE CHARACTERISTICS

A range of bacteria that use food as a carbon and energy source facilitates chemical reactions that cause unpleasant olfactory modifications in foods (Rawat 2015). Spoilage of fresh fish and other fish products is caused by the action of microorganisms, chemical oxidations and autolysis (Gram et al. 2002). Even though a variety of compounds are produced due to chemical oxidation and enzymatic degradation, microbial action and production of microbial metabolites is the principal source of fresh fish spoilage (Gram and Huss 1996). Microbial spoilage of food takes place in different forms, however, all result from microbial growth and activity which manifest itself as changes in the sensory characteristics (Gram et al. 2002). Spoilage can at times, be identified by visual changes before any assessable chemical changes take place.

Lipids and pigments in fat-containing foods may be metabolised producing undesirable off-flavours and development of compounds with aggressive biological effects or changes in colour (Forsythe 2000). Economic consequences resulting from spoilage of food because of off-odours and off-flavours may be severe, it is therefore crucial to have active methods of detecting the origin and being able to initiate counteractive actions with the slightest of delays (Dainty 1996). Sensory and microbiological analyses play an essential role in monitoring the shelf-life and quality of food products (Dainty 1996).

Disease resulting from the action of bacteria is one of the most important threats to the aquaculture industry (Jacobs 2007). Spoilage of fish products is another threat posed by microorganisms after harvest, during packaging, transporting and storage (Jacobs 2007). *Chryseobacterium balustinum* (Harrison 1929; Vandamme et al. 1994), *C. piscium* (de Beer et al. 2006), *C. piscicola* as well as *C. scophthalmum* (Mudarris et al. 1994; Vandamme et al. 1994) were isolated from diseased and spoiled fish.

The spoilage of food is mainly attributed to microorganisms being able to produce lipolytic and/or proteolytic enzymes which have major effects on the sensory characteristics of the food product.

2.4.1 Enzyme Production

Enzymes are biological molecules that accelerate biochemical reactions (Roy et al. 2018). Microbial enzymes are responsible for quality deterioration and food spoilage (Braun et al. 1999). Understanding the enzymatic processes occurring in food is essential for making effective, logical shelf-life assessments (Braun et al. 1999). Several bacteria have been described as being remarkable producers of proteases and lipases (Braun et al. 1999). Psychrotolerant bacteria present serious spoilage challenges to the food industry because of their ability to produce proteolytic and lipolytic enzymes (Vithanage et al. 2016).

2.4.1.1 Lipolytic Enzyme Production

Lipolytic enzymes are carboxyl esterases that catalyse the hydrolysis of acyl glycerols (Chen et al. 2003). Lipolytic enzymes are responsible for both agreeable and disagreeable flavours arising in dairy products (Mielman 2006). The breakdown of triglycerides by lypolytic enzymes results in sour, butyric, or soapy flavours (Chen et al. 2003; Bekker et al. 2016).

Off-flavours described as “oxidised card-boardy” or metallic are caused by the related development of carbonyls, due to oxidation of unsaturated fatty acids (Chen et al. 2003). Heat resistant psychrotolerant bacterial lipases can cause lipolysis in dairy products, even when heat is applied (Venter et al. 1999).

The optimal temperature for extracellular Gram-negative bacterial lipases ranges between 30–40 °C (Mielman 2006). Bekker et al. (2016), after inoculating milk with either *C. joostei* or *Pseudomonas (P.) fluorescens*, observed high levels of lipolysis produced by *C. joostei*. No significant differences were observed between *C. joostei* and *P. fluorescens* at 4 °C, however, *C. joostei* produced significantly higher levels of lipolysis than *P. fluorescens* at 25 °C. It was concluded that abusive storage temperatures prior to pasteurisation could result in intense lipolytic degradation of milk by *C. joostei*.

There is limited evidence concerning lipase production by chryseobacterial species, however, *Chryseobacterium bovis* is another example of a *Chryseobacterium* strain that displayed lipolytic activity in milk (Hantsis-Zacharov et al. 2008a).

2.4.1.2 Proteolytic enzyme production

Proteolytic enzymes are enzymes which breakdown proteins to peptones, polypeptides, and amino acids (Mielman 2006). Proteases can be categorized in terms of their origin, catalytic activity and nature of the reactive group in the catalytic site (Raveendran et al. 2018). Proteases hydrolyse casein fractions and result in defects defined as bitter off-flavours and result in age gelation (Stoeckel et al. 2016). Secreted proteases that degrade host tissues have been detected in some pathogenic bacteria and have been shown to play a role in the virulence of the organisms (Rochat et al. 2019).

Chryseobacterium species from different sources are able to produce proteolytic enzymes. These proteolytic enzymes enhance the pathogenic potential of some strains belonging to the genus *Chryseobacterium*. The fish pathogenic *Chryseobacterium* species, for instance, produce extracellular proteases. These proteases degrade constituents of muscle, cartilage and connective tissue (Bernardet et al. 2006).

Proteolytic enzyme production by *Chryseobacterium* species influences their spoilage capability (Bekker et al. 2015). Some *Chryseobacterium* species have been reported to produce proteolytic enzymes that are pasteurisation stable (Venter et al. 1999; Bernardet et al. 2006). Venter et al. (1999) found that *C. indologenes* Ix9a showed optimal proteolytic activity at a temperature of 50 °C. The heat-resistant protease from *C. indologenes* Ix9a was characterised as a metalloprotease, in that it consists of a catalytic metal ion at its active site, which assists in the metabolism of peptide bonds resulting in protein degradation.

Chryseobacterium balustinum, C. gleum and *C. indologenes* strains which were isolated from Cape marine fish in South Africa displayed varied proteolytic activities including H_2S production (Engelbrecht et al. 1996b). Off-odours produced by these organisms included pungent, stale and fruity odours. It was speculated that these organisms were introduced during the processing of the fish (Engelbrecht et al. 1996b). An important organoleptic effect of proteolysis in marine fish is the softening of the flesh which is followed by a slimy appearance (Engelbrecht 1992).

2.4.2 Sensory spoilage in terms of odour

Microbial growth degrades enormous quantities of food, and this affects the economy negatively and results in loss of substantial nutrient sources (Madigan et al. 2000). Microorganisms recovered from food will be capable of producing spoilage metabolites only when conditions promote uncontrolled growth (Mielman 2006). It is therefore important that determination of spoilage characteristics use assessable methods, since the spoilage potential of an organism is dependent on its ability to release spoilage metabolites (Bekker et al. 2016). These considerations in general, are the execution of a cautious mixture of microbiology, sensory analysis and chemistry (Gram et al. 2002).

2.4.2.1 Sensory analysis

Sensory food tests are important means for ensuring high product quality. Sensory analysis comprises the testing of appearance, taste, odour, consistency and texture of the food product (German Agricultural Society 2017). Foods contain a large number of aroma-active substances that interact with each other and in their interaction, trigger the sensory overall flavour impression in consumers (German Agricultural Society 2017).

Sensory analysis is a method that can be used to detect spoilage in food. Sensory analysis of seafood is an essential technique used by the Federal Drug Administration (FDA) to protect consumers of seafood that has been contaminated due to putrefaction (ORA Laboratory Manual 2013). The consistency of the sensory program is influenced by the integrity of the sensory analysts and the way in which the analyses are directed, described, and understood for regulatory purposes (ORA Laboratory Manual 2013).

Proteolytic and lipolytic enzymes produced by *Chryseobacterium* are commonly associated with off-odour and flavour production (Tsôeu et al. 2016). The type of odour produced by *Chryseobacterium* species can be described using the sniffing method (Bekker et al. 2016). GC-MS together with sensory analysis can be used to identify the compounds causing of off-odours and off-flavours (Bekker et al. 2016).

The organisms which are responsible for spoilage of marine fish are considered to be those which produce off-odours when cultivated in pure cultures on fresh fish or in fish press juice (Engelbrecht 1992). In a study by Engelbrecht (1992), odour production by flavobacterial species was assessed by using a sensory panel of five people to determine the type of odour produced. The odours assessed were: no odour, stale, fruity pungent or sulphidy. Out of 60 flavobacterial species tested, 19 produced no odour, another 19 produced a stale odour, 2 produced fruity, zero produced sulphidy and 20 produced a pungent odour.

2.4.2.2 Gas Chromatography (Volatiles)

Fish are very fragile and for this reason, it is crucial to have consistent spoilage evaluation approaches (Duflos et al. 2006). The freshness of fish can be investigated by the analysis of volatile compounds. Volatile organic compounds produced during spoilage of fish include alcohols, aldehydes, ketones, esters, organic acids and sulphur compounds (Parlapani et al. 2017). Characterization by means of signal pattern attained by headspace/mass spectrometry (HS/MS) is a modern practice which could allow sensory evaluation by means of chemical composition methods (Duflos et al. 2006).

Gas chromatography/mass spectrometry(GC/MS) can be utilised to characterize spoilage by identifying volatile compounds formed during spoilage (Parlapani et al. 2017) and is an ultimate method for approximate and numerical determination of volatile and semi-volatile organic compounds in a set of samples (Sneddon et al. 2007). Gas chromatography can separate many volatile and semi-volatile compounds but not always selectively identify them whereas MS can selectively identify many compounds but not always separate them and hence the two methods are combined to give effective results (Duflos et al. 2006).

The combination of (GC/MS) is most likely the most inclusive instrumental analytical practice that the scientist in food analysis can utilise at present (Gerhardt 1990). The GC/MS method can also be used to measure the free fatty acids responsible for off-flavours and off-odours produced during lipolysis of milk (Wang and Xu 2009).

In a study by Bekker et al. (2016), the determination of volatile compounds in fat-free and full-cream milk was performed by using the GC/MS method. *Chryseobacterium joostei* and *P. fluorescens* incubated at 4 °C and 25 °C were organisms used to conduct this study. *Chryseobacterium joostei* samples incubated at 4 and 25 °C for fat-free milk showed high levels of acetone production compared to that of the *P. fluorescens* samples. Isovaleric acid (3-methylbutanoic acid), was among the three volatile compounds detected in the *C. joostei* samples only whereas, 2=furfuryl alcohol and hexanal were only detected in the samples inoculated with *P. fluorescens*. Butanoic acid, dichloromethane, hexanoic acid, 2-nonanone and 2-pentanone were present in all the inoculated samples for full cream milk.

Each volatile can produce a variety of odours (Bekker et al. 2016). Fatty acids for instance, result in the production of cheesy and sour odours in milk, while isovaleric acid has been linked with sweat-like odour (Amoore 1977; Jooste et al. 1986a; Wang and Xu 2009).

2.5 CONCLUSIONS

The genus *Chryseobacterium* formed part of the *Flavobacteriaceae* family for a long time, but since 2019 belongs to the family *Weeksellaceae* (García-López et al. 2019). This genus has been isolated from a variety of sources which includes clinical, environmental, industrial and food sources. This study highlighted the occurrence of this genus in food, especially fish, where this genus has not only been implicated in pathogenesis of fish, but also fish spoilage.

Developments in techniques of studying prokaryotes resulted in the use of molecular techniques which include 16S rRNA gene sequencing. This gene plays a fundamental role in the classification of microorganisms as it is an outstanding phylogenetic marker for *Bacteria* and *Archaea*. Identification and classification of bacteria can be attained by utilizing a manual or an electronic system based on numerical classification.

The methods that have been utilized to identify strains include conventional (traditional) methods, computerized biochemical analysis, and molecular-based analysis. Even though conventional phenotypic methods form an integral part in species identification, they however, have limitations. Therefore, in order to complete a taxonomical study, the conventional phenotypic methods should be utilised in conjunction with the molecular-based methods.

The members of the genus *Chryseobacterium* include cold-tolerant and enzymatic spoilage microorganisms that are extensively isolated from food sources such as milk, fish, meat and poultry. Food spoilage is characterised by undesirable variations in taste, odour, appearance and texture resulting from microbial growth. The ability of *Chryseobacterium* species to produce proteolytic enzymes enhances their spoilage potential; however, methods such as sensory analysis and GC/MS analysis of the volatile compounds assist in investigating spoilage in terms of odour.

CHAPTER 3

THE CLASSIFICATION OF *Chryseobacterium* SPECIES ISOLATED FROM FISH

ABSTRACT

Eleven yellow-pigmented, Gram-negative bacterial strains isolated in a previous study (Engelbrecht 1992) from fresh Cape marine fish in South Africa were classified and identified in this study. The methods used included molecular-based analysis, conventional methods and automated biochemical analysis. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the unidentified bacterial strains represented members of the genus *Chryseobacterium.* Three *Chryseobacterium* isolates namely; strains SH 23-4, SH 30-1 and SH 28-3 had a high similarity percentage value of 99.8%, and 100% for *C. piscium* indicating that they could be strains of *C. piscium*, while five had a similarity percentage that is lower than 98.7% meaning that they might be novel species, however, all strains require further polyphasic investigations to have full evidence for description as novel species. The 11 bacterial species and three reference strains (*C. gleum, C. balustinum,* and *C. piscium*) were also profiled and identified using the BIOLOG^{TM} Omnilog Gen III identification system. The three reference strains were correctly identified by the Omnilog identification system, seven of the bacterial strains were incorrectly identified as being *C. palustre*, *C. scopthalmum* and *C. ureitylicum* and five of the 11 bacterial isolates had a similarity value that was lower than 0.5 and they had a no identity result. The bacterial isolates were also characterized using conventional phenotypic methods.

3.1 Introduction

Bacterial taxonomy was believed to be an uninteresting field in microbiology, by young or determined scientists (Vandamme et al. 1996). The latest taxonomic techniques and improvements have however, changed this attitude. These developments revolutionized the current perceptions in the phylogeny and taxonomy of all existing organisms. Taxonomy plays a significant role in the classification and identification of organisms (Prakash et al. 2007). It is based on the nomenclature, classification and identification of organisms (Bisen 2012). All genotypic, phenotypic, and phylogenetic information are integrated in polyphasic taxonomy.

The methods that have been used to classify and identify microorganisms include molecular-based analysis, conventional methods and automated biochemical analysis (Morgan et al. 2009). The modern identification methods are as a result of the development in molecular biology, unravelling the structure and function of microorganisms (Khatib et al. 2014). These methods are more effective and the time for microbial identification using molecular-based PCR techniques is much less compared to conventional methods of detection (Senthilraj et al. 2016).

The 16S rRNA gene sequencing technique is an example of a molecular based bacterial identification method. Culture-independent methods for revealing microbial biodiversity are mainly based on the examination of small subunit ribosomal RNA (SSU rRNA) genes from environmental samples (Giovannoni et al. 1990). PCR is a process that can produce more than a million copies of a particular DNA or RNA sequence (Johnson 1991). It is a valuable, positive and rapid technique with high sensitivity and specificity (Joshi and Deshpande 2011). The conventional Sanger sequencing method, or the first-generation sequencing, includes PCR amplification, product qualitative detection and separation by gel electrophoresis, purification of the amplicon through ethanol precipitation, sequencing by an amplification reaction and final capillary electrophoresis (Chen et al. 2014).

Phylogenetic analysis can be obtained by the construction of a phylogenetic tree which is used to determine the genus to which the strain belongs and its closest neighbours (Prakash et al. 2007). Initially, Stackebrandt and Goebel (1994) proposed that a 16S rRNA gene sequence similarity of 97% should become the limit for delineation of prokaryotic species, and bacterial strains which show more than 3% on 16S rRNA gene sequence divergence, were considered to be members of different species (Vandamme et al*.* 1996). However, recently, a cut-off value of 98.7–99%, after inspection of a large amount of published data, was suggested (Stackebrandt and Ebers 2006).

The BIOLOG^{TM} Omnilog is an automated identification method that is designed for the phenotypic identification and characterization of bacteria and fungi (filamentous and yeasts) (Sandle et al. 2013). It identifies microorganisms based on the exchange of electrons generated during respiration when microorganism cells oxidize a carbon source in a 96-well microplate, leading to tetrazolium-based colour changes (Miller et al. 1993; Truu et al. 1999).

The genus *Chryseobacterium* was initially described by Vandamme et al. (1994) as belonging to the family *Flavobacteriaceae*. However, due to recent studies based on the genome taxonomic classification of Bacteroidetes, the genus *Chryseobacterium* has been placed in the family *Weeksellaceae* (García-López et al. 2019). The members of the genus *Chryseobacterium* include cold-tolerant organisms with the ability to cause spoilage by enzymatic degradation of food (proteolysis). Their association with the deterioration of food and food products has gradually increased (de Beer 2005). Some *Chryseobacterium* species associated with fish include *C. arothri*, which was isolated from the kidneys of a pufferfish from the warm tropical waters of the Hawaiian Islands (Campbell et al. 2008). *Chryseobacterium* *balustinum* was isolated from marine fish (Harrison 1929, Bergey and Breed 1948; Engelbrecht et al. 1996a, 1996b), while four strains of *C. piscium* were isolated from fish caught in the South Atlantic Ocean near South Africa (de Beer et al. 2006). Fish and other fish products are easily spoiled because of their high water activity (a_w), neutral pH, and low content of connective tissue (Hassoun and Karoui 2017). The psychrotolerant and proteolytic nature of *Chryseobacterium* species enables some of them to grow and cause spoilage of fish residing in cold aqueous environments (de Beer et al. 2006).

The aims of this study were to identify and classify 11 yellow-pigmented, Gram-negative bacterial strains isolated from fish in a previous study (Engelbrecht 1992) by 16S rRNA sequencing and the BIOLOG^{TM} Omnilog identification system and then to characterize the strains by conventional tests together with the information from the BIOILOG^{TM} Omnilog system.

3.2 Methods and Materials

3.2.1 Cultures used and their maintenance

The strains and the reference strains used in this study are listed in Tables 3.1 and 3.2, respectively. The test strains used in this study were isolated from fresh marine fish obtained from commercial fish retailers in Cape Town, Western Cape Province, South Africa in a previous study (Engelbrecht 1992). Thirty-nine portions of fresh, processed Cape hake and 26 portions of fresh fish species other than hake (Kingklip, monk, angelfish and gurnard) from the processing factory were taken to the laboratory and kept at 4 °C until testing on the same day. The three reference strains used in this study were selected because *C. gleum* is the type strain for the genus *Chryseobacterium* and both *C. piscium* (de Beer et al. 2006) and *C. balustinum* (Harrison 1929) were isolated from fish. The reference strains were obtained from international culture collections (Culture Collection, University of Göteborg, CCUG and National Collection of Type Cultures, NCTC) in a freeze-dried state in ampoules.

For long-term maintenance, the isolates were freeze-dried on 5 mm diameter filter paper discs (Whatmann) in Petri dishes, transferred to small screw capped Wasserman tubes and stored at -20 °C. Prior to usage, the strains were reactivated in 10 ml nutrient broth (Oxoid CM67) and incubated for 48 h at 25 °C. Their purity was checked by streaking on nutrient agar (Oxoid CM003), incubating them at 25 °C for 24 h and performing Gram-staining after incubation. The pure cultures were cultivated on nutrient agar slants and were further stored at 4 °C for short-term maintenance and re-streaked every 4 to 6 weeks.

Table 3.1. The 11 unidentified yellow-pigmented, Gram-negative bacterial strains isolated from fresh South African marine fish (Engelbrecht 1992) which were used in this study.

Chryseobacterium **isolate**	**Source**	**Place of isolation**	**Date of isolation**
SH 23-4	Fresh Marine Fish	Cape Town	1990
SH 11-3(a)	Fresh Marine Fish	Cape Town	1990
SH 20-4	Fresh Marine Fish	Cape Town	1990
SH 30-1	Fresh Marine Fish	Cape Town	1990
SH 28-3	Fresh Marine Fish	Cape Town	1990
IJ 30-2	Fresh Marine Fish	Cape Town	1990
SH 11-3(b)	Fresh Marine Fish	Cape Town	1990
SH 25-4	Fresh Marine Fish	Cape Town	1990
SH 19-2(b)	Fresh Marine Fish	Cape Town	1990
SH 11-4(b)	Fresh Marine Fish	Cape Town	1990
SH 40-3	Fresh Marine Fish	Cape Town	1990

Table 3.2 Reference strains used in this study. NCTC, National Collection of Type Cultures; CCUG, Culture Collection, University of Göteborg.

Genus and species	**Culture collection**	**Source of collection**	**Reference**
Chryseobacterium gleum	NCTC 11432^T	Vaginal swab	Vandamme et al. 1994
Chryseobacterium balustinum	NCTC 11212^T	Heart blood of fresh water fish	Vandamme et al. 1994
Chryseobacterium piscium	CCUG 49271^T	Fresh marine fish	de Beer et al. 2006

3.2.2. DNA Extraction

The freeze-dried filter paper discs containing a specific strain from Tables 3.1 and 3.2 were added to 10 ml of nutrient broth and incubated for 48 h at 25 °C after which the cultures were each streaked onto nutrient agar and incubated at 25 °C for 48 h. After cultivation, single colonies were scraped from the agar using a pipette tip, resuspended into 100 µl NucleoSpin® Microbial DNA (Macherey-Nagel) kit elution buffer and centrifuged for 30 s, the resulting mixture was used for DNA extraction. DNA was extracted by using the NucleoSpin® Microbial DNA (Macherey-Nagel) kit, according to the manufacturer's instructions. The extracted DNA quantity and quality was assessed using the Nanodrop ND-1000 (v3.3.0) spectrophotometer. The extracted DNA was stored at -20 °C.

3.2.3 16S rRNA sequencing

The isolates in Tables 3.1 and 3.2 were exposed to whole-cell polymerase chain reaction (PCR) amplification of the 16S rRNA gene according to the manufacturer's instructions (Thermo-Scientific); dNTPs (2 µl of 10 mM) were added to 0.2 ml PCR tubes, followed by 1 µl of 10 µM forward and reverse primers. The forward primer used was 27F (5‟-AGAGTTTGATCCTGGCTCAG-3”, Integrated DNA Technologies) and the reverse primer was 1492R (5‟-GGTTACCTTGTTACGACTT-3”, Integrated DNA Technologies). Buffer (ThermoPol®; 5 µl of 10x concentration) and 1 µl of the DNA template sample were added to the PCR tubes. In addition, 0.3 µl of 5000 U/ml Taq DNA Polymerase (New England BioLabs Inc.) was added. The reaction volume was adjusted to 50 µl by addition of nuclease-free water and a quick spin of 1 s was done to collect all the liquid at the bottom.

Thermal cycling was conducted using a 2720 Thermocycler (Applied Biosystems) that was programmed as follows: initial denaturation at 94 °C for 5 min, 35 amplification cycles of: denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 115 s. Final elongation was allowed at 72 °C for 5 min and the reaction was kept at 4 °C until further processing.

3.2.4. Visualisation of PCR Amplicons

The PCR amplicons were visualized on a 1% w/v agarose (Seakem® LE Agarose, Lonza) gel to which ethidium bromide stain (~20 mg/ml) was added. The agarose gel was prepared using 1x TAE buffer (containing 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0).

The gel wells were loaded with 1 µl of 6x TriTrack DNA loading dye (10 mM Tris-HCl, pH 7.6; 0.03% bromophenol blue; 0.03% xylene cyanol FF; 0.15% orange G; 60% glycerol and 60 mM EDTA) mixed with 5 µl of the PCR product. The DNA marker Thermo-Scientific O‟gene Ruler™ (3 µl) was also loaded alongside the samples. Electrophoresis was performed for 34 min at 9 V/cm. The PCR amplicons were viewed using a Gel Doc^{TM} EZ Imager (Bio-Rad) and photographed using ImageLab^{TM} software (version 5.0, Bio-Rad).

3.2.5. Amplicon clean-up and sequence reactions

Amplicon lengths of about 1500 bp corresponding to the probable amplicon bands were excised and purified with the Wizard® SV Gel and PCR Clean-Up system (Promega) according to the manufacturer's instructions. These were then used as templates for the subsequent sequencing reactions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The ddNTP chain termination method (Sanger sequencing) was used followed by sequencing clean-up reactions using the EDTA/ethanol precipitation protocol in the BigDye manual. The samples were submitted for Sanger sequencing at the University of the Free State, Bloemfontein, South Africa.

Sequence data was analysed and aligned using Geneious Pro R9 software (http://www.geneious.com, Kearse et al. 2012) and compared with sequences on the EzBioCloud (https://www.ezbiocloud.net/) database (Yoon et al. 2017) to identify closely related validly published species.

Determination of the relationship of the unidentified isolates to that of the type strains of the already validly published 113 *Chryseobacterium* species (http://www.bacterio.net/chryseobacterium.html) were done based on phylogenetic analyses performed using Molecular Evolutionary Genetics Analysis (MEGA) software version 7 (Tamura et al. 2016). Neighbour-joining and maximum likelihood methods with Kimura two (KP2) parameter distance measure aided in the construction of phylogenetic trees (Tamura et al. 2016). Confidence values were estimated from bootstrap analysis of 1000 replicates.

3.2.6. Automated phenotypic tests

3.2.6.1. BIOLOG^{TM} Omnilog Gen III Identification and phenotypic tests

The unidentified strains and reference strains were streaked onto NA plates and incubated at 25 °C for 24 h then profiled using the BIOLOG^{TM} Omnilog Gen III identification system (BIOLOG Inc., Hayward, CA, USA) according to the manufacturer's instructions. After incubation, a sterile swab was used to pick up a single colony from the previously incubated plate. The colony was suspended in an inoculating fluid (IF-A) which is used for organisms that grow at a normal rate and this was done at the recommended (BIOLOG) cell density. A 100 µl of the cell suspension was then inoculated into each of the GEN III MicroPlate wells. An automated pipette was used to dispense the cell suspension into the 96-well colourless microtiter plates which were further incubated in the BIOLOG^{TM} incubator at 25 °C for 24 h to allow the phenotypic fingerprint to form. The RetroSpect^{TM} 2.0 software was used to view the data.

3.2.7 Conventional Phenotypic Tests

Phenotypic tests used were in accordance to the minimal standards for the description of new taxa in the family [*Flavobacteriaceae*] (Bernardet et al. 2002) and the biochemical tests were performed according to Cowan (1974) and MacFaddin (1980).

The isolates were streaked on NA and incubated for 48 h at 25 ^{o}C. Colonial morphology was observed on NA. Gram-staining and cell morphology determination were done according to MacFaddin (1980). The production of oxidase, catalase, lipolytic and proteolytic enzymes was determined according to MacFaddin (1980). Motility was determined by simultaneous stab inoculation into nutrient agar tubes and growth in nutrient broth at 22 ^{o}C and checking for motility with phase-contrast microscopy (Nikon).

For standardization of the inoculum concentration for the rest of the phenotypic tests, strains were revived and cultivated in 10 ml NB after which the cell culture was transferred and cultivated in 100 ml of NB in 500 ml Erlenmeyer flasks at 25 °C for 48 h. The cells were then centrifuged at 1,087 x *g* for 10 min with an Eppendorf 5430 R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was discarded and the cell pellets washed with phosphate buffer (0.1 M, pH 7). Centrifugation was repeated as mentioned before. Cell pellets were re-suspended in 10 ml of phosphate buffer (0.1 M, pH 7) and standardized in comparison with a McFarland number 2 density standard (Difco 0691326). This served as the inoculum for the different biochemical tests.

The resulting series of phenotypic tests were performed according to Cowan (1974) and MacFaddin (1980): growth in 0 to 10% (w/v) sodium chloride; growth at 4, 10, 15, 25, 30, 35, 40 and 42 °C; growth on nutrient agar, MacConkey agar (Oxoid CM0007), trypticase soy agar (Oxoid CM0131), R2A agar (Oxoid CM0906)) and standard plate count agar (Oxoid CM0463); production of hydrogen sulphide (Kliger iron agar; Oxoid CM0033), indole (Kovac's reagent; Merck 1.09293), urease on Christensen's urea agar (Richard and Kiredjian 1995); and hydrolysis of casein, gelatine (plate method), and lecithin (egg yolk medium).

3.3 Results and discussion

3.3.1 PCR amplicons

Figure 3.1 shows the electropherogram of the PCR amplicons for the 11 isolates' 16S rRNA regions. All the strains gave bands of ~1500 bp. The edited 16S rRNA sequences acquired from Sanger sequencing were blasted on the NCBI GenBank (http://www.ncbi.nlm.nih.gov) and EzBioCloud (http://www.ezbiocloud.net/) databases and the results confirmed that all of the 11 strains belonged to the genus *Chryseobacterium* based on their high similarity values (Table 3.3).

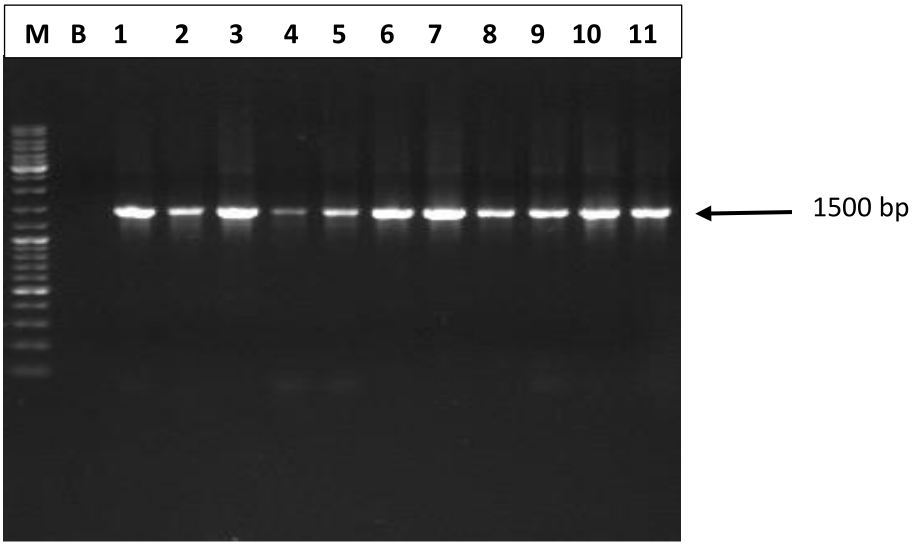

Fig. 3.1. Electropherogram of the ~1500 bp PCR products of the 16S rRNA regions of the 11 isolates. M, DNA molecular marker; B, Negative control; 1, strain SH 23-4; 2, strain SH 11-3(a); 3, strain SH 20-4; 4, strain SH 30-1; 5, strain SH 28-3; 6, strain IJ 30-2; 7, strain SH 11-3(b); 8, strain SH 25-4; 9, strain SH 19-2(b); 10, strain SH 11-4(b); 11, strain SH 40-3.

3.3.2. 16S rRNA gene sequence analysis

The BLAST search results for the 16S rRNA consensus sequences differentiated by using sequences of recognised *Chryseobacterium* type species on the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) are shown in Table 3.3. The maximum percent identity (Max. % identity) relates to a match of the subject sequence with the highest percentage of identical bases.

According to these results, strain SH 23-4 is closely related to *C. piscium* LMG 23089^T with a similarity of 99.89% and a query coverage of 100%. *Chryseobacterium balustinum* NBRC 15053^T was the second most closely related species to strain SH23-4 with 99.20% similarity and a query coverage of 99%. Isolates SH 30-1 and SH 28-3 had a similarity of 100% to *C. piscium* LMG 23089^T and a query coverage of 100%. *Chryseobacterium balustinum* NBRC 15053^T was also the second most closely related relative of both these isolates with similar similarities of 99.78% and query coverages of 100%. It can therefore, be concluded that all three these strains are not novel species of the genus *Chryseobacterium,* but could be strains of *C. piscium*.

Strain SH 11-3(a) showed the highest similarity of 97.72% to *C. yonginense* HMD1043^T with a query coverage of 99%. *Chryseobacterium antarcticum* AT1013^T was the second closest relative with a similarity of 97.51% and a query coverage of 100% (Table 3.3).

Chryseobacterium yonginense HMD1043^T was also closely related to strain SH 20-4 with a similarity of 97.35% and a query coverage of 97%, followed by *C. chaponese* Sa 1147-46^T with a similarity of 96.89% and a query coverage of 100%.

Chryseobacterium type strains that shared a similarity greater than 97.00% to isolate IJ 30-2 were *C. carnis* G81^T (99.67%), *C. treverense* IMMIB L-1519^T (97.63%) and *C. solincola* 1YB-R12^T (97.53%) and had query coverages of 99.67, 97.63 and 97.53%, respectively (Table 3.3). This strain, therefore, most likely belongs to *C. carnis*.

Isolate SH 11-3(b) had a similarity of 97.55% to *C. yonginense* HMD1043^T with a query coverage of 99% and also shared 97.33% similarity with *C. carnis* G81^T.

Isolate SH 25-4 was most closely related to *C. chaponese* Sa 1147-46^T at 97.03% similarity and had a query coverage of 100% and it's second most closely related species was *C. carnis* G81^T with a sequence similarity of 97.70% at 97% query coverage.

Isolates SH 19-2(b), SH 11-4(b) and SH 40-3 were also closely related to *C. chaponese* Sa 1147-46^T and *C. carnis* G81^T with similarity values of 97.70, 97.25, 96.92% and query coverages of 100, 99, and 100%, respectively.

Percentage similarities of the 11 isolates to existing *Chryseobacterium* species ranged from 96.47–100% (Table 3.3). Not all of them exceeded the commonly recognized threshold value of 97.0%, suggesting a possible novel species. Wayne et al. (1987) and Stackebrandt and Goebel (1994), proposed that 16S rRNA gene sequence similarity values greater than 97% need DNA-DNA hybridization (DDH) methods to approve individual species status. It can therefore be concluded that 8 of the 11 isolates require DDH studies. Strains SH 20-4, SH 11-4(b) and SH 40-3 had gene sequence similarity values that were lower than 97.0%. This may indicate that these three strains are a novel species or more than one novel species of *Chryseobacterium*.

Stackebrandt and Ebers in 2006 re-assessed the relationship between 16S rRNA gene sequence similarity and DNA re-association values and suggested a threshold value of 98.7%. Taking this cut-off value into consideration, and basing classification only on 16S rRNA gene sequence similarity values, it therefore can be concluded that strains SH 11-3(a), SH 11-3(b), SH 25-4 and SH 19-2(b) together with SH 20-4, SH 11-4(b) and SH 40-3 represent possible new species, while strains SH 23-4, SH 30-1, SH 28-3 and IJ 30-2 do not represent new species since their closest relatives (*C. piscium,* 99.89%; *C. balustinum,* 99.20%; *C. piscium,* 100% and *C. carnis,* 99.67%) have similarity values that are higher than the threshold value. Other high value 16S rRNA gene sequence similarities have also been reported. The discussion of the correlation between 16S rRNA gene sequence similarity and DNA re-association values has been controversial and as a result the 16S rRNA gene cannot be used alone as a standard for bacterial delineation (Clarridge 2004).

3.3.3 Phylogenetic Analysis

A phylogenetic tree is an evaluation of the relationships between taxa (or sequences) and their proposed common ancestors (Felsenstein 2004; Hall 2011). These days, most phylogenetic trees are constructed from molecular information such as, DNA or protein sequences. At first, the objective of most molecular phylogenetic trees was to assess the relations between the species characterized by those sequences; however, the objectives have diversified lately (Hall et al. 2009).

Figure 3.2 shows the neighbour joining phylogenetic analysis of the 11 test strains in this study compared to type species of the genus *Chryseobacterium*. Another phylogenetic tree was constructed using the maximum likelihood in MEGA version 7 software package (Kumar et al. 2016) (Figure 3.3). It was evident from the trees that all the 11 test strains belonged to the genus *Chryseobacterium*.

Table 3.3: GenBank BLAST results for 16S rRNA gene sequences for all the 11 test strains.

Isolate	Description	Query Cover%	E-Value	Max% Identity	Accession number
SH 23-4	*Chryseobacterium piscium* LMG 23089^{T} 16S ribosomal RNA, partial sequence	100	0.0	99.89 (1620/1620)	042410.1
	Chryseobacterium balustinum strain NBRC 15053^{T} 16S ribosomal RNA, partial sequence	99	0.0	99.20 (1585/1585)	113721.1
	Chryseobacterium scophthalmum strain LMG 13028^{T} 16S ribosomal RNA, partial sequence	99	0.0	98.75 (1567/1567)	025386.1
SH 11-3(a)	*Chryseobacterium yonginense* strain HMD1043^{T} 16S ribosomal RNA, partial sequence	99	0.0	97.72 (1583/1583)	108572.1
	Chryseobacterium antarcticum strain AT1013^{T} 16S ribosomal RNA, partial sequence	100	0.0	97.51 (1576/1576)	025809.1
	Chryseobacterium chaponense strain Sa 1147-06^{T} 16S ribosomal RNA, partial sequence	100	0.0	97.29 (1572/1572)	117501.1
SH 20-4	*Chryseobacterium chaponense* strain Sa 1147-06^{T} 16S ribosomal RNA, partial sequence	100	0.0	96.89 (1561/1561)	117501.1
	Chryseobacterium yonginense strain HMD1043^{T} 16S ribosomal RNA, partial sequence	97	0.0	97.35 (1537/1537)	108572.1
	Chryseobacterium carnis strain G81^{T} 16S ribosomal RNA, partial sequence	97	0.0	97.24 (1533/1533)	126255.1
SH 30-1	*Chryseobacterium piscium* strain LMG 23089^{T} 16S ribosomal RNA, partial sequence	100	0.0	100 (2477/2477)	042410.1
	Chryseobacterium balustinum strain NBRC 15053^{T} 16S ribosomal RNA, partial sequence	100	0.0	99.78 (2460/2460)	113721.1
	Chryseobacterium scophthalmum strain LMG 13028^{T} 16S ribosomal RNA, partial sequence	100	0.0	98.88 (2398/2398)	025386.1

Isolate	**Description**	**Query Cover%**	**E-Value**	**Max% Identity**	**Accession number**
SH 28-3	*Chryseobacterium piscium* strain LMG 23089^T 16S ribosomal RNA, partial sequence	100	0.0	100 (2388/2388)	042410.1
	Chryseobacterium balustinum strain NBRC 15053^T 16S ribosomal RNA, partial sequence	100	0.0	99.77 (2372/2372)	113721.1
	Chryseobacterium scophthalmum strain LMG 13028^T 16S ribosomal RNA, partial sequence	100	0.0	98.84 (2309/2309)	025386.1
IJ 30-2	*Chryseobacterium carnis* strain G81^T 16S ribosomal RNA, partial sequence	96	0.0	99.67 (1646/3283)	126255.1
	Chryseobacterium treverense strain IMMIB L-1519^T 16S ribosomal RNA, partial sequence	100	0.0	97.63 (1594/3180)	104497.1
	Chryseobacterium solincola strain 1YB-R12^T 16S ribosomal RNA, partial sequence	100	0.0	97.53 (1589/3169)	116343.1
SH 11-3(b)	*Chryseobacterium yonginense* strain HMD1043 ^{T} 16S ribosomal RNA, partial sequence	97	0.0	97.55 (1531/1531)	108572.1
	Chryseobacterium carnis strain G81^T 16S ribosomal RNA, partial sequence	97	0.0	97.33 (1528/1528)	126255.1
	Chryseobacterium chaponense strain Sa 1147-06^T 16S ribosomal RNA, partial sequence	100	0.0	97.21 (1526/1526)	117501.1
SH 25-4	*Chryseobacterium jejuense* strain JS17-8^T 16S ribosomal RNA, partial sequence	100	0.0	98.93 (1395/1395)	044300.1
	Chryseobacterium lactis strain KC1864^T 16S ribosomal RNA, partial sequence	99	0.0	98.39 (1452/1452)	126256.1
	Chryseobacterium nakagawai strain G41^T 16S ribosomal RNA, partial sequence	98	0.0	98.29 (1376/1376)	126257.1

Isolate	**Description**	**Query Cover%**	**E-Value**	**Max% Identity**	**Accession number**
SH 19-2(b)	*Chryseobacterium chaponense* strain Sa 1147-06^T 16S ribosomal RNA, partial sequence	100	0.0	97.03 (2320/2320)	117501.1
	Chryseobacterium carnis strain G81^T 16S ribosomal RNA, partial sequence	100	0.0	97.70 (2311/2311)	126255.1
	Chryseobacterium jeonii strain AT1047^T 16S ribosomal RNA, partial sequence	100	0.0	96.85 (2283/2283)	025810.1
SH 11-4(b)	*Chryseobacterium chaponense* strain Sa 1147-06^T 16S ribosomal RNA, partial sequence	98	0.0	96.80 (1568/3130)	117501.1
	Chryseobacterium yonginense strain HMD1043 ^{T} 16S ribosomal RNA, partial sequence	96	0.0	97.25 (1537/3069)	108572.1
	Chryseobacterium antarcticum strain AT1013^T 16S ribosomal RNA, partial sequence	99	0.0	96.26 (1535/3063)	025809.1
SH 40-3	*Chryseobacterium chaponense* strain Sa 1147-06^T 16S ribosomal RNA, partial sequence	100	0.0	96.47 (1547/1547)	117501.1
	Chryseobacterium yonginense strain HMD1043 ^{T} 16S ribosomal RNA, partial sequence	97	0.0	96.92 (1592/1592)	108572.1
	Chryseobacterium carnis strain G81^T 16S ribosomal RNA, partial sequence	97	0.0	96.81 (1515/1515)	126255.1

Strain IJ 30-2 clustered tightly together with *C. carnis* which was isolated from human clinical sources (Holmes et al. 2013) and this was supported by a high bootstrap value of 99% (Fig. 3.2). It also sub-clustered with *C. solincola* isolated from soil (Benmalek et al. 2010) and *C. treverense* isolated from human blood (Yassin et al. 2010). The stability of this phylogeny was also supported by the maximum likelihood tree (Fig. 3.3). The GenBank BLAST results for 16S rRNA gene sequences for these organisms in Table 3.3 correlated with the phylogenetic results in that closely related species of the genus *Chryseobacterium* clustered tightly together with the unidentified strains demonstrating very close relationships.

The 16S rRNA gene sequence similarity values of 99.8, 100 and 100% between SH 23-4, SH 30-1 and SH 28-3 respectively, supported their affiliation to the same species, *Chryseobacterium piscium,* and this was also demonstrated on the two phylogenetic trees (Figures 3.2 and 3.3).

Strains SH 11-3(a) and SH 11-3(b) clustered tightly together which was supported by a bootstrap value of 89%. These strains also formed a sub-cluster with strains SH 20-4, SH 19-2(b), SH 11-4(b) and SH 40-3 suggesting that they could belong to the same species even though some of them had very low bootstrap values. All six isolates clustered with *C. yonginense* isolated from a mesotrophic artificial lake (Joung and Joh 2011) as their nearest phylogenetic neighbour. The 16S rRNA sequencing analysis also indicated that these six strains might be regarded one or more novel species based on similarity percentages that were lower than 98.7%.

Strains SH 23-4, SH 30-1 and SH 28-3 clustered tightly with *C. piscium* (Fig. 3.2 and Fig. 3.3) which was isolated from fresh marine fish (de Beer et al. 2006). The 16S rRNA sequencing data also indicated that these strains may be regarded as *C. piscium* because of their high similarity values (99.89, 100 and 100%, respectively) to *C. piscium* (Table 3.3). *Chryseobacterium balustinum* and *C. scophthalmum* both isolated from diseased fish (Harrison 1929; Mudarris et al. 1994; Vandamme et al. 1994) formed a sub-cluster with *C. piscium* (Fig. 3.2 and Fig. 3.3), however, it was weakly supported since the bootstrap re-sampling values were lower than 70%.

Strain SH 25-4 clustered into the clade of the genus *Chryseobacterium* by forming a clear and separate progeny amongst the most closely related species such as *C. jejuense* isolated from soil samples (Weon et al. 2008), *C. nakagawai* isolated from clinical sources (Holmes et al. 2013) and *C. aurantiacum* isolated from a freshwater pond used for Murray cod culture (Luo et al. 2018). Strain SH 25-4 was stably close to *C. nakagawai* and *C. jejuense* in both the NJ and ML trees (Fig. 3.2 and Fig. 3.3), and the two type strains also showed highest percentage similarity to strain 8_SH 25-4 on the basis of 16S rRNA gene sequences (Table 3.3). According

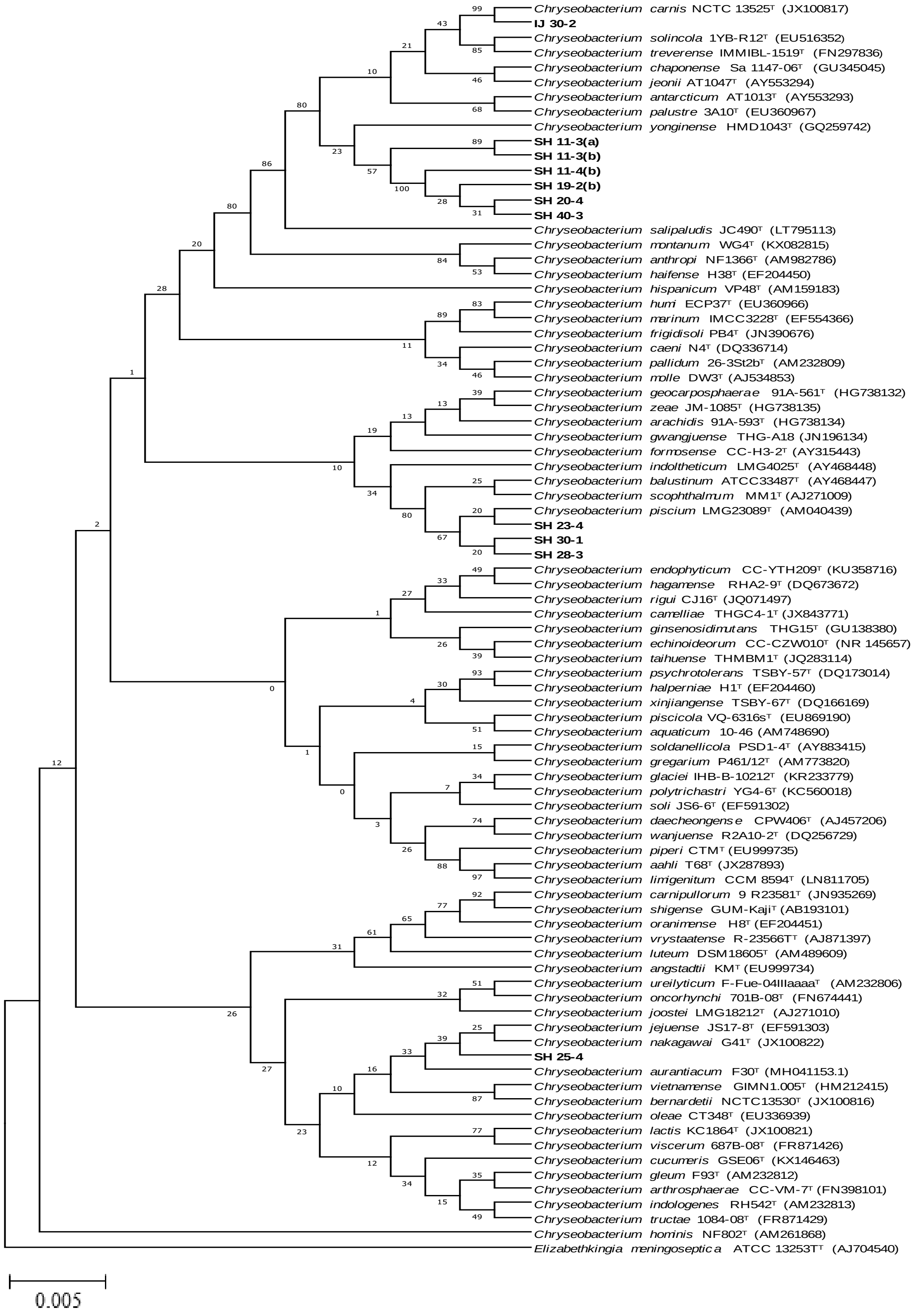

Fig. 3.2. Phylogenetic analysis of the 11 *Chryseobacterium* strains isolated from fish, nearest *Chryseobacterium* type species and outgroup (*Elizabethkingia meningoseptica*) based on 16S rRNA gene sequences available from the GenBank database (accession numbers are given in parentheses). Multiple alignments were performed and evolutionary distances were computed using the Kimura 2-parameter method. Clustering was determined using the neighbour-joining method in the MEGA version 7 software package (Tamura et al. 2016). Bootstrap values >70%, based on 1000 replications, are given as percentages at the branching points. Bar, 0.0050 substitutions per nucleotide position.

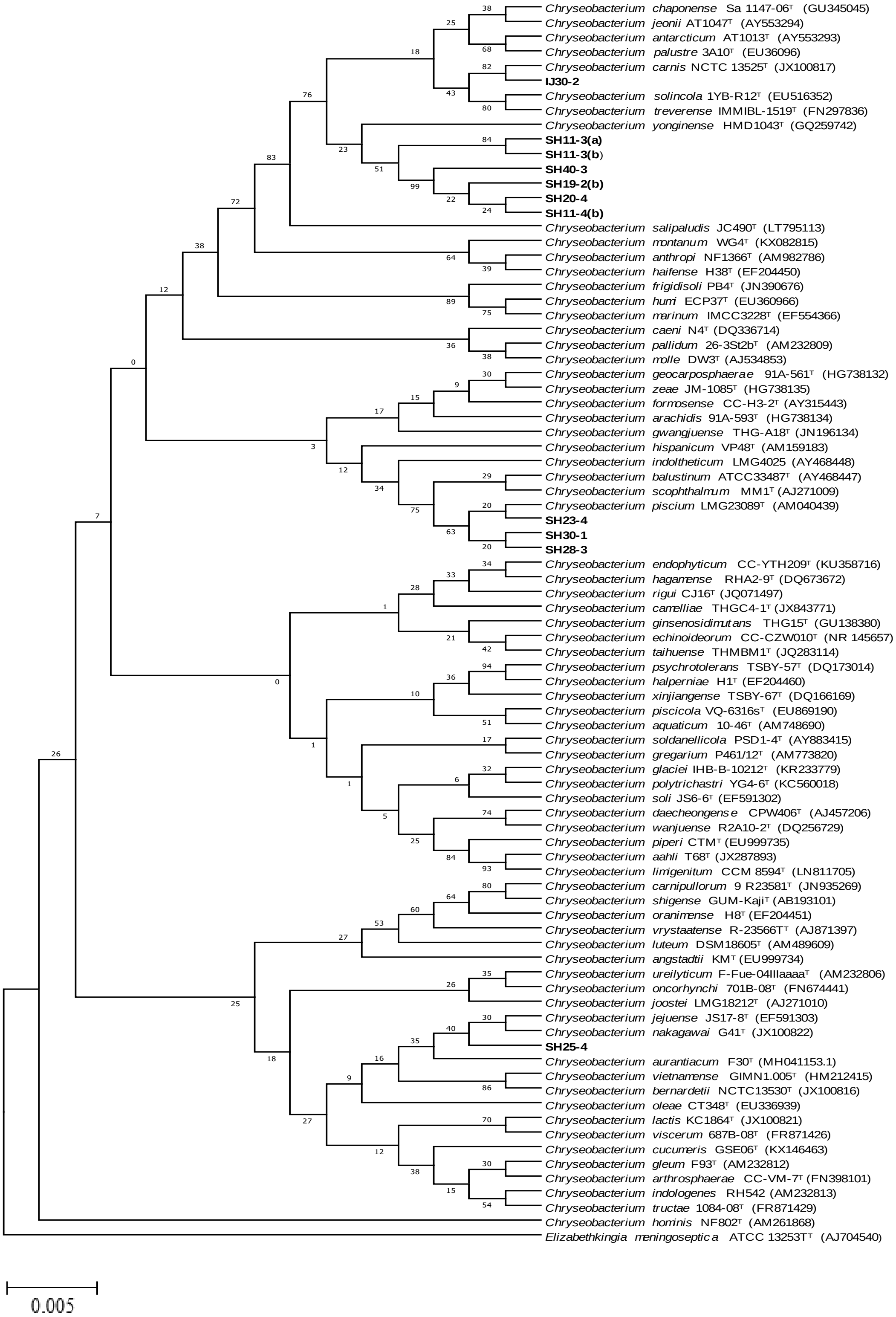

Fig. 3.3. Phylogenetic analysis of the 11 *Chryseobacterium* strains isolated from fish, nearest *Chryseobacterium* type species and outgroup (*Elizabethkingia meningoseptica*) based on 16S rRNA gene sequences available from the EzBioCloud database (accession numbers are given in parentheses). Multiple alignments were performed and evolutionary distances were computed using the Kimura 2-parameter method. Clustering was determined using the maximum likelihood method in the MEGA version 7 software package (Tamura et al. 2016). Bootstrap values >70%, based on 1000 replications, are given as percentages at the branching points. Bar, 0.0050 substitutions per nucleotide position.

to 16S rRNA gene sequence similarity, the closest neighbours of SH 25-4 included *C. jejuense* (98.93%), *C. lactis* (98.39%) and *C. nakagawai* (98.29%), however on the phylogenetic trees, *C. lactis* did not group very close to strain SH 25-4. This, therefore, shows that there are particular characteristics of relations that are better illustrated by phylogenetic trees than by percentage similarity.

It can be concluded that the evaluation of the 16S rRNA gene sequences assisted in the characterisation of organisms at the genus level through all major phyla of bacteria. This is because, the 16S rRNA gene sequence analysis can differentiate far more effeciently between strains of bacteria than is possible with phenotypic methods, it can allow a more precise identification of poorly described, rarely isolated, or phenotypically different strains, and also allows the classification of strains at multiple levels, including species and subspecies level (Clarridge 2004).

3.3.4 Phenotypic differentiation: BIOLOG^{TM} Omnilog Gen III

The BIOLOG^{TM} Omnilog identification system is an innovative device that is used for identifying and characterizing organisms. It uses the microbe's capability to oxidise particular carbon sources from a 71-substrate assay and its sensitivity to 23 inhibitory chemicals. This allows the formation of a metabolic fingerprint (BIOLOG 2008). The fingerprint data is imported into the Omnilog data collection software, which examines the database and makes an identification call.

Table 3.4 shows the phenotypic identification of the 11 bacterial isolates in this study and the three reference strains using the BIOLOG^{TM} Omnilog Gen III identification system. The SIM value is a crucial identification call factor and has to range between 0.5–1.0 after at least 8 h of cultivation for an identification to be made (BIOLOG 2008).

The percentage of strains identified to species level was 64.29% (9/14) and the SIM values ranged from 0.501 to 0.873 (Table 3.4). About 35.71% of the strains (5/14) could not be identified by the BIOLOG^{TM} identification system because they had low SIM values, which ranged from 0.115–0.436. This might be because the *Chryseobacterium* species are not present in the BIOLOG^{TM} system's present database and as a result, only the genus name was identified.

The BIOLOG^{TM} Omnilog identification system was able to correctly identify all the reference strains used for this study, however, all the test strains whose SIM values were below 0.5 were incorrectly identified to species level. Strains SH 23-4, SH 30-1 and SH 28-3 were identified as belonging to the same species being *C. piscium*/*C. scophthalmum* (Table 3.4). These

Table 3.4. Phenotypic identification of the 11 isolates and three reference strains using the BIOLOG^{TM} Omnilog Gen III identification system. PROB, Probability; SIM, similarity; ID, identification.

Strain	PROB	SIM	ID
C. balustinum NCTC 11212^T	0.714	0.714	*C. balustinum*
C. gleum NCTC 11432^T	0.803	0.803	*C. gleum/C. joostei*
C. piscium CCUG 49271^T	0.734	0.699	*C. piscium/C. scophthalmum*
SH 23-4	0.649	0.526	*C. piscium/C. scophthalmum*
SH 11-3(a)	-	0.387	No ID
SH 20-4	0.873	0.873	*C. palustre*
SH 30-1	0.787	0.787	*C. piscium/C. scophthalmum*
SH 28-3	0.712	0.655	*C. piscium/C. scophthalmum*
IJ 30-2	0.579	0.501	*C. palustre*
SH 11-3(b)	-	0.436	No ID
SH 25-4	0.600	0.505	*C. ureilyticum*
SH 19-2(b)	-	0.301	No ID
SH 11-4(b)	-	0.205	No ID
SH 40-3	-	0.115	No ID

results were in accordance to the 16S rRNA sequencing results by similarities (Table 3.3) and by the phylogenetic trees (Figures 3.1 and 3.2). They had SIM values ranging from 0.526–0.787 with probability values ranging from 0.649–0.787.

Strains SH 20-4 and IJ 30-2 were both identified as belonging to *C. palustre* (Table 3.4). Their SIM values ranged from 0.501–0.873 and their probability values ranged from 0.579–0.873, respectively. According to the 16S rRNA gene sequencing results (Table 3.3) and the phylogenetic trees (Figures 3.1 and 3.2), strain SH 20-4 could be regarded a novel species while strain IJ 30-2 was identified as a possible strain of *C. carnis*.

The blast gene sequence results were very helpful in assessing the overall phylogenetic relationship between the 11 unidentified test strains and nearest *Chryseobacterium* type species. Although the BIOLOG^{TM} method can be used for identification purposes, the tool was only able to identify a few *Chryseobacterium* species that were present in its database: *C. gleum, C. balustinum, C. indologenes, C. piscium/C. scophthalmum, C. palustre, C. ureilyticum* and *C. joostei*. The BIOLOG^{TM} method could as a result, only confirm that the test strains belonged to the genus *Chryseobacterium*. The BIOLOG^{TM} Omnilog identification system yielded a “No identification” result where it could not identify the isolates either to the genus or species level. It was stated in a study conducted by Chojniak et al. (2015) that the BIOLOG^{TM} system could be a valuable complement to other methods used for strain verification. However, using it as a single method for identification could be misleading hence it is important to use it together with other alternative identification methods.

3.3.5 Phenotypic differentiation: Conventional phenotypic tests

All strains in this study produced yellow, smooth colonies with a fruity/spoiled odour. The cells were Gram--negative and non-motille, which are typical characteristics of the genus *Chryseobacterium* (Hugo et al. 2019).

The growth characteristics of the 11 *Chryseobacterium* strains in this study as well as the reference strains in terms of temperature are indicated in Table 3.5. Most *Chryseobacterium* species grow at temperatures between 30-37 °C, while the majority are psychrotolerant and can grow at even lower temperatures (Hugo et al. 2019). In this study, all the strains were able to grow from 4 to 35 °C, however, only a few could grow at 42 °C. Strains SH 23-4 and SH 11-4(b) can be differentiated from both *C. balustinum* and *C. piscium* in that they can grow at 42 °C together with *C. gleum*. This may be an indication that these strains have pathogenic characteristics since *C. gleum* is a recognised human pathogen (Vandamme et al. 1994). The ability of these strains to grow at 4 °C is also a characteristic of some members of the genus *Chryseobacterium* which are cold-tolerant with the ability to grow at 7 °C or lower irrespective of their optimal growth temperature (Tsôeu et al. 2016).

Table 3.5. Growth characteristics of the 11 *Chryseobacterium* isolates and the reference strains at different temperatures. All data are from this study. +, positive; -, negative; w, weakly positive.

Strain	Growth Temperature (°C)					
	4	**10**	**15**	**25**	**35**	**42**
C. balustinum NCTC 11212^T	+	+	+	+	+	-
C. gleum NCTC 11432^T	+	+	+	+	+	+
C. piscium CCUG 49271^T	+	+	+	+	+	-
SH 23-4	+	+	+	+	+	+
SH 11-3(a)	+	+	+	+	+	-
SH 20-4	+	+	+	+	+	-
SH 30-1	+	+	+	+	+	-
SH 28-3	+	+	+	+	+	w
IJ 30-2	+	+	+	+	+	-
SH 11-3(b)	+	+	+	+	+	w
SH 25-4	+	+	+	+	+	-
SH 19-2(b)	+	+	+	+	+	-
SH 11-4(b)	+	+	+	+	+	+
SH 40-3	+	+	+	+	+	-

Table 3.6 indicates the growth characteristics of the 11 *Chryseobacterium* strains in this study as well as the reference strains in terms of growth media. All the strains could grow on all the different media except for strain SH 11-4(b), which could be differentiated from both *C. balustinum* and *C. piscium* in that it could not grow on MacConkey agar (Table 3.6). MacConkey agar is a selective and differential medium containing the selective agents, crystal violet and bile salts which hinder the growth of Gram-positive bacteria but allow growth of non-fastidious Gram-negative bacteria. The Gram-negative bacteria that do not grow on MacConkey agar, are differentiated from other Gram-negative bacteria because of their inability to ferment lactose and are more likely to be pathogenic (Bentz et al. 2018). In Table 3.5, strain SH 11-4(b) was also one of the few strains that could grow at 42 °C which is another indication that this strain might be pathogenic towards humans or animals/fish or both.

Table 3.6. Growth characteristics of the 11 *Chryseobacterium* isolates and the reference strains on different growth media. All data are from this study. +, positive; -, negative; w, weakly positive; NA, nutrient agar; TSA, trypticase soy agar; MCA, MacConkey agar; R2A, Reasoner's2 agar; SPCA, standard plate count agar.

Strain	Growth media				
	NA	**TSA**	**MCA**	**R2A**	**SPCA**
C. balustinum NCTC 11212^T	+	+	+	+	+
C. gleum NCTC 11432^T	+	+	w	+	+
C. piscium CCUG 49271^T	+	+	+	+	+
SH 23-4	+	+	+	+	+
SH 11-3(a)	+	+	w	+	+
SH 20-4	+	+	+	+	+
SH 30-1	+	+	+	+	+
SH 28-3	+	+	+	+	+
IJ 30-2	+	+	+	+	+
SH 11-3(b)	+	+	w	+	+
SH 25-4	+	+	+	+	+
SH 19-2(b)	+	+	+	+	+
SH 11-4(b)	+	+	-	+	+
SH 40-3	+	+	+	+	+

Growth was observed with 0–3% NaCl in most of the strains and reference strains (Table 3.7). Strain SH 11-4(b) was the only *Chryseobacterium* test strain which could readily grow in 5% NaCl inclusion level and it shared this characteristic with *C. piscium* which also displayed growth in 5% NaCl inclusion level in a study conducted by de Beer and colleagues in 2006. It was also concluded that *C. piscium* was a possible spoilage organism because of its ability to convert urea to ammonia due to the action of the enzyme, urease. The ability of these isolates to grow at 5% NaCl inclusion level therefore suggests that they may be able to cause spoilage of food which have been preserved with salt.

Table 3.7. Growth characteristics of the 11 *Chryseobacterium* isolates and the reference strains at different salt concentrations. +, positive; -, negative; w, weakly positive.

	% NaCl										
Strain	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**
C. balustinum NCTC 11212^T	+	+	+	+	+	+	+	-	-	-	-
C. gleum NCTC 11432^T	+	+	+	+	+	+	+	+	-	-	-
C. piscium CCUG 49271^T	+	+	+	+	+	+	+	+	+	+	+
SH 23-4	+	+	+	+	+	+	+	+	+	-	-
SH 11-3(a)	+	+	+	+	+	+	+	+	-	-	-
SH 20-4	+	+	+	+	+	+	+	+	w	-	-
SH 30-1	+	+	+	+	+	+	+	w	-	-	-
SH 28-3	+	+	+	+	+	+	+	w	-	-	-
IJ 30-2	+	+	+	+	+	+	+	+	w	-	-
SH 11-3(b)	+	+	+	+	+	+	+	+	-	-	-
SH 25-4	+	+	+	+	+	+	+	-	-	-	-
SH 19-2(b)	+	+	+	+	+	+	+	-	-	-	-
SH 11-4(b)	+	+	+	+	+	+	+	+	+	+	+
SH 40-3	+	+	+	+	+	+	+	-	-	-	-

The phenotypic characteristics of the 11 *Chryseobacterium* isolates in this study as well as the reference strains in terms of spoilage are indicated in Table 3.8. Enzyme production (lipase, protease, gelatinase) varied individually. All the *Chryseobacterium* isolates except for SH 20-4, IJ 30-2 and SH 19-2(b) tested positive for the enzyme protease and this could be attributed to the fact that most of the bacterial species isolated from fish and fish products are proteolytic in nature (Venugopal 1990). All strains were positive for the production of oxidase and catalase enzymes (Table 3.8) which are characteristic of the genus *Chryseobacterium* (Hugo et al. 2019).

The presence of particular enzymes is an essential parameter for identification and characterization of bacteria and differentiation of related organisms (Busse et al. 1996). The ability of most of the strains to produce lipolytic (lecithinase) and proteolytic (caseinase, gelatinase) enzymes can be linked with the fact that members of the genus *Chryseobacterium* have been linked with strong proteolytic and lipolytic activity (Bernadet et al. 2011). *Chryseobacterium* strains with lipolytic and proteolytic activity have also been recognized as common spoilers of protein-rich foods, and food under low temperature storage, such as, processed meats, chilled fish, shellfish and dairy products (Gonza´lez et al. 2000; Hugo et al.

1999, 2003). The activities of heat-stable proteolytic and lipolytic enzymes originating from psychrotolerant microorganisms play a significant role in limiting the maintenance of the flavour quality of milk and its products (Hantsis-Zacharov and Halpern 2007b).

Eight out of the 11 chryseobacterial test strains were able to produce indole from tryptophan and they shared this characteristic with *Chryseobacterium piscium*. These organisms have the enzyme, tryptophanase which breaks down tryptophan into indole, pyruvic acid, and ammonia. The formation of indole from tryptophan has been associated with “unclean” flavours (Ayres et al. 1980). Indole is used as a spoilage indicator in shrimp and crustaceans because in high concentrations it imparts putrid and musty odours (Sarnoski et al. 2010).

All the *Chryseobacterium* isolates and the reference strains except strain SH 25-4 and SH 19-2(b) were able to produce ammonia from urea. The release of large amounts of ammonia also contributes to the development of spoilage odours (Nychas et al. 1998). None of the 11 strains and the reference strains were able to produce H_2S (Table 3.8). Several volatile odour compounds like volatile basic nitrogen compounds, volatile acids and H_2S are produced in fish as a result of bacterial spoilage (Venugopal 1990).

Table 3.8. Production of enzymes and odours of the 11 *Chryseobacterium* isolates and the reference strains. +, positive; -, negative; w, weakly positive.

Characteristic	*C. balustinum*	*C. gleum*	*C. piscium*	SH 23-4	SH 11-3(a)	SH 20-4	SH 30-1	SH 28-3	IJ 30-2	SH 11-3(b)	SH 25-4	SH 19-2(b)	SH 11-4(b)	SH 40-3
Enzyme production:														
Caseinase	+	+	+	+	+	-	+	+	-	+	+	-	+	+
Gelatinase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lecithinase	w	+	+	-	+	+	-	-	+	-	-	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of odours:														
Indole	-	-	+	+	+	+	+	+	-	-	+	-	+	+
Hydrogen sulphide	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ammonia	+	+	+	+	+	+	w	+	+	w	-	-	+	+

3.4 Conclusions

The 11 bacterial strains that were isolated from a previous study (Engelbrecht 1992) were evaluated by molecular (16S rRNA sequencing) and phenotypic (conventional and automated BIOLOG^{TM} Omnilog Gen III) methods in order to classify and characterise them.

Based on prokaryotic delineation, strains are considered novel when they have 16S rRNA sequence similarity values less than 98.7%. Strain IJ 30-2 was identified as *C. carnis* while strains SH 23-4, SH 30-1 and SH 28-3 belonged to *C. piscium*. It was concluded that strains SH 11-3(a), SH 20-4, SH 11-3(b), SH 19-2(b), SH 11-4(b), and SH 40-3 might be novel species of *Chryseobacterium*. However, more genotypic and chemotypic studies have to be done to confirm that they are new members of the genus *Chryseobacterium.*

The BIOLOG^{TM} system did identify all three reference strains correctly and also indicated, like the 16S rRNA gene sequencing data, that strains SH 23-4, SH 30-1 and SH 28-3 belonged to *C. piscium*. The BIOLOG^{TM} method can therefore, also be used for identification purposes, however, because of its limited database, some isolates could not be identified at either genus or species level and therefore it is very important to use the BIOLOG^{TM} Omnilog identification system in conjunction with other identification methods.

The bacterial test strains were also characterized in their ability to grow at different temperatures, on different growth media and in different concentrations of NaCl. Most of the test strains were able to grow at temperatures between 4 and 37 °C and their ability to grow at 4 °C can be linked with the psychrotolerant nature of the members of the genus *Chryseobacterium*. In this study, strain SH 11-4(b) indicated that it is a candidate novel species of *Chryseobacterium* and also indicated possible pathogenic characteristics in terms of not being able to grow on MacConkey agar, growing at 42 °C and having a high salt tolerance.

The ability of the members of the genus *Chryseobacterium* to produce proteolytic and lipolytic enzymes have been linked with spoilage potential. The role of protease and lipase secretion in relation to fish spoilage has not received the same attention as compared to spoilage and lipase secretion in relation to milk and milk products and should be investigated further in future.

CHAPTER 4

THE DETERMINATION OF THE SPOILAGE CHARACTERISTICS OF *Chryseobacterium* SPECIES ISOLATED FROM FISH

ABSTRACT

The spoilage characteristics of 11 *Chryseobacterium* isolates isolated from fish were investigated by their ability to utilise BIOLOG^{TM} carbon sources, odour production by sensory analysis and volatile compound production by GC/MS. Strains SH 23-4 and SH 11-4 utilised the most carbon sources on the BIOLOG^{TM} Omnilog system. No significant differences were observed in terms of odour production during sensory analysis, but odours such as smelly feet, cabbage-like, fruity, sour and putrid-sewage were noted for the 11 *Chryseobacterium* isolates from fish and their reference strains. A total of 87 volatile compounds were detected by GC/MS and the most detected volatile compounds were 2-ethyl-1-hexanol, indole, dimethyl disulphide, and 2-phenylethanol. The detected volatile compounds were most prevalent in *Chryseobacterium gleum, C. piscium* and strain SH 30-1 and least prevalent in strains SH 19-2(b) and SH 11-4(b).

4.1. Introduction

Spoilage of food is any visible modification in a food product that makes it undesirable to the consumer. Specific microorganisms are associated with food spoilage and resultant metabolic activities resulting in slime production or the production of ammonia and sulphur compounds, are responsible for undesirable flavours and odours (Gram et al. 2002). Specific spoilage organisms (SSO), are organisms that are present in a product during spoilage and can grow without causing unpleasant changes (Gram and Huss 1996; Gram et al. 2002). Specific spoilage organisms may exist in low numbers at first and only contribute to a small portion of the processed seafood microbiota at the beginning of the storage period (Mace et al. 2013). Comparing the impact of bacterial isolates on the chemical and sensory characteristics of the food products has been used by some authors to identify the specific spoilage organisms present in a product at the time of spoilage (Mace et al. 2013).

Growth and survival of microorganisms in foods are influenced by the nutrient status of the food, physiological attributes of the contaminant microorganisms, and extrinsic factors, e.g., temperature, gas environment, relative humidity and processing factors (Mossel 1983). Several microorganisms growing on meat and fish muscle produce a wide range of hydrolytic enzymes, mainly proteases, which are made up of endoproteases or proteinases and exoproteases comprising of amino peptidases and carboxypeptidases (Venugopal 1990). Protein, fat and polysaccharide degradation can result in changes in the texture of foods while partial breakdown of amino acids and fatty acids and fermentations of simple sugars can promote changes in flavour (Banwart 1989).

As part of the Gram-negative rod shaped, psychrotolerant, proteolytic group, the genus *Chryseobacterium* harbours active food spoilage organisms (Hugo and Jooste 2003; Bernardet et al. 2006). The members of the genus *Chryseobacterium* are widely distributed in food sources such as milk, fish, meat and poultry (Hugo et al. 2003; de Beer et al. 2005, 2006; Charimba et al. 2013). Psychrotolerant bacteria, specifically Gram-negative rods such as *Pseudomonas, Alteromonas, Flavobacterium/Chryseobacterium, Moraxella, Acinetobacter* and *Vibrio* are mainly responsible for limiting the quality of fish, however, not all bacteria that are found on fish can be regarded as active spoilers (Cousin 1982; Engelbrecht 1992). *Chryseobacterium* species that have been isolated from fish include *Chryseobacterium scophthalmum* which was isolated from diseased turbot (*Scophthalmus maximus*) in Scotland (Mudarris et al. 1994), *Chryseobacterium shigense* isolated from rainbow trout (*Oncorhynchus mykiss*) during fish disease outbreaks in Spain (Zamora et al. 2012b), *Chryseobacterium piscicola* responsible for mortality in farmed Atlantic salmon (*Salmo salar*) in Chile (Ilardi and Avendaño-Herrera 2008; Ilardi et al. 2009) and*Chryseobacterium balustinum* which showed pathogenicity in halibut (Harrison 1929).

The freshness of fish is believed to be the most significant quality parameter as it is closely related to the sensory qualities perceived by consumers such as appearance, texture, odour, and taste (Hassoun and Karoui 2017). Sensory analysis and GC/MS are methods that are used to measure characterising flavours and odours in food products (Krüsemann et al. 2019). The BIOLOG^{TM} Omnilog is not only used for identification purposes, but also plays an important role in determining the spoilage potential of microorganisms through carbon source substrate oxidation. Sensory analysis of seafood is an essential method used by the Federal Drug Administration (FDA) to protect customers from seafood that has undergone putrefaction (ORA Laboratory Manual 2013), while gas chromatography (GC) and mass spectrometry (MS) play a significant role in detecting food spoilage and volatile compounds responsible for the production of off-odours (Bekker 2011).

In the present study, the spoilage potential of the 11 *Chryseobacterium* test strains was determined by using the BIOLOG^{TM} Omnilog MicroPlates to identify the specific carbon sources metabolised by the *Chryseobacterium* species, because the degradation of similar carbon sources in foods by microorganisms can result in the production of microbial metabolites, associated with potential spoilage defects. Sensory analysis by using the sniffing method and GC/MS by evaluating production of volatile compounds.

4.2. Materials and methods

4.2.1. Cultures used and their maintenance

Eleven isolates from fish namely, SH 23-4, SH 11-3 (a), SH 20-4, SH 30-1, SH 28-3, IJ 30-2, SH 11-3 (b), SH 25-4, SH 19-2 (b), SH 11-4 (b) and SH 40-3 which were all obtained from a previous study (Engelbrecht 1992) were used in this section of the study. *Chryseobacterium balustinum* NCTC 11212^T, *Chryseobacterium piscium* CCUG 49271^T and *C. gleum* NCTC 11432^T, were obtained from international culture collections (CCUG and NCTC) and were maintained and cultivated as described previously (Chapter 3), were the reference strains used in this study. *Chryseobacterium balustinum* and *C. piscium* were selected as reference strains because they are both isolated from fish and were the closest phylogenetic neighbours to the isolates in this study (see Chapter 3). *Chryseobacterium gleum* was also chosen as a reference strain because it is the type strain of the genus *Chryseobacterium.*

4.2.2. Utilisation of carbon sources for spoilage potential determination

The isolates were reactivated in 10 ml nutrient broth (Oxoid CM67) and incubated at 25 °C for 48 h. They were then streaked on nutrient agar (Oxoid CM003) and incubated at 25 °C for 24 h. The isolates were inoculated into the BIOLOG^{TM} GENIII microplates (BIOLOG Inc. Hayward, California), consisting of 96 different oxidizable carbon sources, according to the manufacturer’s protocol. These carbon sources could be divided into carbohydrates, amino acids, carboxylic acids and polymers.

4.2.3 Preparation of the fish juice broth for sensory analysis and GC/MS analysis

The fish juice broth (FJB) was prepared according to the method described by Parlapani and colleagues (2017) and Chandrasekaran and colleagues (1985), with some modifications.

Frozen hake fillets (250 g) were washed with sterile tap water, cut in pieces and homogenized in 250 ml distilled water in a sterile 750 ml WhirlPak^{TM} bag (Lasec, Bloemfontein, SA), using a stomacher (AME Stomacher Lab-Blender 400, JHB) for 2 min. The juice was separated from the fish solids through cheese cloth and boiled for 5 min in a microwave oven. It was then left for 15 min at ambient temperature and filtered using Whatman No. 1 paper filters to remove the coagulated proteins. After filtration, 200 ml of the filtrate was divided into 100 ml quantities in 1000 ml Schott bottles (Lasec, Bloemfontein, SA) and 900 ml 0.10 M phosphate buffer (0.056 M KH_2PO_4 and 0.044 M K_2HPO_4) was added to each of the 100 ml quantities. The pH was adjusted to 6.5 with NaOH or HCl. Finally, the FJB was autoclaved for 15 min at 121 °C, pH was checked and the broth was stored at 4 °C until use.

4.2.4 Sensory analysis

The FJB inoculated with the individual *Chryseobacterium* isolates and reference strains in this study, was exposed to sensory analysis for off-odour detection. The sensory panel was made up of ten semi-trained staff and students from the Agricultural Faculty of the University of the Free State. The age of the panellists ranged from 24 to 68 years with one male and nine female panellists. During training, the panellists were asked to differentiate between an inoculated FJB sample and an uninoculated FJB sample by performing a sniffing test. The inoculated sample was prepared by inoculating 10 ml of the FJB with the 11 *Chryseobacterium* test isolates and the three reference strains and incubating at 25 °C for 72 h. The control sample was uninoculated FJB. Panellists were also requested not to wear any perfumed personal care products on the day of analysis.

Inoculation of the samples for sensory analysis was carried out by transferring 10 ml volumes of the FJB aseptically into sterile test tubes with screw-on lids to avoid any loss of volatile compounds. One milliliter of a 48-h old nutrient broth culture of each of the 11 *Chryseobacterium* test strains and the three reference strains were each inoculated in duplicate into individual 10 ml FJB. One set of the FJB samples was incubated at 25 °C for 72 h while the other set of samples was incubated at 4 °C for 10 days. These temperatures were chosen to mimic the optimal growth temperature of *Chryseobacterium* species (25 °C) and the refrigeration temperature of cooked fish (4 °C), respectively. An uninoculated sample of the FJB was used as the control. The samples were all heated at 50 °C for 30 min just before sensory evaluations for a better assessment of the volatile fractions produced by the individual test isolates in the FJB. These samples were presented to the panel members in the same test tubes in which the inoculation had been done. Samples were coded with three-digit numbers and served randomly in white test tube racks.

Panellists were presented with the inoculated and control samples in individual booths. The sample tubes were opened, sniffed and closed instantly to avert olfactory exhaustion. Panellists were advised to smell their own skin should olfactory exhaustion occur.

The intensity of spoilage was defined by categorising the spoilage into three levels namely: level 1 = no spoilage; level 2 = weak spoilage; and level 3 = strong spoilage. Panellists were also instructed to give a description of the type of odour, if any, produced by the different organisms. The results were then analysed statistically.

4.2.5 Determination of volatile compounds

Ten milliliter amounts of nutrient broth inoculated with the 11 *Chryseobacterium* test strains and the reference strains (*C. balustinum*, *C. gleum* and *C. piscium*) were incubated for 48 h at 25 °C. Atwenty milliliter volume of the FJB were inoculated with 2 ml of the broth culture after cultivation. The samples were then incubated for 14 days at 4 °C and 7 days at 25 °C in order to allow spoilage to develop. After incubation, samples were kept in a frozen state until volatile analysis could be carried out by the solid-phase microextraction gas chromatographic-mass spectrometric (SPME-GC/MS) procedure.

SPME-GC/MS analysis

Headspace (HS) solid phase micro-extraction (SPME) gas chromatography/mass spectrometry (GC/MS) was used for volatile compound analysis. The detection of volatile compounds was performed according to the technique described by Marsili (1999) with some adjustments as described in the following paragraph.

A 75 µm sorbent thickness manual SPME fibre holder and carboxen fibre assembly was used (Supelco, Belafonte). The depth on the holder was set to 1.5 for sorption and 3.5 for desorption. Ten millilitres of the sample were dispensed into a 20 ml solid phase-micro extraction tube and was heated for 30 min at 50 °C for equilibration. An SPME fibre (divinylbenzene/carboxen/polydimethylsiloxane, Sigma Aldrich) was exposed to the headspace above the sample for 60 min. Desorption was done in the injection port of the gas chromatograph at 250 °C for 1 min in the splitless mode. Samples were regenerated for 10 min at 250 °C and re-used. A ThermoElectron Trace 1310 GC fixed to an ISQ 7000 single quadrupole mass spectrometer was employed for analysis. The analytical column was a Factor Four VF5 ms (length: 60 m, inner diameter: 0.32 mm, film thickness: 0.25 µm, Agilent) and helium (99.999%), at a flow rate of 5 ml/min, was the carrier gas.

The oven program was as follows:

Rate (°C/min)	**Temperature (°C)**	**Hold time (min)**
Initial	40	2
5	300	10

Mass spectral data were collected in total ion count mode from 45–550 m/z. Fragmentation was achieved with electron impact ionization at 70 eV. Data collection and processing was carried out with Xcalibur 4.0 software (ThermoElectron) using the NIST 17 spectral data library.

4.2.6 Statistical analysis

The results for the sensory analysis were captured in spreadsheets in Microsoft Excel 2016. An ANOVA (NCSS Statistical Software package, version 11.0.20) was used to determine the effect of different organisms on spoilage of the FJB at 4 °C and 25 °C. The Tukey-Kramer multiple comparison test (α = 0.05) was carried out to identify significant differences between the treatment means (NCSS Statistical Software package, version 11.0.20).

4.3 Results and Discussion

4.3.1 Estimation of spoilage by substrate oxidation

The estimation of the spoilage potential of the 11 *Chryseobacterium* isolates in this study as well as the reference strains in terms of the BIOLOG^{TM} Omnilog Gen III substrate oxidation, are indicated in Table 4.1a–4.1d. The substrates were divided into the following substrate guilds: carbohydrates; amino acids; carboxylic acids and esters and polymers. The results will now be discussed with regard to these substrate categories.

4.3.1.1 Oxidation of carbohydrates

The carbohydrates were oxidised in varying degrees by specific isolates. Gentibiose, D-mannose and glucose were oxidised by almost all the isolates excluding strain SH 25-4 and SH 19-2(b) for gentibiose, strains SH 11-3(a), SH 20-4, IJ 30-2 and SH 11-3(b) for D-mannose and strains SH 11-3(a), SH 20-4, IJ 30-2, SH 40-3 and SH 11-3(b) for glucose (Table 4.1a). The substrates that were oxidised by only a few isolates were D-cellobiose and sucrose. Strain SH 23-4 can be differentiated from all the reference strains and the other 10 test strains because it was the only isolate that was able to utilize D-cellobiose. Strains 23-4 and SH 19-2(b) were the only isolates with the ability to utilise sucrose.

Table 4.1a. Heat map of the carbohydrate metabolism of the 11 *Chryseobacterium* isolates and the reference strains indicating spoilage potential according to BIOLOG^{TM} substrate oxidation. All data are from this study. Values in coloured blocks indicate well colour development: >50, positive; <30 negative; 30-49 weakly positive.

Substrate	**SH 11-4(b)**	**SH 40-3**	**SH 20-4**	**SH 11-3(a)**	**IJ 30-2**	**SH 19-2(b)**	**SH 11-3(b)**	**SH 30-1**	**SH 23-4**	**SH 28-3**	**SH 25-4**	*C. balustinum*	*C. gleum*	*C. piscium*
D-maltose	87	6	22	13	20	47	6	61	74	17	76	10	70	13
D-trehalose	71	9	12	16	16	36	9	8	67	12	95	88	76	9
D-cellobiose	6	7	13	14	14	24	6	5	72	10	7	5	2	5
Gentiobiose	77	52	13	16	45	89	67	59	98	82	92	81	70	51
Sucrose	5	8	10	10	10	84	7	4	72	8	6	6	2	6
α-D-glucose	76	21	16	20	16	48	14	53	94	64	82	65	61	38
D-mannose	65	59	5	7	8	48	5	34	85	43	85	54	70	55
D-fructose	55	9	13	10	8	47	10	7	64	7	98	90	96	9
D-galactose	60	9	6	12	8	5	12	8	73	7	48	9	51	9
D-arabitol	52	19	11	11	8	15	11	8	64	7	30	6	44	9
D-glucose-6-PO$_4$	42	100	90	14	26	20	31	7	98	7	12	13	90	13

Bacteria will initially metabolise low molecular weight components such as carbohydrates which serve as nutrients for their growth (Venugopal 1990). Bacterial types with the ability to oxidise a particular carbohydrate will utilise it as a carbon source to promote their growth (Tsôeu et al. 2016). The metabolism of carbohydrates by microorganisms is not expected to result in food spoilage because their oxidation does not lead to the production of spoilage indicator metabolites, but to the production of CO_2 and water (Tsôeu et al. 2016). It is only when other simple carbohydrates such as glucose become exhausted, that the metabolism of other substrates such as amino acids begins (Dainty 1996). Off-odour production will then result because of the shift from metabolising simple carbohydrates to amino acids, as the breakdown of amino acids results in the production of metabolites such as ammonia, amines, dimethyl sulphide and hydrogen sulphide (Dainty 1996).

Other metabolic products produced from the oxidation of carbohydrates, e.g., D-trehalose and gentiobiose, include organic acids, alcohols, carbon dioxide (CO_2), hydrogen gas (H_2) and water (H_2O) (Banwart, 1989). Only psychrotolerant facultative anaerobes and obligate anaerobes can metabolize glucose to produce lactic acid which will result in sour flavours (Dilbaghi and Sharma 2007).

4.3.1.2 Oxidation of amino acids

Table 4.1b gives an indication of the metabolism of amino acids by the *Chryseobacterium* isolates. The most oxidised amino acid was L-glutamic acid and all the 11 *Chryseobacterium* test strains as well as thereference strains were able to metabolise it. This was followed by L-aspartic acid which was metabolised by all the isolates except strains SH 30-1 and SH 28-3*.* It was interesting to note that none of the isolates were able to metabolize D-aspartic acid which is the structural isomer of L-aspartic acid. The other two least metabolised amino acids were L-alanine which was only metabolised by *C. gleum* and strains SH 11-4(b) and L-serine which was metabolised by strains SH 11-4(b) and SH 25-4.

Growth of facultative anaerobic microorganisms on meat promotes putrefaction because of the degradation of amino acids which result in the production of amines, ammonia, methylsulfides, and mercaptans (Dilbaghi and Sharma 2007). Greening of the meat also occurs because of the production of small amounts of H_2S by microorganisms. The metabolism of amino acids e.g., leucine and valine, results in the production of volatile fatty acids like isovaleric and isobutyric acids which produce a cheesy flavour in meat (Dilbaghi and Sharma 2007).

The amino acid, glutamic acid may produce acetic acid, butyric acid, carbon dioxide, ammonia and hydrogen and butyric acids when metabolised by microorganisms.Butyric acid is associated with rancid odours in food (Banwart 1989).

Table 4.1b. Heat map of the amino acid metabolism of the 11 *Chryseobacterium* isolates and the reference strains indicating spoilage potential according to BIOLOG^{TM} substrate oxidation. All data are from this study. Values in coloured blocks indicate well colour development: >50, positive; <30 negative; 30-49 weakly positive.

Substrate	**SH 11-4(b)**	**SH 40-3**	**SH 20-4**	**SH 11-3(a)**	**IJ 30-2**	**SH 19-2(b)**	**SH 11-3(b)**	**SH 30-1**	**SH 23-4**	**SH 28-3**	**SH 25-4**	*C. balustinum*	*C. gleum*	*C. piscium*
Glycyl-L-proline	6	5	7	11	17	10	6	42	25	37	95	87	42	80
L-alanine	37	6	6	8	8	9	5	8	0	7	24	7	39	9
L-arginine	88	10	5	14	8	23	23	62	93	8	73	36	40	80
D-aspartic acid	6	7	5	7	8	3	5	9	0	7	4	10	8	9
L-aspartic acid	100	100	80	99	96	72	99	12	73	7	56	41	91	38
L-glutamic acid	100	100	72	100	97	100	99	63	91	55	47	92	93	80
L-histidine	39	11	5	7	8	9	7	8	50	7	43	8	45	7
L-pyroglutamic acid	16	90	44	12	8	15	7	45	62	7	11	11	31	9
Guanidine HCl	61	14	9	11	11	4	11	63	57	78	76	83	62	89
D-serine	55	16	12	14	11	24	13	14	8	13	90	16	73	14
L-serine	71	12	5	7	8	3	8	12	0	7	62	28	16	22

4.3.1.3 Oxidation of carboxylic acids and esters

Production of lactic, acetic, and formic acids andCO_2 by some organisms leads to curdling and souring of milk and this happens because the acids lower the pH of milk which in turn destabilise the milk protein casein as it is susceptible to a pH lower than 4.6 (Dilbaghi and Sharma 2007). All the isolates except strain SH 11-3 (b) and SH 40-3 were able to metabolise acetic acid while none of the isolates were able to metabolise α-keto-butyric acid (Table 4.1c). Acetic acid contributes vinegar-like and pungent aromas to fish sauce (Mohamed et al. 2012).

Esters are commonly used flavouring agents and appreciated for the fruity aromas they produce (Longo and Sanroman 2006). Short chain fatty acids, ethyl or methyl esters usually result in fruity flavours during cheese production, while thioesters derived from thiols are linked with cabbage or sulphur aromas (Longo and Sanroman 2006).

Table 4.1c. Heat map of the carboxylic acid metabolism of the 11 *Chryseobacterium* isolates and the reference strains indicating spoilage potential according to BIOLOG^{TM} substrate oxidation. All data are from this study. Values in coloured blocks indicate well colour development: >50, positive; <30 negative; 30-49 weakly positive.

Substrate	**SH 11-4(b)**	**SH 40-3**	**SH 20-4**	**SH 11-3(a)**	**IJ 30-2**	**SH 19-2(b)**	**SH 11-3(b)**	**SH 30-1**	**SH 23-4**	**SH 28-3**	**SH 25-4**	*C. balustinum*	*C. gleum*	*C. piscium*
Citric acid	64	27	14	13	11	8	33	44	72	7	70	14	82	14
Propionic acid	38	42	35	39	8	64	20	22	58	47	64	29	4	13
Acetic acid	79	27	58	67	89	88	27	99	96	71	99	99	97	93
Formic acid	52	41	35	30	8	3	21	41	68	37	86	24	17	23
α-keto-butyric acid	15	6	11	8	7	16	13	7	18	9	20	17	21	25

4.3.1.4 Oxidation of polymers

The exhaustion of lower molecular weight nutrients results in protein catabolism as survival of the microorganisms depend on it and therefore, only those organisms which secrete proteases can survive after low molecular weight components have been depleted (Venugopal 1990). The breakdown of proteins results in an increase in the production of amino acids and volatile compounds such as H_2S (Venugopal 1990). All the isolates were able to metabolise gelatine (Table 4.1d) and the ability of microorganisms to hydrolyse gelatine has been linked to proteolytic activity, since the enzyme degrading gelatine (gelatinase) is a protease. Pectin was the least metabolised polymer, however, Strains SH 23-4, SH 30-1 and SH 28-3 can be differentiated from *C. gleum* because of their ability to metabolise pectin. Pectin is a structural protein and the degradation of pectin by microorganisms because of the enzyme pectinase results in textural changes in food products which is also a characteristic of spoilage. Pectolytic enzymes, particularly those associated with soft rot in vegetables, are produced by a wide range of microorganisms (Banwart 1989).

Table 4.1d. Heat map of the polymer metabolism of the 11 *Chryseobacterium* isolates and the reference strains indicating spoilage potential according to BIOLOG^{TM} substrate oxidation. All data are from this study. Values in coloured blocks indicate well colour development: >50, positive; <30 negative; 30-49 weakly positive.

Substrate	**SH 11-4(b)**	**SH 40-3**	**SH 20-4**	**SH 11-3(a)**	**IJ 30-2**	**SH 19-2(b)**	**SH 11-3(b)**	**SH 30-1**	**SH 23-4**	**SH 28-3**	**SH 25-4**	*C. balustinum*	*C. gleum*	*C. piscium*
Dextrin	79	66	82	74	65	15	45	85	99	87	16	20	92	99
Pectin	28	21	9	38	14	24	23	64	74	50	11	44	8	34
Gelatine	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Tween-40	37	38	22	36	49	25	24	63	100	70	91	86	99	86

Most isolates were able to metabolize dextrin with the exception of strains SH 25-4, SH 19-2(b) and *C. balustinum*. Complex polysaccharides are normally broken down to simple sugars prior to utilisation by microorganisms. This is a spoilage indicator because oxidation of these polysaccharides results in the production of spoilage metabolites products such as organic acids, alcohols and CO_2 which may produce off-odours, sourness and bitter defects (Banwart 1989).

Almost all the isolates were able to metabolise Tween 40. Hydrolysis of Tween 40 serves as a lipase and esterase production indicator (Harrigan and McCance 1976). Lipolytic enzymes lead to both acceptable and unacceptable flavours in dairy products, due to the hydrolyzation of milk triacylglycerols (Mielman 2006). The oxidation of unsaturated fatty acids resulting from lipolysis gives rise to aldehydes and ketones, which impart off-flavours defined as “oxidised card-boardy” or metallic (Chen et al. 2003).

Altogether 31 of the 96 carbon sources in the BIOLOG^{TM} system could be used to differentiate between the isolates and the reference strains. These carbon sources included 11 carbohydrates, 11 amino acids, 5 carboxylic acids, and 4 polymers. For the reference strains, *C. gleum* oxidised 20 carbon sources, followed by *C. balustinum* with 13 and *C. piscium* with 12. Strains SH 23-4 and SH 11-4(b) oxidised 25 carbon sources, followed by strain SH 25-4 with 20, strain SH 30-1 with 16, strain SH 11-3(a) with 15, strain SH 28-3 with 13, strain SH 19-2(b) with 12, strain SH 20-4 with 10, strain IJ 30-2 with 9 and strain SH 11-3(b) with only 7 carbon sources. Both strains SH 23-4 and SH 11-4(b) oxidised 25 out of the 31 carbon sources tested, followed by *C. gleum* which oxidised 21 out of 31 carbon sources. This could be an indication that these strains are more likely to cause more prevalent types of spoilage than the other species tested.

4.3.2 Sensory analysis

Table 4.2 gives an indication of the demographic profile of the sensory panel members that were used in this study. Ninety percent of the sensory panel consisted of female panellists. The age of the panellists ranged between 20 and 59 years of which 40% of the panellists were between the ages of 20–29 years.

Table 4.2: Demographic profile of sensory panel.

Gender	% of Total	Age	% of Total
Female	90	20-29	40
Male	10	30-39	10
		40-49	20
		50-59	30

Estimation of spoilage in terms of odour production was identified using sensory analysis and the results obtained in this study are indicated in Tables 4.3 and 4.4. A one-way ANOVA between subjects was conducted to determine the degree of spoilage of the 11 *Chryseobacterium* isolates from fish and to compare their spoilage potential to the spoilage potential of the three reference strains used in this study (Table 4.3). There were no significant differences between the degree of spoilage of the 11 *Chryseobacterium* isolates and the reference strains at 4 °C and 25 °C. It was noted that most of the strains produced stronger odours at 25 °C than at 4 °C. Statistically significant ($p < 0.001$) differences were, however, only observed between the uninoculated control samples and the inoculated samples. This indicated that the sensory panel was able to detect that the control sample was significantly ($p < 0.001$) less spoiled than the inoculated samples, but that they were not able to detect significant spoilage differences between the inoculated samples.

Table 4.3: Analysis of variance on degree of spoilage at 4 °C and 25 °C

Organism	Degree of spoilage at 4°C	Degree of spoilage at 25°C
Control	$1.13^{a} \pm 0.40$	$1.10^{a} \pm 0.30$
C. balustinum	$2.20^{b} \pm 0.63$	$2.40^{b} \pm 0.52$
C. gleum	$2.30^{b} \pm 0.48$	$2.70^{b} \pm 0.48$
C. piscium	$2.50^{b} \pm 0.53$	$2.30^{b} \pm 0.48$
SH 20-4	$2.00^{b} \pm 0.67$	$2.40^{b} \pm 0.52$
SH 23-4	$2.60^{b} \pm 0.52$	$2.50^{b} \pm 0.53$
SH 25-4	$2.40^{b} \pm 0.70$	$2.50^{b} \pm 0.53$
SH 28-3	$2.80^{b} \pm 0.42$	$2.60^{b} \pm 0.52$
SH 40-3	$2.10^{b} \pm 0.57$	$2.40^{b} \pm 0.52$
SH 30-1	$2.60^{b} \pm 0.52$	$2.60^{b} \pm 0.52$
IJ 30-2	$2.60^{b} \pm 0.52$	$2.50^{b} \pm 0.53$
SH 11-3(a)	$2.50^{b} \pm 0.53$	$2.50^{b} \pm 0.53$
SH 11-3(b)	$2.40^{b} \pm 0.84$	$2.30^{b} \pm 0.67$
SH 11-4(b)	$2.80^{b} \pm 0.42$	$2.40^{b} \pm 0.70$
SH 19-2(b)	$2.30^{b} \pm 0.67$	$2.50^{b} \pm 0.53$
Significance level	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly.

The percentage frequency of descriptive odours that were observed by the panellists for the different samples, are given in Table 4.4. The odours that were observed for *C. balustinum* at both 4 and 25 °C were egg, sickening sweet, smelly feet, cabbage-like, rotten fruit and sour. The highest % frequency was at 4 °C for the smelly feet odour with 40%, followed by sickening sweet odour with a 30% frequency. Smelly feet and sour odour were both used at 30% frequency for *C. balustinum* incubated at 25 °C.

Odours that were described for *C. piscium* were fruity, sickening sweet, smelly feet, rotten fruit, sulphur, sweet, cabbage-like, sour and putrid sewage. Fruity odour had a 40% frequency at 4 °C, and 30% at 25 °C followed by sickening sweet odour with a 30% frequency at 4 °C and 20% at 25 °C.

The odour that stood out for *C. gleum* samples incubated at 4 °C were smelly feet at a 30% frequency, followed by fish and sour odours which were both at 20% frequency. Sickening sweet and smelly feet odours shared a frequency of 20% which was the highest for the odours that were described at 25 °C.

Table 4.4: Frequency of descriptions of odours in fish juice samples by 10 semi-trained panellists at 4ºC and 25ºC

	Incubation at 4 ºC		Incubation at 25 ºC	
Organism	**Descriptor**	**Frequency of use (%)**	**Descriptor**	**Frequency of use (%)**
Control	Ammonia	15	Ammonia	12.5
	Cabbage-like	2.5	Earthy	5
	Fruity	7.5	Fishy	2.5
	No smell	62.5	Fruity	7.5
	Nothing	10	No smell	65
	Sickening-sweet	2.5	Sickening sweet	2.5
			Smelly feet	5
C. balustinum	Egg	10	Cabbage-like	10
	No smell	10	Rotten fruit	10
	Sickening sweet	30	Sickening sweet	20
	Smelly feet	40	Smelly feet	30
	Smelly feet, cabbage-like	10	Sour	30
C. piscium	Fruity	40	Cabbage-like	10
	Fruity, sickening sweet	10	Fruity	30
	Rotten fruit	10	Putrid sewage	10
	Sickening sweet	10	Rotten fruit	10
	Smelly feet	10	Sickening sweet	20
	Sulphur	10	Smelly feet	10
	Sweet	10	Sour	10
C. gleum	Cabbage-like, rotten fruit	10	Smelly feet	10
	Fishy	20	Cabbage-like	10
	Sickening sweet	10	Earthy	10
	Smelly feet	30	Rotten fruit	10
	Sour	20	Sickening sweet	20
	Spoiled	10	Smelly feet	20
			Sour	10
			Sulphur	10
SH 20-4	Ammonia	10	Cabbage-like	10
	Cabbage-like	30	Fishy	10
	Earthy	20	Fruity	10
	No smell	10	Rotten fruit	30
	Putrid sewage	10	Smelly feet	10
	Sickening sweet	10	Sour	20
	Smelly feet	10	Sulphur	10
SH 23-4	Ammonia	10	Cabbage-like	20
	Cabbage-like	10	Cabbage-like, smelly feet	10
	Egg	10	Fishy/Earthy	10
	Fishy	10	Rotten fruit	20
	Putrid sewage	10	Sickening sweet	30
	Rotten fruit	10	Sour	10

	Rotten fruit, egg	10		
	Smelly feet	30		
SH 25-4	Cabbage-like	30	Cabbage-like	20
	No smell	10	Cabbage-like, smelly feet	10
	Rotten fruit	10	Fishy/Earthy	10
	Sickening sweet	10	Fruity	10
	Smelly feet	40	Putrid sewage	10
			Sickening sweet	20
			Smelly feet	10
			Sour	10
SH 28-3	Cabbage-like	10	Cabbage-like	20
	Fishy	10	Fruity	10
	Putrid sewage	20	Rotten fruit	10
	Rotten fruit	20	Sickening sweet	10
	Sickening sweet	10	Smelly feet	30
	Smelly feet	20	Sour	20
	Sour	10		
SH 40-3	Ammonia	10	Cabbage-like	10
	Cabbage-like	40	Earthy	10
	Earthy	20	Fruity	10
	Fishy	10	Putrid sewage	20
	Smelly feet	20	Rotten fruit	10
			Sickening sweet	10
			Smelly feet	30
SH 30-1	Cabbage-like	20	Cabbage-like	30
	Fruity	20	Fruity	20
	Putrid sewage	10	Rotten fruit	10
	Rotten fruit	10	Sickening sweet	10
	Rotten fruit, cabbage-like	10	Smelly feet	20
	Sickening sweet	10	Sulphur	10
	Smelly feet	20		
IJ 30-2	Ammonia	10	Cabbage-like	10
	Cabbage-like	30	Fruity	10
	Fruity	10	Sickening sweet	20
	Sickening sweet	10	Smelly feet	40
	Smelly feet	20	Sour	20
	Sour	10		
	Sulphur, egg	10		
SH 11-3(a)	Ammonia	10	Ammonia	10
	Cabbage-like	10	Cabbage-Like	10
	Putrid sewage	10	Earthy	10
	Rotten fruit, cabbage-like	10	Fruity	10
	Sickening sweet	10	Rotten fruit	10
	Smelly feet	30	Sickening sweet	20
	Sour	10	Smelly feet	20
	Sulphur	10	Sour	10

SH 11-3(b)	Ammonia	10	Cabbage-like	30
	Cabbage-like	10	Fruity	10
	Fruity	10	No smell	10
	No smell	10	Putrid sewage	20
	Putrid sewage	10	Sickening sweet	10
	Rotten fruit, cabbage-like	10	Sour	20
	Smelly feet	40		
SH 11-4(b)	Cabbage-like	30	Cabbage-like	10
	Egg	10	Cabbage-like, smelly feet	10
	Rotten fruit	20	Earthy	10
	Rotten fruit, cabbage-like	10	Fruity	20
	Smelly feet	20	Fruity, smelly feet	10
	Sulphur	10	No smell	10
			Sickening sweet	10
			Smelly feet	10
			Spoiled	10
SH 19-2(b)	Cabbage-like	50	Cabbage-like	40
	Fishy	10	Fruity	10
	Fruity	10	Rotten fruit	10
	Sickening sweet	10	Sickening sweet	10
	Smelly feet	10	Smelly feet	10
	Spoiled	10	Smelly feet, Sour	10
			Sour	10

Strain SH 20-4 had the highest frequency of 30% for cabbage-like odour which was followed by earthy odour with a frequency of 20% at 4 °C. Samples that were stored at 25 °C showed the highest frequency of 30% for rotten fruit odour, followed by sour odour with a frequency of 20%.

Smelly feet was the most described odour for strain SH 23-4 at 4 °C with a frequency of 30%. Sickening sweet was the most described odour at 25 °C with a frequency of 30%, followed by the rotten fruit odour with a frequency of 20%.

Strain SH 25-4 was characterised by a 40% frequency for smelly feet odour at 4 °C and it shared this characteristic with *C. balustinum.* The second most described odour at 4 °C was cabbage-like at 30% frequency. Both cabbage-like and sickening sweet odours were the most often described at 25 °C with a frequency of 20%, respectively.

Putrid sewage, rotten fruit and smelly feet odours shared a frequency value of 20% for strain SH 28-3 incubated at 4 °C. Smelly feet was the most described odour at 25 °C with a frequency value of 30%, followed by cabbage-like and sour odour which shared a frequency of 20%.

The odours that were described for strain SH 40-3 were cabbage-like, ammonia, earthy, fishy, smelly feet, putrid sewage, sickening sweet and rotten fruit respectively. Cabbage-like was the most described odour with a frequency of 40% at 4 °C and smelly feet was the most distinctive odour at 25 °C with a frequency of 30%, followed by putrid sewage odour with a frequency of 20%.

Cabbage-like was the most distinctive odour for strain SH 30-1 samples incubated at both 4 and 25 °C. The only difference was the frequency of use which was higher for the 25 °C samples.

Strain SH 30-1 had the highest frequency of 30% for cabbage-like odour at 25 °C, while smelly feet, fruity and cabbage-like shared a frequency value of 20% at 4 °C.

Cabbage-like was the most described odour for strain IJ 30-2 at 4 °C which was supported by a frequency value of 30%, followed by smelly feet odour with a frequency of 20%. Smelly feet was the most characteristic odour at 25 °C, followed by the sour odour.

Smelly feet was again the most described odour with a frequency value of 30% for strain SH 11-3(a) incubated at 4 °C. Both sickening sweet and smelly feet odours had a frequency of 20% at 25 °C.

Strain 11-3(b) had the highest frequency of 40% for the smelly feet odour at 4 °C, and the most distinctive odour at 25 °C was cabbage-like with a frequency of 30%. Other described odours included sour, sickening sweet and putrid sewage.

Cabbage-like was the most outstanding odour at 4 °C for strain SH 11-4(b) which was supported by a frequency of 30%, followed by rotten fruit and smelly feet. Fruity odour was the most characteristic odour at 25 °C with a frequency of 20%.

Strain SH 19-2(b) had the highest frequency of 50% for cabbage-like odour at 4 °C and 40% at 25 °C. The most described odours were smelly feet, cabbage-like, fruity, sour and putrid sewage.

Engelbrecht (1992) stated that organisms which are responsible for spoilage of marine fish are considered to be those which produce off-odours when cultivated in pure cultures on fresh fish or in fish press juice. It can therefore be concluded that all the isolates were potential spoilage organisms of marine fish because they were able to produce off-odours in FJB. Proteolytic and lipolytic enzymes produced by *Chryseobacterium* are normally the major cause of off-odours and flavours (Tsôeu et al. 2016).

In a study by Bekker (2011), smelly feet was also one of the odours produced by *C. joostei* which was associated with the production of isovaleric acid. It was also stated in a study by

Banwart (1989) that the breakdown of complex polysaccharides results in the formation of organic acids, alcohols and CO_2 which will produce off-odours, sourness and bitter defects.

4.3.3 Volatile compound determination

4.3.3.1 Volatiles produced

The results of the determination of volatile compounds produced at 25 and 4 °C by the *Chryseobacterium* strains in this study are given in Table 4.5. A total of 87 volatile compounds were detected in the head-space of the FJB inoculated with the *Chryseobacterium* strains. The identified volatile compounds that were produced included 20 alcohols, 15 carbonyls (ketones and aldehydes), 11 esters, 6 amines, 7 ring compounds, 4 sulphide, 3 acids, 3 aliphatic, 3 aromatic, 3 thioesters, 2 ethers, 1 halide and 9 unidentified compounds. Allmost all volatile compound from esters are linked with pleasant odours such as fruity and sweet. Most of the amine, alcohol, sulphide, aldehyde and acidic volatile compounds are associated with unpleasant odours (Table 4.5). The concentration of the 87 volatile compounds in terms of area of the peaks varied between the strains at 4 °C and 25 °C (Tables 4.6 and 4.7, respectively). Volatiles were expressed in both tables as the peak area of each volatile present in the strain. The most produced compounds at 4 °C and 25 °C were (listed in decreasing order): 2-ethyl-1-hexanol; indole; dimethyl disulphide; 2-phenylethanol; S-methyl 3-methylbutanethioate; ethylene glycol mono-tert-butyl ether; 2-nonanone; 2,4-di-tert-butyl phenol; 3-methoxy-3-methylbutanol; 1,3-di-tert-butylbenzene; 2,4-dimethylheptan-1-ol; 2,4-dimethylheptan-2-one; methyl mercaptan; 3-hydroxy-2,2,4-trimethylpentyl isobutyrate; 4-methyl heptanone; 2-nonanol and trimethylamine.

4.3.3.2 Concentration of the volatiles produced

Alcohols, ketones, aldehydes, sulphide and nitrogen-containing compounds have been revealed as the main source of fishy odours in aquatic animals (Campo et al. 2003; Duflos et al. 2006). *Chryseobacterium gleum* is the only strain which did not produce trimethylamine at both 4 and 25 °C (Tables 4.6 and 4.7). The concentration of trimethylamine was a bit lower at 4 °C in all the other strains than at 25 °C. Ethyl 2-methyl-2-butenoate and 3-methylbutyl 2-methylbutanoate were only detected in *C. gleum* at both 4 and 25 °C. Methyl mercaptan, a sulphide compound associated with foul and spoiled odours was present in all the strains except strain SH 20-4.

Table 4.5: A list of all the detected volatile compounds produced by the *Chryseobacterium* strains in this study, and their possible descriptive odours.

Compound	Compound type	Odour properties	Reference
2-Ethyl-1-hexanol	Alcohol	Raw fish, green, chalky,	Tao et al. 2014; Bartsch et al. 2016
2-Phenylethanol	Alcohol	Rose, honey	Bartsch et al. 2016
2,4-Di-tert-butylphenol	Alcohol		
3-Methoxy-3-methylbutanol	Alcohol		
2,4,-Dimethylheptan-1-ol (diastereomere 1)	Alcohol		
2,4,-Dimethylheptan-1-ol (diastereomere 2)	Alcohol		
2-Nonanol	Alcohol		
2-Methyl-2-nonanol	Alcohol		
1-Nonanol	Alcohol	Dusty, oily	Giri et al. 2010
3-Methyl-2-heptanol	Alcohol		
2-Dodecanol	Alcohol		
3-Methyl-1-butanol	Alcohol	Burnt, balsamic	Fukami et al. 2002; Giri et al. 2010
1-Methyl-4-(1-methylethyl)-cyclohexanol	Alcohol	Irritatant	Schiffman et al. 2001
2-Methyl-1,3-pentanediol	Alcohol		
2-Methyl-1-butanol	Alcohol	Fossil oil	Giri et al. 2010
3,5-Dimethyl-2-octanol	Alcohol		
2-Decanol	Alcohol		
2-Heptanol	Alcohol	Mushroom like	Giri et al. 2010
4-Methyl-2-heptanol	Alcohol		
2-Dodecanol	Alcohol		
2-Nonanone	Carbonyl	Floral, grassy	Wang and Xu 2009
2,4,-Dimethylheptan-2-one	Carbonyl		
4-Methyl heptanone	Carbonyl		
Acetophenone	Carbonyl	Cheesy, Floral	Fukami et al. 2002; Bartsch et al. 2016

2,6-Nonandienal	Carbonyl	Green, waxy, roast	Giri et al. 2010; Tao et al. 2014
2-Decanone	Carbonyl	Irritant	Schiffman et al. 2001
2-Hexadecanone	Carbonyl	Sweet	Queiroga et al. 2019
2,6-Di-tert-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	Carbonyl		
2-Undecanone	Carbonyl	Tallow, musty	Giri et al. 2010
2-Decanone	Carbonyl	Irritant	Schiffman et al. 2001
4-Methyl-2-Hexanone	Carbonyl		
2-Tetradecanone	Carbonyl		
3-Octen-2-one	Carbonyl		
2-Pentadecanone	Carbonyl		
2-Methyl butyraldehyde	Carbonyl	Irritant	Schiffman et al. 2001
Ethyl 2-methyl-2-butenoate	Ester		
Ethyl 3-methyl butyrate	Ester	Sweet	Li et al. 2019
3-Hydroxy-2,2,4-trimethylpentyl isobutyrate	Ester		
Ethyl-2-methyl butanoate	Ester		
Isobutyl 3-hydroxy-2,2,4-trimethylpentanoate	Ester		
2,2,4-Trimethyl-1,3-pentanediol di-isobutyrate	Ester		
Ethyl acetate	Ester	Fruity, Sweet, Pineapple	Giri et al. 2010, Li et al. 2019
Methyl benzoate	Ester	Sweet/Fruity	Amenduni et al. 2016
3-Methylbutyl 3-methylbutanoate	Ester	Fruity	Fan and Quin 2006
3-Methylbutyl 2-methylbutanoate	Ester	Fruity	Fan and Quin 2006
2-Methyl-2-butyl acetate	Ester		
Trimethylamine	Amine	Fishy	Fukami et al. 2002; Campo et al. 2003
2,5-Dimethylpyrazine	Amine	Fruity	Fukami et al. 2002
3-Ethyl-2,5-dimethylpyrazine	Amine	Irritant, intense	Schiffman et al. 2001
5-Ethyl-2-methylpyridin-4-amine	Amine		
Trimethyl pyridine	Amine		
Diphenylamine	Amine		

2-Methyl-2-isobutyloxirane	Heterocyle		
1,3-Dioxane	Heterocyle		
2,2,5,5-Tetramethyl furan	Heterocyle		
2,5-dimethyl-5-isopentyl pyrazine	Heterocyle		
3-Methyl butyl oxirane	Heterocyle		
2,5-Dimethyl-3-(2-methylpropyl)-pyrazine	Heterocyle		
Dimethyl disulfide	Sulfide	Cooked cabbage	Giri et al. 2010
Methyl mercaptan	Sulfide	Foul, spoiled	German Agricultural Society 2017
Dimethyl sulfide	Sulfide	Putrid, decayed vegetables	Schiffman et al. 2001
tert-Buthyl methyl sulfide	Sulfide		
Pentadecanoic acid	Acid		
Hexadecanoic acid	Acid	Marine, fatty, fruity	Variet et al. 2006
2-Methyl butyric acid	Acid		
2-Methoxy decane	Aliphatic		
2,4-Dimethyl-1-heptene	Aliphatic		
1,3,5-Cycloheptatriene	Aliphatic		
1,3-Di-tert-butylbenzene	Aromatic		
Di-isobutyl phthalate	Aromatic		
Dibutyl phthalate	Aromatic		
S-Methyl 3-methylbutanethioate	Thioester		
Methyl thioacetate	Thioester		
Methyl thiobutyrate	Thioester		
Ethylene glycol mono-tert-butyl ether	Ether		
tert-Butyl ethyl ether	Ether		
1H-Indole	Cyclic alcohol	Faecal, nauseating	Schiffman et al. 2001
Chloroform	Hallide		
Unidentified_6	Unknown		
Unidentified_2	Unknown		

Unidentified_7	Unknown
Unidentified_3	Unknown
Unidentified_4	Unknown
Unidentified_5	Unknown
Unidentified_1	Unknown
Unidentified_8	Unknown
Unidentified_8	Unknown

Table 4.6: Peak area values in terms of concentration of compounds detected in *Chryseobacterium* strains cultured in fish juice broth incubated at 25 °C. RT, retention time; *C.*, *Chryseobacterium*; N/F, not found.

Compound	**RT**	**IJ 30-2**	**S 19-2(b)**	**SH 11-3(a)**	**SH 11-3(b)**	**SH 11-4(b)**	**SH 20-4**	**SH 25-4**	**SH 28-3**	**SH 30-1**	**SH 40-3**	*C. balustinum*	*C. gleum*	*C. piscium*	**Average**
2-Ethyl-1-hexanol	14.74	72816	22088	21760	72583	8057	22777	60158	181750	245241	26932	145589	31694	91863	77178
1H-Indole	22.34	25246	4562	35482	54433	6846	2951	19391	44404	54101	11968	28139	26205	53718	28265
Dimethyl disulfide	6.34	15740	2200	19036	29182	9838	30246	91853	47623	24792	5794	3330	4917	22707	23635
2-Phenylethanol	17.50	6604	4594	5634	13788	2602	1644	452	33252	53771	12383	38987	14058	47053	18063
Ethyl 2-methyl-2-butenoate	11.83	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	11598	N/F	11598
S-Methyl 3-methylbutanethioate	11.95	2613	901	2060	7014	1292	1973	5537	2778	5189	1462	941	87483	2996	9403
Ethylene glycol mono-tert-butyl ether	13.71	6748	4417	4893	11893	3719	5808	5194	6188	8973	6963	3969	26757	7338	7912
2-Nonanone	16.68	2139	597	1248	1820	350	402	16452	5012	6658	1634	8366	42649	5563	7145
Ethyl 3-methyl butyrate	9.17	N/F	13	19	11	182	N/F	484	N/F	155	297	N/F	43486	N/F	5581
2,4-Di-tert-butylphenol	26.25	4006	2595	3588	5296	4241	1895	3421	7722	7444	4750	7934	8182	10750	5525
3-Methoxy-3-methylbutanol	15.21	3787	2048	2551	3405	577	898	2510	8457	13825	3906	11563	5169	9473	5244
1,3-Di-tert-butylbenzene	21.12	8442	1845	3687	4019	1785	944	2912	5639	6094	2542	9116	7160	9197	4876
Methyl mercaptan	3.15	827	4902	4919	9220	9317	N/F	517	666	3960	4327	5819	8079	5322	4823
2,4,-Dimethylheptan-1-ol (diastereomer 1)	16.38	2477	2106	1947	1889	446	535	2165	7756	13519	4014	10902	5595	9238	4815
2,4,-Dimethylheptan-2-one	15.49	2404	490	1316	1667	362	504	6884	6803	7806	1491	9036	13526	6753	4542
3-Hydroxy-2,2,4-trimethylpentyl isobutyrate	23.85	2491	521	977	2587	947	823	1103	2980	2946	1071	11773	6479	20564	4251
2,4,-Dimethylheptan-1-ol (diastereomer 2)	16.53	2473	1158	1373	1490	313	413	2098	7013	11988	2461	10241	5177	7905	4162
4-Methyl heptanone	11.77	3893	3536	3096	5809	2023	5148	3756	3687	5513	4770	2062	6897	3792	4152

Trimethylamine	3.24	3681	5601	1711	9220	4169	2149	644	1783	4462	11108	1835	N/F	1786	4012
2-Nonanol	16.99	923	581	720	1220	151	207	2298	2359	4992	1375	4103	26800	3196	3763
Ethyl-2-methyl butanoate	9.05	24	504	19	2	5	530	71	67	162	404	68	44692	36	3583
Acetophenone	16.06	2476	1401	2148	3284	485	888	1750	7219	7070	3515	8377	3113	4310	3541
2,6-Nonandienal	16.78	2474	1823	2602	2451	479	697	2549	3819	8299	3975	6640	3148	6149	3470
2-Decanone	18.64	1482	323	891	1412	400	360	3206	4845	4932	1077	4777	13198	8003	3454
2-Hexadecanone	30.08	N/F	N/F	N/F	45	28	N/F	4324	72	63	48	81	24335	83	3231
2-Methyl-2-nonanol	14.99	1397	1082	1043	1019	202	264	1348	2693	5426	1512	4615	17527	3633	3212
2-Methoxy decane	13.17	2567	1565	2086	6038	1458	3024	2173	3066	3392	2188	1412	10395	2305	3205
Unidentified_6	19.12	2210	977	2046	2376	567	N/F	N/F	3599	4465	3475	4523	3392	5682	3028
Isobutyl 3-hydroxy-2,2,4-trimethylpentanoate	23.39	1903	544	1005	2327	927	1090	1076	3113	2564	1131	7056	4228	11982	2996
2,5-Dimethylpyrazine	11.04	2669	1359	2049	4722	1684	3006	1849	3437	4345	3027	1258	6279	2589	2944
1-Nonanol	19.08	2211	977	2046	2376	567	1313	3287	4076	4465	3475	4523	3392	4820	2887
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	27.54	1801	55	114	280	161	130	1016	393	171	168	12201	5251	15506	2865
2,6-Di-tert-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	25.41	1783	215	1554	3410	1567	1336	287	5362	5767	3154	8409	1309	3081	2864
Unidentified_2	15.79	933	3930	1636	1031	294	N/F	2063	3503	4846	1302	5003	3305	4855	2725
3-Ethyl-2,5-dimethylpyrazine	16.27	1788	558	855	1770	325	403	968	5928	8384	1176	6996	1552	4193	2684
Ethyl Acetate	2.99	1678	2400	899	1600	2266	12993	775	3527	1471	875	1823	1926	1690	2609
3-Methyl-2-heptanol	12.33	954	293	574	1436	534	539	3867	1638	1697	573	773	15846	1630	2335
2-Dodecanol	19.70	1563	838	1077	1585	364	396	501	2225	4470	1761	3794	2895	5237	2054
3-Methyl-1-butanol	6.07	1489	935	1987	3215	1498	6198	1060	1526	3018	1264	1823	895	1684	2046
Dimethyl sulfide	3.40	381	N/F	N/F	N/F	N/F	6320	N/F	N/F	N/F	N/F	566	N/F	712	1995
Methyl benzoate	16.93	274	356	291	490	70	105	172	2305	2359	981	1862	13013	1534	1832
3-methylbutyl 3-methylbutanoate	17.14	155	316	221	578	53	2776	1912	3019	1543	1052	865	10422	801	1824
1-Methyl-4-(1-methylethyl)-cyclohexanol	19.40	640	846	843	1101	240	307	527	1950	4791	1979	2942	2548	4665	1798
Methyl thioacetate	5.40	584	29	679	885	4702	N/F	1434	3085	3359	104	4370	708	1432	1781

3-methylbutyl 2-methylbutanoate	17.19	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	1712	N/F	1712
2-Methyl-1,3-pentanediol	14.11	1469	863	1083	2637	848	1245	838	1373	1833	1300	735	6218	1762	1708
2-Undecanone	22.11	718	95	464	720	330	176	769	1036	1088	285	2185	11925	2247	1695
5-ethyl-2-methylpyridin-4-amine	15.63	849	489	541	958	131	256	1408	2957	5492	906	3052	1345	3164	1658
Unidentified_7	19.26	1029	391	799	975	307	213	1276	2838	2865	564	3216	2601	4192	1636
Unidentified_3	18.77	407	153	637	1398	169	N/F	884	1629	5066	573	958	2312	5194	1615
Unidentified_4	18.80	782	154	637	1398	142	41	428	1507	5069	573	2293	5566	2216	1600
2-Decanone	23.46	701	N/F	437	767	1027	N/F	979	1465	1792	177	2387	3509	4196	1585
Di-isobutyl phthalate	31.47	1785	77	96	808	437	894	2021	1054	1014	700	4223	3050	4010	1551
2-Methyl-1-butanol	6.16	1959	862	1856	3162	1480	4228	609	904	1216	1263	1087	440	579	1511
Unidentified_5	19.00	453	194	1099	1230	288	41	390	2718	3741	437	1517	1810	4872	1445
Trimethyl pyridine	13.49	1047	190	737	2278	308	1030	1209	1709	1739	181	297	5927	1102	1366
Unidentified_1	14.00	1134	447	651	2459	621	948	446	758	1374	821	498	6107	1347	1355
2-Methyl-2-isobutyloxirane	13.83	946	638	639	1487	496	811	561	775	1180	989	477	5885	1028	1224
4-Methyl-2-Hexanone	10.29	342	204	238	272	302	131	4844	264	385	265	266	7072	346	1149
3,5-Dimethyl-2-octanol	19.57	916	348	604	906	225	108	293	846	2462	811	2022	2036	2816	1107
1,3-Dioxane	15.85	731	521	579	483	118	198	418	2149	3635	838	1909	711	1771	1082
Unidentified_8	19.36	383	554	463	462	148	173	473	1084	2654	1262	1765	1418	2627	1036
Diphenylamine	28.32	412	130	1446	2064	2215	484	19	411	3658	2060	97	175	201	1029
2-Decanol	18.94	274	220	1099	396	288	47	316	2718	1463	604	1411	2926	1431	1015
2,2,5,5-Tetramethyl furan	6.68	1049	813	830	1212	553	1658	1644	1318	1503	1016	305	401	851	1012
tert-Butyl methyl sulfide	7.65	1221	184	554	1098	605	1910	1126	1143	2469	595	391	311	937	965
2-Heptanol	10.63	119	3820	104	90	66	237	1481	54	221	151	72	5875	113	954
2-Tetradecanone	27.08	360	108	279	432	459	134	469	5481	756	384	1061	1116	1268	947
4-Methyl-2-heptanol	12.38	814	261	451	1109	405	318	N/F	N/F	1697	545	770	2750	N/F	912
2,5-dimethyl-5-isopentyl pyrazine	22.58	348	158	617	1401	449	145	69	1310	1850	450	669	669	3268	877
Unidentified_8	25.65	414	131	579	752	368	513	338	1145	747	478	2003	555	2353	798
Pentadecanoic acid	30.85	1030	333	250	415	618	268	282	333	208	424	1081	3326	839	724

3-Methyl butyl oxirane	11.68	7	217	864	350	99	586	2171	1046	156	72	249	2757	615	707
3-Octen-2-one	20.23	179	N/F	285	346	63	176	322	727	1998	378	367	1104	2513	705
Methyl thiobutyrate	9.13	10	N/F	12	39	N/F	N/F	4646	27	81	N/F	N/F	N/F	26	692
tert-Butyl ethyl ether	4.20	461	1287	616	774	756	144	43	262	576	2092	554	348	344	635
2,5-Dimethyl-3-(2-methylpropyl)-pyrazine	19.88	296	205	276	455	412	992	235	769	1280	478	612	556	1287	604
Hexadecanoic acid	30.97	1208	330	276	506	691	534	108	180	142	351	902	1956	578	597
2,4-Dimethyl-1-heptene	8.75	1557	441	776	342	615	808	470	291	481	429	362	469	628	590
Dibutyl phthalate	32.69	448	110	98	158	91	689	630	511	640	648	1256	821	1249	565
2-Dodecanol	27.20	411	78	282	471	579	867	218	676	406	317	1024	862	919	547
Chloroform	4.34	651	N/F	528	581	454	1220	341	263	502	N/F	423	380	375	520
2-Methyl-2-butyl acetate	7.84	27	53	44	2135	19	N/F	8	1667	45	59	11	11	27	342
2-Methyl butyric acid	9.11	N/F	547	N/F	N/F	182	N/F	N/F	N/F	N/F	283	N/F	N/F	NF	337
1,3,5-Cycloheptatriene	6.84	240	33	90	432	47	450	82	344	306	149	22	49	63	177
2-Pentadecanone	29.17	176	N/F	102	192	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	157
2-Methyl butyraldehyde	4.84	N/F	19	233	20	12	138	76	10	N/F	21	N/F	N/F	N/F	66

Table 4.7: Area values in terms of concentration of compounds detected in fish juice media samples inoculated with *Chryseobacterium* strains incubated at 4 °C. RT, retention time; *C.*, *Chryseobacterium*; N/F, not found

Compound	**RT**	**IJ 30-2**	**SH 19-2(b)**	**SH 11-3(a)**	**SH 11-3(b)**	**SH 11-4(b)**	**SH 20-4**	**SH 25-4**	**SH 28-3**	**SH 30-1**	**SH 40-3**	*C. balustinum*	*C. gleum*	*C. piscium*	**Average**
2-Ethyl-1-hexanol	14.74	49867	16891	16047	63888	7715	17606	55410	144586	230406	22434	107071	30585	87926	65418
1H-Indole	22.34	18730	4058	26429	46148	6789	2557	14664	32033	38866	8934	26191	22615	43019	22387
Dimethyl disulfide	6.34	11374	1640	17641	23742	9514	24138	75519	32762	21481	4489	2330	4701	24131	19497
2-Phenylethanol	17.50	5978	3171	4982	10945	2292	1214	386	31172	43527	9617	36502	14522	41359	15821
Ethyl 2-methyl-2-butenoate	11.83	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	10276	N/F	10276
S-Methyl 3-methylbutanethioate	11.95	2267	862	1901	6477	1151	1441	4409	1946	4585	1134	666	87570	2679	9007
Ethylene glycol mono-tert-butyl ether	13.71	4852	3743	4056	10971	2958	5425	3964	5493	8345	5543	3054	26918	7038	7105
2-Nonanone	16.68	1510	471	923	1441	307	380	15154	3566	6306	1352	7503	34503	5325	6057
Ethyl 3-methyl butyrate	9.17	N/F	11	17	8	148	N/F	440	N/F	115	260	N/F	39746	N/F	5093
2,4-Di-tert-butylphenol	26.25	2926	2042	2702	4204	3510	1882	2994	6757	6556	4460	5864	6382	9482	4597
3-Methoxy-3-methylbutanol	15.21	2859	1500	2227	2911	565	777	2131	6154	13304	2916	10476	4466	8346	4510
2,4,-Dimethylheptan-1-ol (diastereomere 1)	16.38	2012	1926	1822	1479	403	461	1622	7236	9994	3215	9367	4868	8561	4074
1,3-Di-tert-butylbenzene	21.12	6019	1501	3566	3006	1282	765	2665	4797	4968	2051	7373	5535	9410	4072
Methyl mercaptan	3.15	637	3911	4735	7858	6720	N/F	482	452	2886	3251	5155	7441	4581	4009
3-Hydroxy-2,2,4-trimethylpentyl isobutyrate	23.85	1844	420	723	1779	773	804	840	2592	2799	867	9446	6790	20643	3871
2,4,-Dimethylheptan-2-one	15.49	1906	351	939	1260	329	372	6258	5215	7000	1487	6438	10929	7094	3814
4-Methyl heptanone	11.77	3045	2730	2306	5228	1607	4595	3343	2516	4002	4678	1825	6104	3160	3472
2-Nonanol	16.99	752	432	639	1071	117	204	2041	1953	4055	1207	3292	26210	2761	3441

2,4,-Dimethylheptan-1-ol (diastereomere 2)	16.53	1830	963	1313	1193	242	348	1614	4951	10728	2289	7287	4442	6571	3367
Trimethylamine	3.24	2524	4931	1353	6381	3256	1694	496	1215	4158	9825	1572	N/F	1893	3275
Ethyl-2-methyl butanoate	9.05	17	455	17	2	5	441	58	55	157	349	48	39642	35	3175
2,6-Nonandienal	16.78	2179	1499	2430	1809	446	539	2024	3404	7175	3485	5249	2965	5648	2989
Acetophenone	16.06	2252	1062	1917	2246	375	796	1474	6115	5971	2832	7205	2546	3981	2982
2-Decanone	18.64	1300	294	688	1264	345	286	2940	3648	4512	955	3951	10638	7327	2934
2-Methoxy decane	13.17	1979	1406	1835	4440	1143	2504	1808	2838	2511	2088	1277	10353	2400	2814
2-Methyl-2-nonanol	14.99	1189	752	906	918	158	211	1239	2332	5067	1139	3267	15178	3510	2759
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	27.54	1637	46	83	192	143	127	818	319	151	165	9913	4463	15660	2594
2-Hexadecanone	30.08	N/F	N/F	N/F	32	25	N/F	4480	50	53	36	63	18470	74	2587
2,5-Dimethylpyrazine	11.04	2046	978	1466	4297	1290	2266	1441	3051	4239	3039	1203	6047	2168	2579
Isobytyl 3-hydroxy-2,2,4-trimethylpentanoate	23.39	1385	455	740	1747	660	817	994	2825	2304	1121	5655	3869	10629	2554
Unidentified_6	19.12	1717	793	1462	2034	429	N/F	N/F	2821	4284	3219	3803	2853	4377	2527
2,6-Di-tert-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	25.41	1307	173	1083	2959	1411	986	292	4081	5145	2676	7565	1138	2918	2441
1-Nonanol	19.08	1741	892	1936	2170	512	1200	2509	3198	3936	2716	3600	3138	3679	2402
Unidentified_2	15.79	691	2735	1385	864	269	N/F	1583	3306	3918	1040	4707	2634	4711	2320
3-Ethyl-2,5-dimethylpyrazine	16.27	1534	518	597	1365	274	324	802	5541	6660	997	4811	1386	3515	2179
Ethyl Acetate	2.99	1192	1851	775	1086	2062	9529	727	3010	1311	714	1454	1462	1359	2041
3-Methyl-2-heptanol	12.33	725	237	510	1066	384	476	3210	1376	1619	502	642	12708	1340	1907
3-Methyl-1-butanol	6.07	1184	728	1808	2636	1332	5975	1098	1268	2357	1262	1724	805	1588	1828
2-Dodecanol	19.70	1335	705	830	1444	287	362	452	1525	4157	1327	3058	2904	5257	1819
3-methylbutyl 2-methylbutanoate	17.19	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	1751	N/F	1751
3-methylbutyl 3-methylbutanoate	17.14	112	303	176	496	47	2127	1568	2061	1404	945	742	10568	832	1645
Dimethyl sulfide	3.40	327	N/F	N/F	N/F	N/F	4897	N/F	N/F	N/F	N/F	540	N/F	697	1615
1-Methyl-4-(1-methylethyl)-cyclohexanol	19.40	452	673	801	748	174	269	421	1477	4224	1620	2719	2500	4721	1600
Methyl benzoate	16.93	208	293	255	425	59	100	130	2007	1860	769	1306	11894	1467	1598

2-Undecanone	22.11	529	66	365	676	244	175	651	767	843	254	1867	11138	2395	1536
Methyl thioacetate	5.40	453	25	587	712	3775	N/F	1137	2616	2856	105	3839	704	1426	1520
2-Methyl-1,3-pentanediol	14.11	1083	672	1013	2255	611	980	744	1277	1494	1253	638	5789	1470	1483
Unidentified_7	19.26	885	319	582	920	277	186	1307	2045	2319	537	2684	2731	4289	1468
5-ethyl-2-methylpyridin-4-amine	15.63	705	398	500	904	106	236	1428	2392	4654	830	2606	1154	2897	1447
Unidentified_3	18.77	372	134	535	1302	163	N/F	923	1244	3817	521	696	2319	4998	1419
Unidentified_4	18.80	593	124	586	1246	116	30	338	1421	3661	540	1827	5510	2319	1409
2-Decanone	23.46	633	N/F	409	587	936	N/F	907	1185	1423	137	1871	3579	3752	1402
2-Methyl-1-butanol	6.16	1469	794	1559	2470	1256	3945	517	720	1042	1088	978	424	572	1295
Di-isobutyl phthalate	31.47	1616	61	75	702	344	787	1542	883	880	572	3133	2425	3325	1257
Unidentified_1	14.00	879	321	597	2244	595	953	386	545	1355	758	345	5331	1229	1195
Unidentified_5	19.00	395	180	1022	835	253	39	347	2187	2954	382	1379	1405	3902	1175
Trimethyl pyridine	13.49	844	157	704	1620	265	891	959	1415	1310	167	227	4931	1129	1125
2-Methyl-2-isobutyloxirane	13.83	745	591	616	1345	428	621	435	650	966	794	410	5002	807	1032
4-Methyl-2-Hexanone	10.29	295	166	183	243	289	119	4462	230	336	222	210	5509	316	968
3,5-Dimethyl-2-octanol	19.57	675	262	499	784	210	108	246	731	1991	642	1676	1989	2147	920
2-Decanol	18.94	249	172	1061	274	285	38	290	2526	1115	594	1342	2516	1487	919
Diphenylamine	28.32	358	112	1169	1683	2064	432	19	344	3319	1970	92	173	200	918
1,3-Dioxane	15.85	533	497	505	378	90	170	405	1673	3343	635	1360	629	1663	914
Unidentified_8	19.36	329	488	362	415	122	136	380	909	2556	1103	1269	1251	2378	900
2,2,5,5-Tetramethyl furan	6.68	739	684	696	877	422	1214	1697	1000	1249	753	220	391	750	822
tert-Buthyl methyl sulfide	7.65	825	142	499	931	539	1543	849	1048	2343	463	275	266	799	809
2-Tetradecanone	27.08	273	91	213	404	367	131	401	4712	591	359	806	1125	1046	809
2-Heptanol	10.63	91	3486	89	67	52	218	1382	44	195	145	54	4547	93	805
4-Methyl-2-heptanol	12.38	625	240	417	875	369	287	N/F	N/F	1659	453	700	2252	N/F	788
Methyl thiobutyrate	9.13	9	N/F	11	36	N/F	N/F	4873	19	73	N/F	N/F	N/F	24	721
2,5-dimethyl-5-isopentyl pyrazine	22.58	313	131	453	984	368	123	69	937	1496	440	600	555	2806	713
Unidentified_8	25.65	342	90	532	630	306	422	302	1049	729	406	1686	506	2178	706

Pentadecanoic acid	30.85	934	261	179	352	443	238	237	243	163	311	892	3273	731	635
3-Methyl butyl oxirane	11.68	6	185	837	270	89	567	1963	900	146	60	214	2448	557	634
3-Octen-2-one	20.23	154	N/F	244	266	58	141	260	684	1862	366	275	869	2199	615
tert-Butyl ethyl ether	4.20	394	1004	508	706	734	129	40	186	427	2078	384	288	345	556
Hexadecanoic acid	30.97	1050	305	262	468	666	403	108	139	135	257	720	1672	521	516
2,5-Dimethyl-3-(2-methylpropyl)-pyrazine	19.88	245	161	225	409	322	931	217	545	985	463	579	484	1097	513
Dibutyl phthalate	32.69	369	90	79	135	77	697	579	439	548	558	954	746	1187	497
2,4-Dimethyl-1-heptene	8.75	1057	395	572	316	581	721	473	223	464	352	260	425	510	488
2-Dodecanol	27.20	343	68	197	419	569	862	173	535	337	291	777	868	706	473
Chloroform	4.34	461	N/F	366	512	445	1140	351	208	359	N/F	358	361	389	450
2-Methyl buryric acid	9.11	N/F	446	N/F	N/F	156	N/F	N/F	N/F	N/F	221	N/F	N/F	N/F	274
2-Methyl-2-butyl acetate	7.84	19	44	32	1635	14	N/F	7	1213	37	54	8	11	24	258
1,3,5-Cycloheptatriene	6.84	196	28	68	358	42	353	86	243	287	122	15	37	58	146
2-Pentadecanone	29.17	125	N/F	91	158	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	N/F	125
2-Methyl buteraldehyde	4.84	N/F	18	165	14	12	100	68	7	N/F	16	N/F	N/F	N/F	50

The compound dimethyl sulphide was only detected in strains SH 20-4, IJ 30-2, *C. balustinum* and *C. piscium* at both 4 and 25 °C.

2-ethyl-1-hexanol was detected in all the samples and was more prevalent in strain SH 30-1 than in the other samples both at 4 and 25 °C. More compounds were detected in *C. gleum* than in any other strain incubated at both 4 and 25 °C. *Chryseobacterium gleum, C. piscium* and strain SH 30-1 had the most detected volatile compounds while strains SH 19-2(b) and SH 11-4(b) had the least detected compounds. When the area of the peaks (concentration) were compared for incubation at 4 and 25 °C, higher peak values were evident at 25 °C. Although the samples were incubated for 14 days at 4 °C and 7 days at 25 °C, the 25 °C results still showed higher values which is an indication that spoilage in terms of volatile production will be more pronounced at 25 °C than at 4 °C.

4.3.3.3 Composition of volatiles produced at 4 and 25 °C

Strain SH 30-1 at 4 and 25°C (Figures 4.1a and 4.1b) had the largest composition of volatile compounds 1–22 which was followed by *C. gleum.* However, in terms of the number of volatile compounds detected, *C. gleum* produced the most volatile compounds when compared to all the *Chryseobacterium* isolates used in this study at both 4 and 25 °C (Tables 4.6 and 4.7).

The alcohol, 2-ethyl-1-hexanol, was detected in all the 14 strains in this study at 4 and 25 °C however, it was most prevalent in strains SH 30-1, SH 28-3 and *C. gleum* (Figures 4.1a and 4.1b). It is associated with odours such as, raw fish, green, and chalky. The raw fish odour detected by GC/MS also correlates with the fishy odour described for strains SH 28-3 and *C. gleum* by the sensory analysis panellists (Table 4.3).

The second most produced compound at 4 and 25 °C was indole and it was most prevalent in strains SH 30-1, SH 28-3, SH 11-4(b) and *C. piscium* (Figures 4.1a and 4.1b). Indole is a spoilage indicator which results from the microbial degradation of the amino acid tryptophan. The production of indole from tryptophan results in disagreeable odours that may be faecal and nauseating (Schiffman et al. 2001). Microorganisms particularly consume glucose and avoid degradation of amino acids until glucose is exhausted. However, microorganisms are capable of using most of the naturally occurring amino acids for growth (Jacoby 1964). Microbial degradation of amino acids while growing in meat results in the production of amines, ammonia, methylsulfides, and mercaptans which are associated with putrefactive odours (Dilbaghi and Sharma 2007).

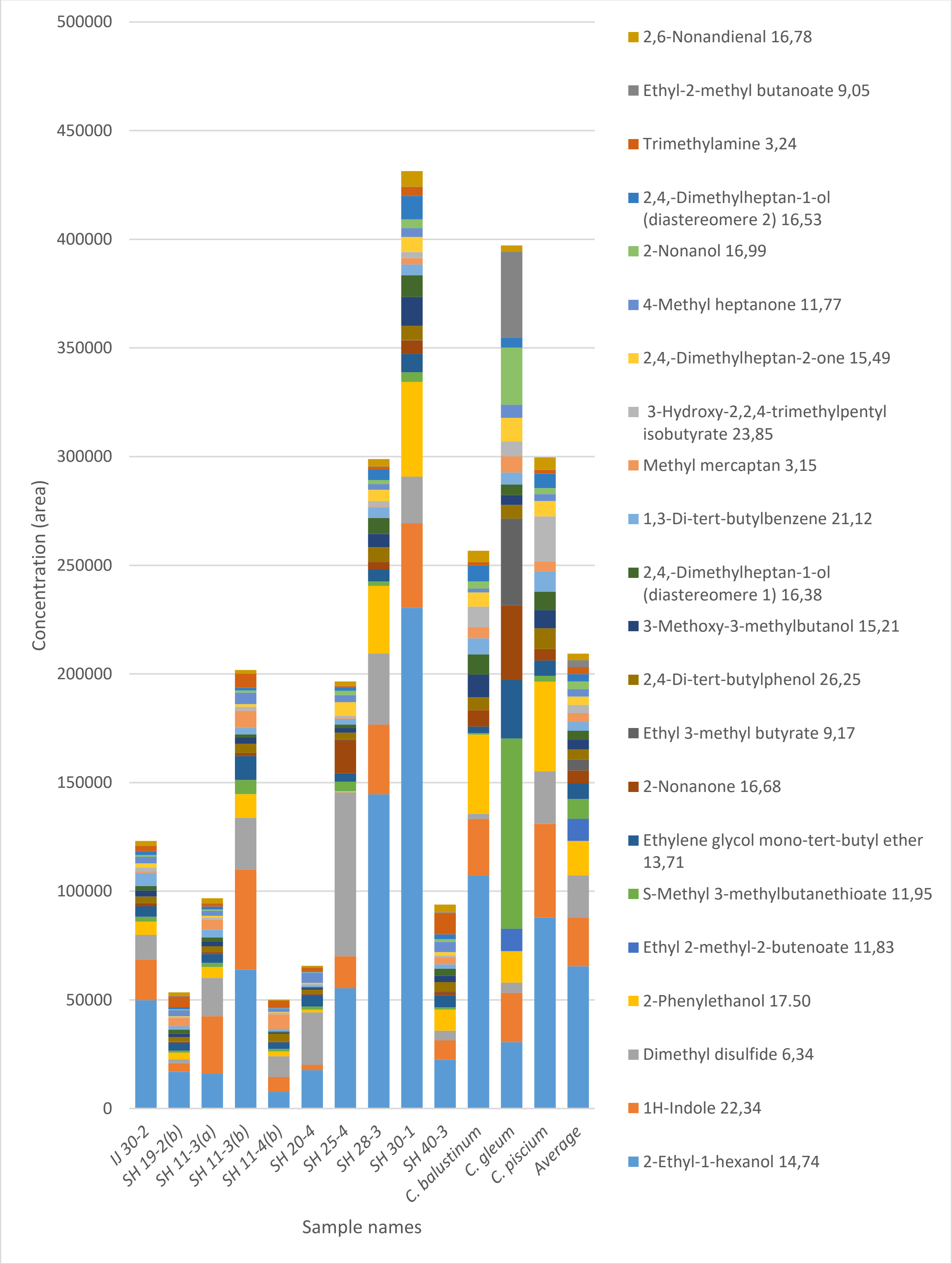

Figure 4.1a: A stacked column diagram of volatile compounds 1–22 (Table 4.7) produced by the *Chryseobacterium* strains in this study incubated at 4 °C.

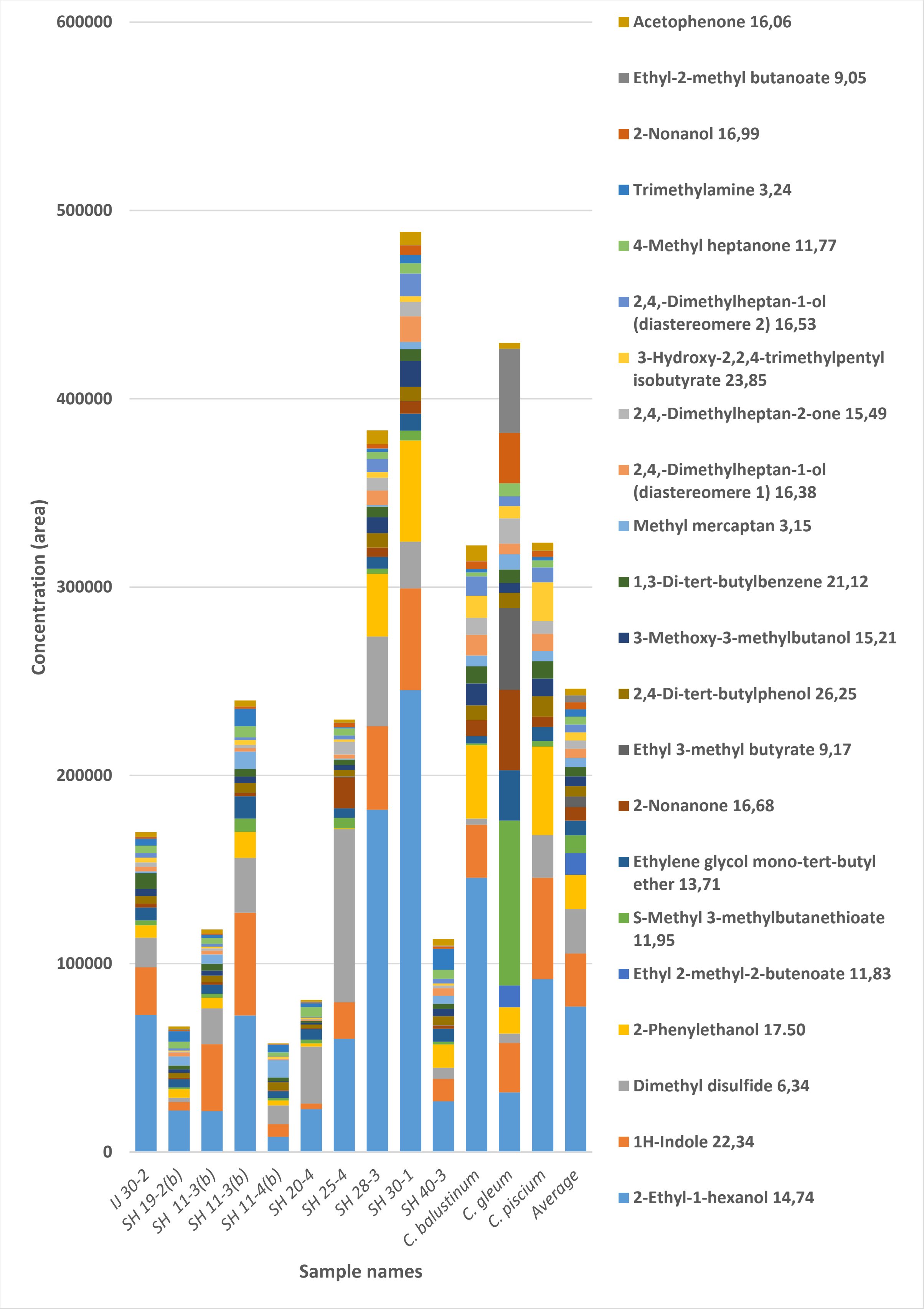

Figure 4.1b: A stacked column diagram of volatile compounds 1–22 (Table 4.6) produced by the *Chryseobacterium* strains in this study incubated at 25 °C.

Dimethyl disulphide was the third most detected compound at 4 and 25 °C and was most prevalent in strains SH 25-4, SH 30-1 and SH 20-4 (Fig. 4.1a and Fig. 4.1b). The degradation of sulfur-containing amino acids such as cystine or methionine results in the formation of hydrogen sulphide, mercaptans, organic disulfides, and other sulfur-containing (thio) compounds (Banwart 1989). Table 4.5 shows some of the odours associated with volatile compounds and the odours such as cooked cabbage, foul, putrid and decayed vegetables were linked with sulphur containing compounds (Schiffman et al. 2001; Giri et al. 2010).

Production of alcohols, aldehydes and ketones such as 2-methyl-1-butanol, ethanol, 2-methylbutanal, 3-methylbutanal, 2, 3-pentanedione, 2-heptanone, 3-hydroxy-2-butanone, has been linked with metabolic activity of microorganisms that spoil fish and meat (Nychas et al. 2008; Parlapani 2014). One of the most significant aroma-related alcohols is 2-phenylethanol which imparts a rose-like odour (Longo and Sanromán 2006). 2-Phenylethanol was the fourth most detected compound and its concentration was most prevalent in strain SH 30-1, *C. balustinum* and *C. piscium* at 4 and 25 °C (Figures 4.1a and 4.1b). The odour imparted by this compound can also be linked with the sickening-sweet and fruity odours that were described for FJB inoculated with *C. piscium* by sensory analysis panellists (Table 4.3).

Biogenic amines are biologically active nitrogenous compounds of low molecular weight which result from the decarboxylation of amino acids and their presence in fish gives an indication of the degree of spoilage as they are associated with bacterial spoilage (Veciana-Nogués et al. 1997). Trimethylamine, which is associated with the fishy odour, is derived from the breakdown of trimethylamine oxide (TMA-O) by bacterial enzymes (Li et al. 2013). Trimethylamine was present in all the samples at 4 and 25 °C except the one sample containing *C. gleum*. It was most prevalent in samples inoculated with strains SH 11-3(b) and SH 40-3 (Figures 4.1a and 4.1b).

Aldehydes have a great impact on the overall fresh fish flavour (Leduc et al. 2012). These compounds mostly come from enzymatic catabolism of unsaturated fatty acids (Yasuhura and Shibamoto, 1995; Kawai, 1996). The compound 2,6-Nonadienal, was present in all the samples however its composition was extremely low in strains SH 11-4(b) and SH 20-4 at 4 °C (Fig. 4.1a) and at 25 °C (Fig. 4.2b). It is produced from the autoxidation of lipids and is characterised by roasty and waxy odours (Giri et al. 2010; Tao et al. 2014). Most of the C6–C9 aldehydes, ketones and alcohols are aroma compounds characterizing fresh fish (Kawai, 1996).

Esters are products which result from microbial degradation of lipids (Li et al. 2013). Esters are normally used as flavouring agents and they are valued for the fruity aromas they provide (Longo and Sanromán 2006). The odours that were associated with most ester compounds detected in this study were sweet and fruity (Table 4.5.). The composition of compounds from the samples that were incubated at both 4 and 25°C did not differ much. The compound, ethyl-3-methyl butyrate (Fig. 4.1a and Fig. 4.1b) and methyl benzoate (Fig. 4.2a and Fig. 4.2b) which is associated with fruity and sweet flavours, was more prevalent in *C. gleum* and detectectable in trace amounts in other samples. Strains IJ 30-2, SH 20-4 and SH 28-3 can be differentiated from both *C. balustinum* and *C. piscium* in that they did not produce ethyl-3-methyl butyrate at 4 and 25°C (Fig. 4.1a and Fig. 4.1b).

Pyrazines are heterocyclic, nitrogen-containing compounds which are associated with nutty and roasted flavours (Longo and Sanromán 2006). A limited number of microorganisms are able to synthesize pyrazines (Longo and Sanromán 2006). Some bacteria are able to produce tetramethylpyrazine from the metabolism of amino acids (Demain et al. 1967). 2,5-Dimethyl pyrazine, 3-ethyl-2,5-dimethyl pyrazine, 2,5-dimethyl-3-(2-methylpropyl)-pyrazine and 2,5-dimethyl-5-isopentyl pyrazine were pyrazines detected in this study at 4 °C (Fig. 4.2a and Fig. 4.4a) and 25 °C (Figure 4.2b and Fig. 4.4b). 2,5-Dimethyl pyrazine is associated with fruity odours (Fukami et al. 2002), and the odours imparted by 3-ethyl-2,5-dimethyl pyrazine are irritating and intense (Schiffman et al. 2001). 3-Ethyl-2,5-dimethyl pyrazine was detected in all the samples but, was most prevalent in samples inoculated with strains SH 30-1, SH 28-3 and *C. balustinum* (Figures 4.2a, 4.4a, 4.2b and 4.4b).

Ketones such as 2-heptanone, 2-nonanone, and 2-undecanone, are the major contributors of decayed flavours in UHT milk (Badings et al. 1981). Low-carbon saturated ketones have a special aroma, and many ketones have been found in cheese, which are the main volatile constituents of cheese with a unique flavour (Bertolino et al. 2011). 2-Hexadecanone was most prevalent in samples inoculated with *C. gleum,* and absent in strains SH 19-2(b), IJ 30-2, SH 11-3(b) and SH 20-4 at 4 and 25 °C (Fig. 4.3a and Fig. 4.3b). 2-undecanone, a ketone linked with musty odours, was also more prevalent in *C. gleum* and almost absent in strain SH 19-2(b) at 4 and 25 °C (Fig. 4.3a and Fig. 4.3b).

Acids are normally oxidised to carbohydrates and/or other simple organic acids (Mielman 2006). A total of three acids were detected in this study with two of them being pentadecanoic acid and hexadecenoic acid associated with marine and fatty odours were more prevalent in *C. gleum* and IJ 30-2 at both 4 and 25 °C (Figures 4.4a and 4.4b).

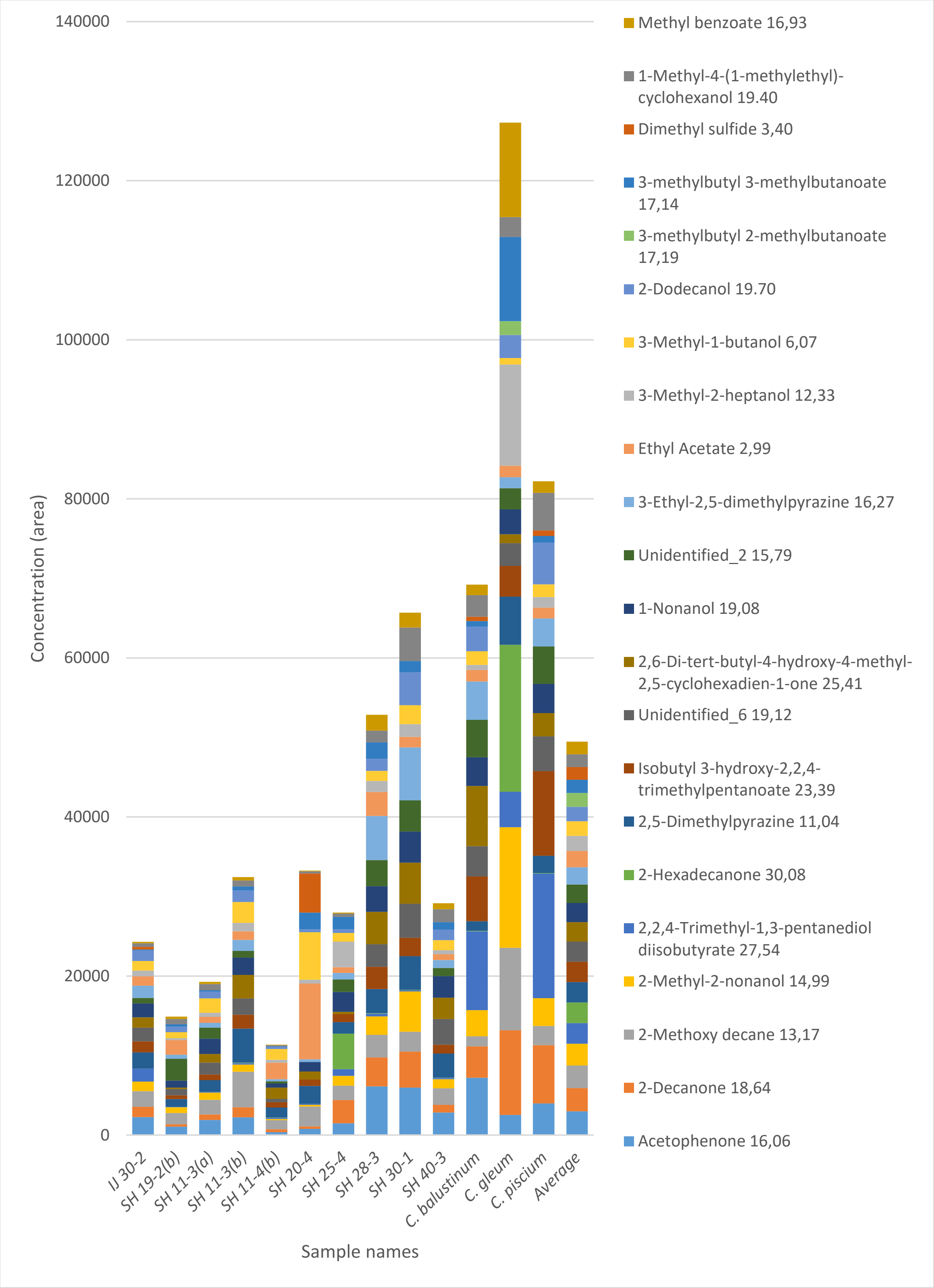

Figure 4.2a: A stacked column diagram of volatile compounds 23–44 (Table 4.7) produced by the *Chryseobacterium* strains in this study incubated at 4 °C.

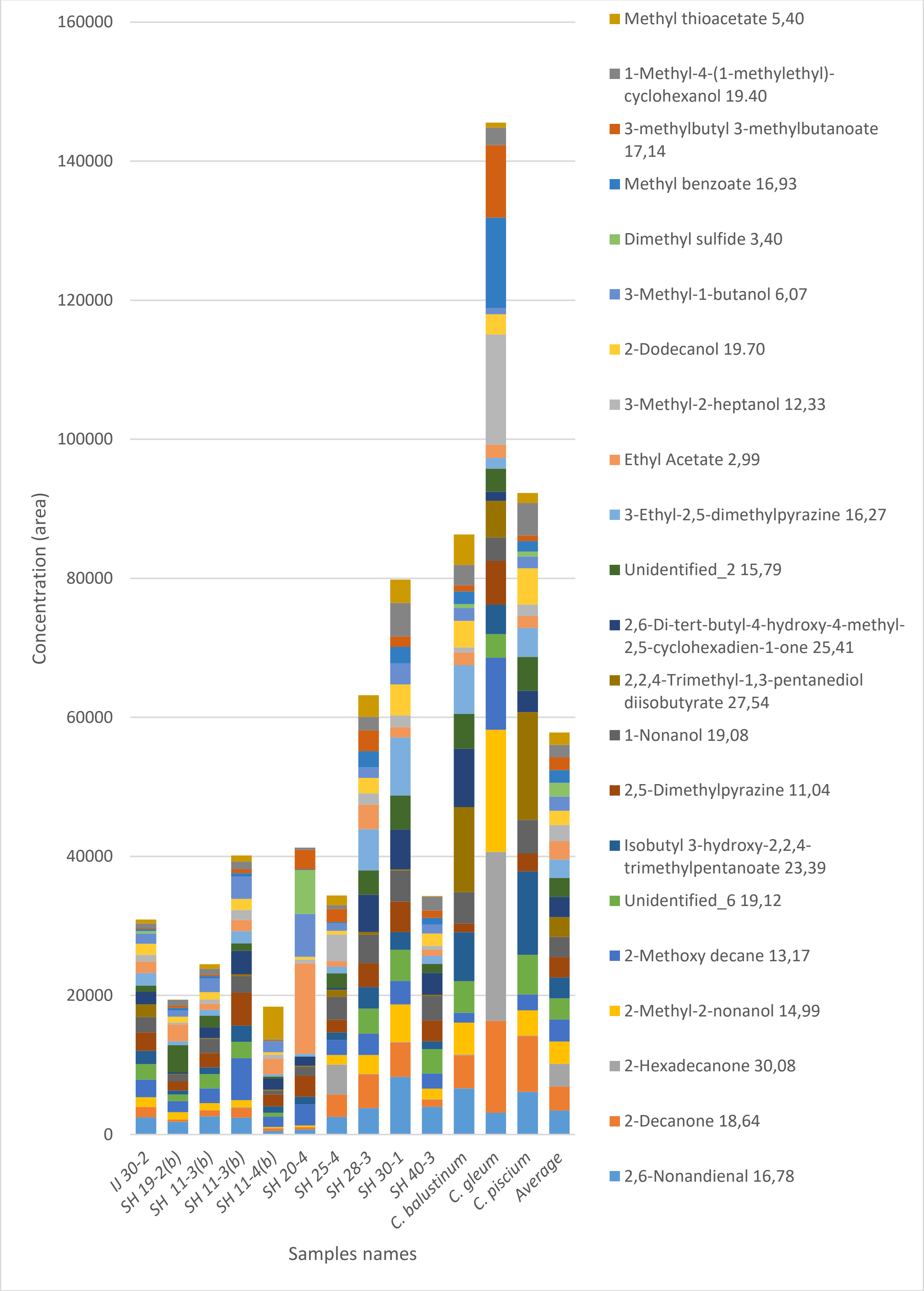

Figure 4.2b: A stacked column diagram of volatile compounds 23–44 (Table 4.6) produced by the *Chryseobacterium* strains in this study incubated at 25 °C.

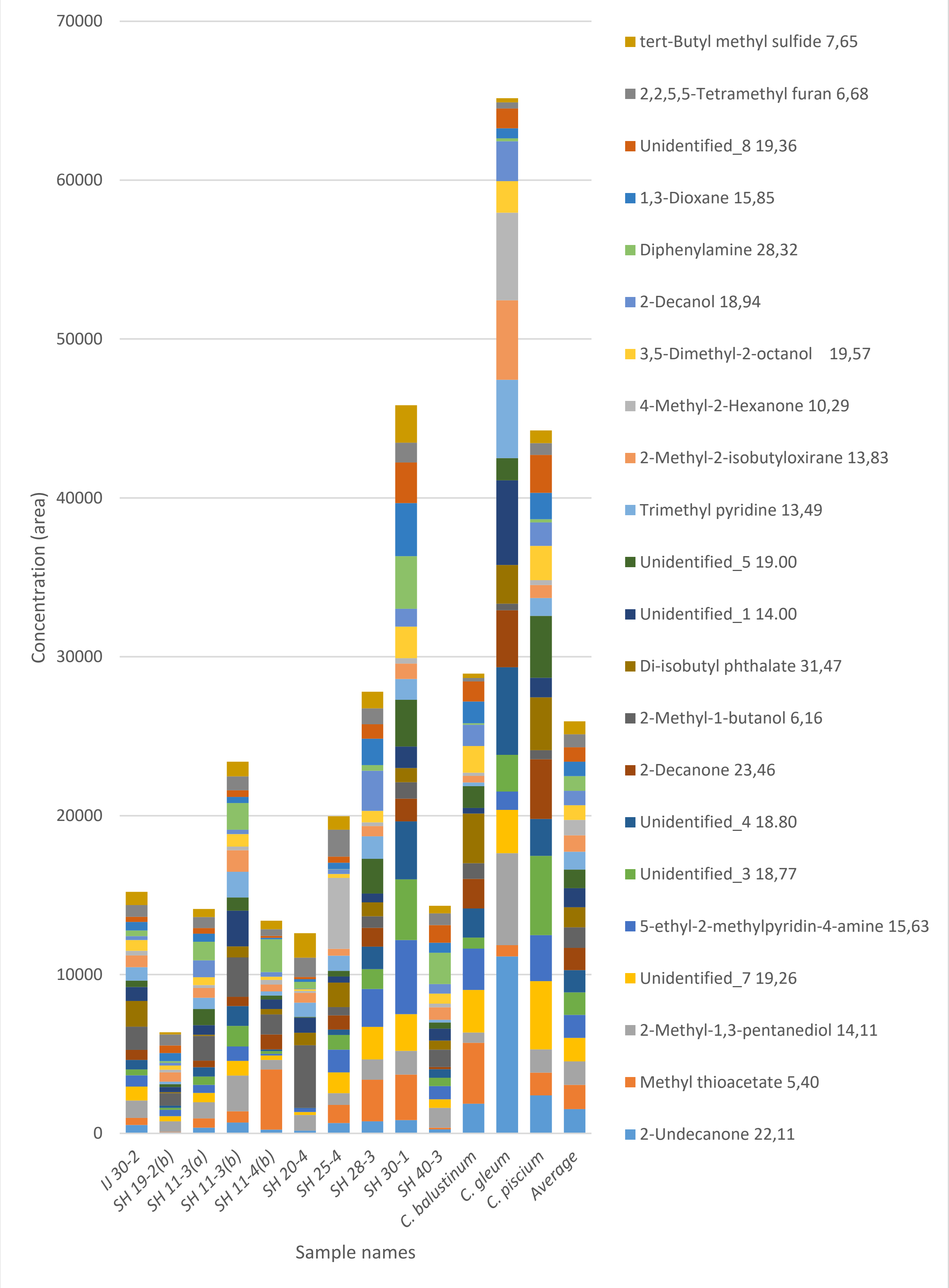

Figure 4.3a: A stacked column diagram of volatile compounds 45–66 (Table 4.7) produced by the *Chryseobacterium* strains in this study incubated at 4 °C.

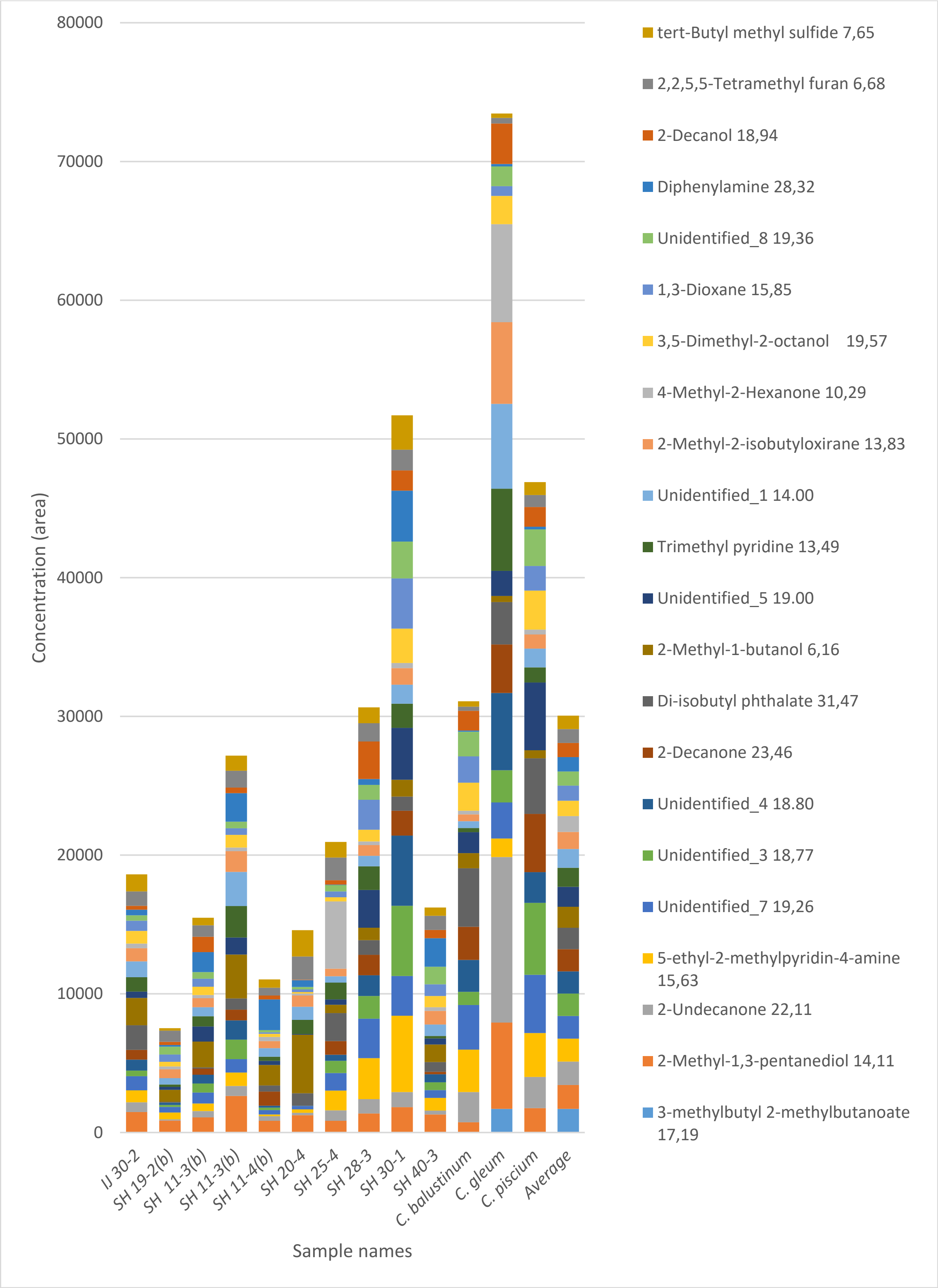

Figure 4.3b: A stacked column diagram of volatile compounds 45–66 (Table 4.6) produced by the *Chryseobacterium* strains in this study incubated at 25 °C

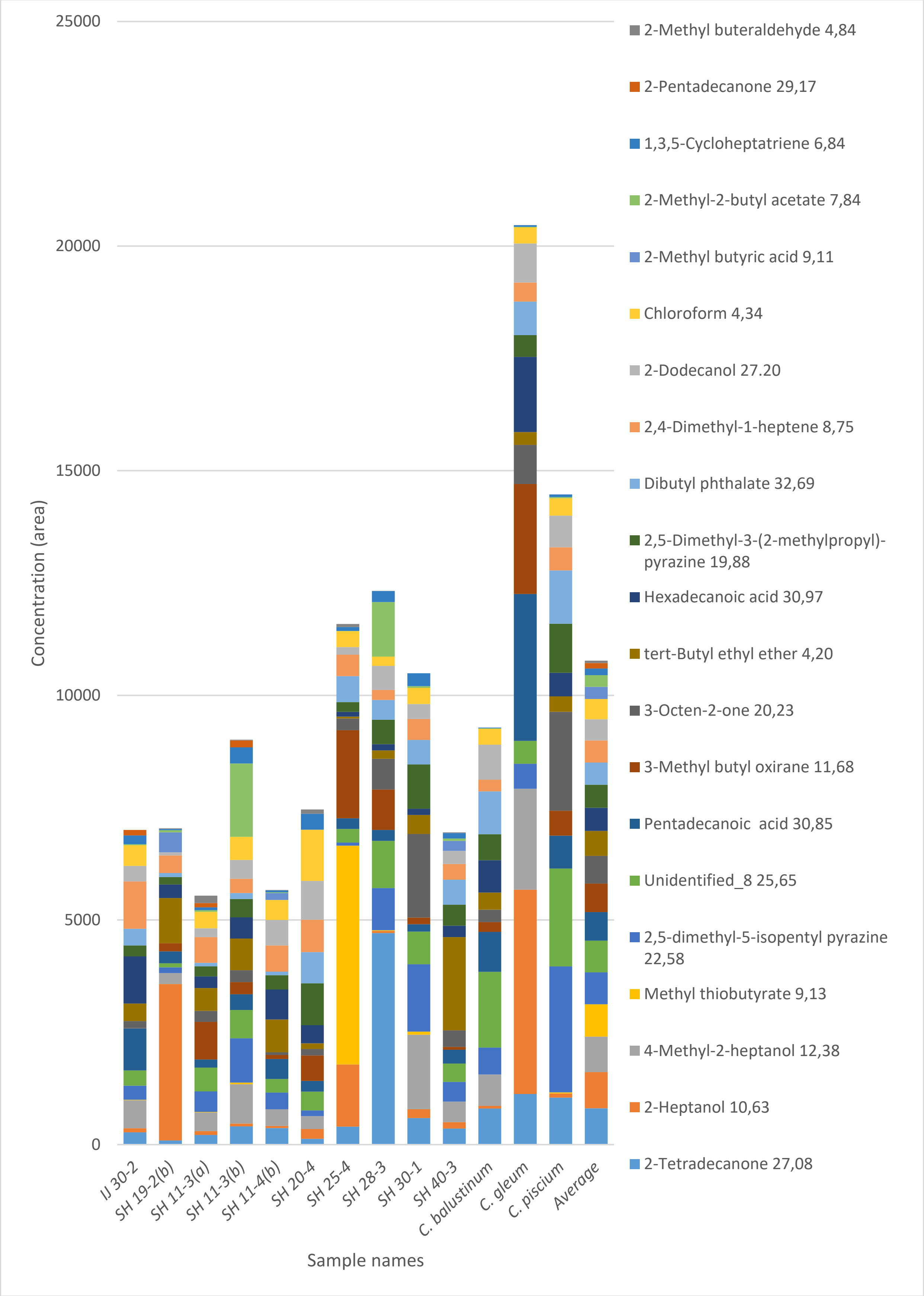

Figure 4.4a: A stacked column diagram of volatile compounds 67–87 (Table 4.7) produced by the *Chryseobacterium* strains in this study incubated at 4 °C.

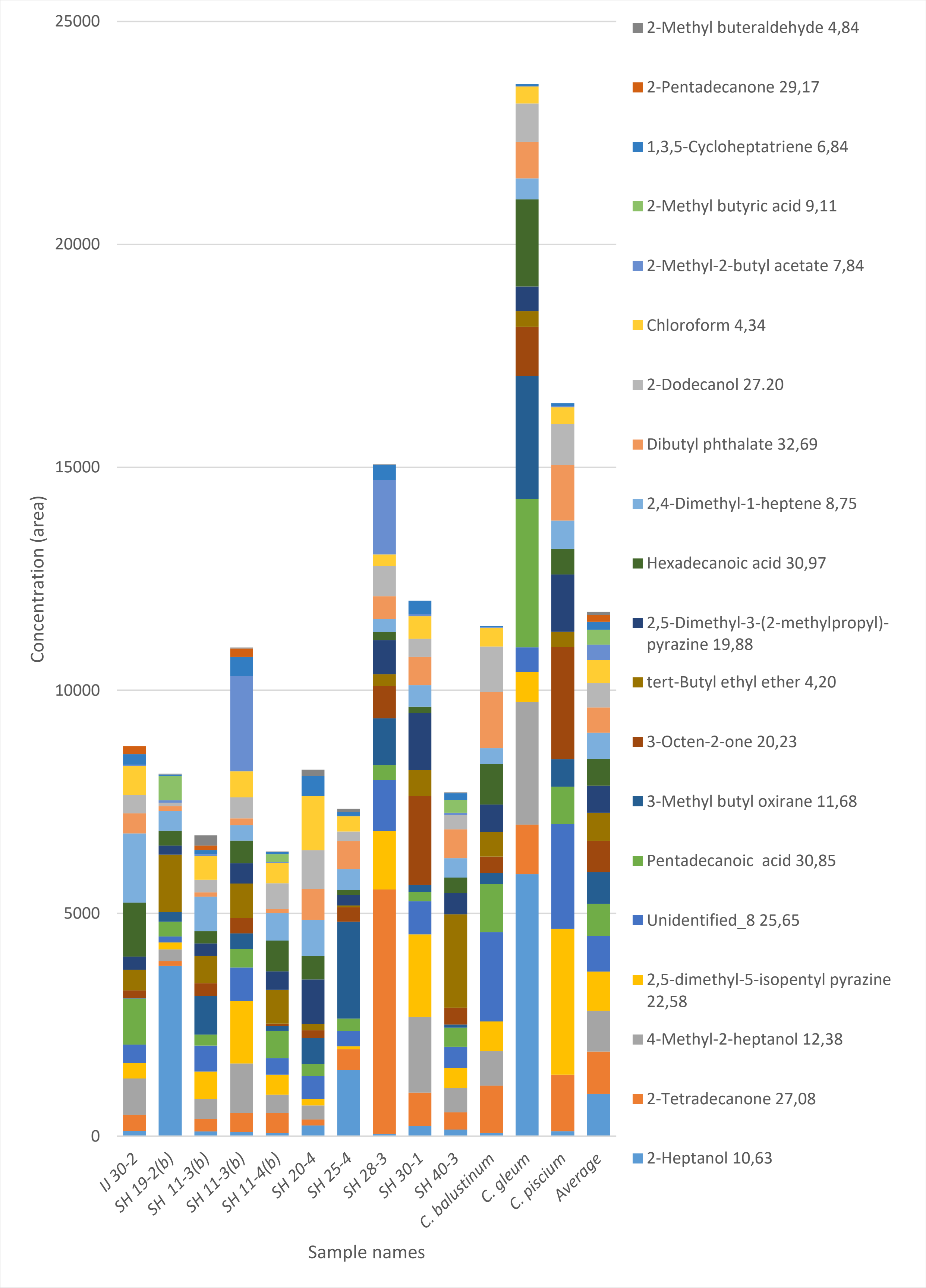

Figure 4.4b: A stacked column diagram of volatile compounds 67–87 (Table 4.4b) produced by the *Chryseobacterium* strains in this study incubated at 25 °C.

4.4 Conclusions

The spoilage potential of the *Chryseobacterium* isolates was determined by using the BIOLOG^{TM} Omnilog system to evaluate the ability of the isolates to oxidise carbon sources. Thirty-one carbon sources made it possible to differentiate between the species of *Chryseobacterium* studied. Strains SH 23-4 and SH 11-4(b) utilised 25/31 carbon sources, followed by *C. gleum* which utilised 21/31 carbon sources. These findings suggest that these strains are capable of causing more detectable spoilage in fish than all the other strains tested in this study. Amino acids and carbohydrates were the most utilised carbon sources. Not all microorganisms can produce unacceptable metabolites from carbohydrates, however, the metabolism of amino acids results in the formation of spoilage associated metabolites. All the strains were able to degrade gelatine which is also a spoilage indicator since this is an action carried out by bacterial proteolytic enzymes normally associated with spoilage potential.

Sensory evaluation of fish juice samples inoculated with *Chryseobacterium* strains showed no significant differences between all the tested samples, it did however result in the identification of odour descriptors, such as cabbage-like, smelly feet, rotten fruit, fruity, and sour. It can therefore be concluded that all the isolates and reference strains in this study are potential spoilage organisms of marine fish because they were able to produce off-odours in FJB.

A total of 87 volatile compounds were detected using the GC/MS analysis. More compounds were detected in the FJB inoculated with *C. gleum* than in any other strain incubated at both 4 and 25 °C while samples inoculated with strains SH 19-2(b) and SH 11-4(b) had the least detected compounds. When the area of the peaks (concentration) were compared for incubation at 4 and 25 °C, higher peak values were evident at 25 °C. Although the samples were incubated for 14 days at 4 °C and 7 days at 25 °C, the 25 °C results still showed higher values which is an indication that spoilage in terms of volatile production will be more pronounced at 25 °C than at 4 °C. Alcohols, ketones, aldehydes, sulphide and nitrogen-containing compounds have been revealed as the main source of fishy odours in aquatic animals. Some of the odours detected by GC/MS were also described for the same strains by the sensory analysis panellists. Sensory analysis and GC/MS and the BIOLOG^{TM} Omnilog were therefore useful in estimating the spoilage potential of the *Chryseobacterium* species through odour production. The volatile compounds which resulted in spoilage odours in this study were most prevalent in strains SH 30-1, SH 28-3, SH 25-4 and *C. gleum*. In conclusion, the BIOLOG^{TM} Omnilog, Sensory analysis and GC/MS were therefore useful in estimating the spoilage potential of the *Chryseobacterium* species.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Food spoilage is any visible alteration in food that is considered unacceptable by the consumer (Ayres et al. 1980). Fish is a food product that spoils quickly due to microbial growth using a wide variety of carbon sources which may result in the production of metabolites and volatile compounds causing spoilage characteristics such as off-odours and off-flavours (Zhang et al. 2008; Kuley et al. 2017).

Members of the genus *Chryseobacterium* are Gram-negative rods which have been isolated from a variety of clinical, environmental, industrial and food sources (Jooste and Hugo 1999; Bernardet et al. 2006). *Chryseobacterium* species are commonly isolated from food production and processing environments (Hugo et al. 2019). They have also been identified from aquatic environments and they form part of the bacterial community isolated from aquatic animals (Hugo et al. 2019). For instance, *Chryseobacterium balustinum* (Harrison 1929; Vandamme et al. 1994), *C. piscium* (De Beer et al. 2006), *C. piscicola* as well as *C. scophthalmum* (Mudarris et al. 1994; Vandamme et al. 1994) were isolated from fish. *Chryseobacterium balustinum* was first isolated from the scales of halibut (*Hippoglossus hippoglossus*) freshly harvested from the Pacific Ocean and it was considered a spoilage organism rather than a pathogen (Austin and Austin 1999).

In a study of Engelbrecht (1992) at the University of the Free State, several yellow-pigmented bacteria were isolated from Cape marine fish. Many of these bacteria were identified as members of the *Flavobacteriaceae* family (Engelbrecht et al. 1996a) and some of these isolates produced H_2S and hydrolyzed several substrates such as casein and gelatine which are spoilage indicators (Engelbrecht et al. 1996b). Later, in 2006, some of these isolates were identified as a new species, *Chryseobacterium piscium,* which was proposed to be a spoilage organism because of the activities of the enzyme urease and phenylalanine deaminase (de Beer et al. 2006). Several of these flavobacterial isolates, however, remained unidentified.

In this study, 11 of the flavobacterial isolates from the study of Engelbrecht (1992) were evaluated. A polyphasic approach was followed to classify the 11 strains by using conventional phenotypic characterisation, 16S rRNA gene sequence analysis and the BIOLOG^{TM} Omnilog Gen III identification system in comparison with closely related type strains of the species of the genus *Chryseobacterium* in Chapter 3.

Phylogenetic analysis based on 16S rRNA gene sequences was obtained by the construction of phylogenetic trees which were then used to determine the genus to which the strains belonged to and their closest neighbours. It was confirmed that the unidentified bacterial strains used in this study represented members of the genus *Chryseobacterium.* The GenBank BLAST results for 16S rRNA gene sequences for these organisms correlated with the phylogenetic results in that closely related species of the genus *Chryseobacterium* clustered tightly together with the unidentified strains demonstrating very close relationships. Three *Chryseobacterium* isolates namely; SH 23-4, SH 30-1 and SH 28-3 had a high similarity percentage value of 99.8%, and 100% with *C. piscium* indicating that they could be strains of *C. piscium*. Seven strains (SH 11-3(a), SH 11-3(b), SH 20-4, SH 25-4, SH 40-3, SH 19-2(b) and SH 11-4(b)) had a similarity percentage that was lower than 98.7%, and since a cut-off value of 98.7 was proposed in 2006 by Stackebrandt and Ebers for species delineation, it is possible that these strains might be novel species, however, all strains require further polyphasic investigations to have full evidence for description of novel species.

Conventional phenotypic tests and the BIOLOG^{TM} Omnilog Gen III identification system were also used. The BIOLOG^{TM} system was able to correctly identify all three reference strains and also indicated, like the 16S rRNA gene sequencing data and phylogenetic analysis, that strains SH 23-4, SH 30-1 and SH 28-3 belonged to *C. piscium*. The BIOLOG^{TM} method can therefore, also be used for identification purposes, however, because of its limited database, some isolates could not be identified at either genus or species level and therefore it is very important to use the BIOLOG^{TM} Omnilog identification system together with other identification methods.

The growth characteristics of the 11 *Chryseobacterium* strains in this study as well as the reference strains were investigated by including a battery of additional metabolic activity tests. All the strains tested produced yellow, smooth colonies with a fruity/spoiled odour. The strains were negative for Gram-reaction and motility and yielded positive reactions for the enzymes catalase and oxidase and these features are characteristic of the members belonging to the genus *Chryseobacterium* (Hugo et al. 2019).

A better understanding of the spoilage characteristics of the 11 *Chryseobacterium* isolates and reference strains used in this study was obtained by estimating their spoilage potential by determining their growth characteristics at different temperatures, in different sodium chloride concentrations and on different culture media and their ability to produce proteolytic and lipolytic enzymes and to produce odours from specific substrates.

Some of the *Chryseobacterium* strains were able to produce ammonia from urea, hydrolyse casein, gelatine and lecithin and produce indole from tryptophan. The ability of microorganisms to degrade casein, gelatine and lecithin is used as an indication of their protease and lipase activity. Lipolytic and proteolytic enzyme production by members of the genus *Chryseobacterium* has been linked with their spoilage potential (Bernardet et al. 2006). The release of large amounts of ammonia contributes to the development of spoilage odours (Nychas et al. 1998) while the production of indole from tryptophan has been associated with "unclean" flavours (Ayres et al. 1980). Indole is also used as a spoilage indicator in shrimp and crustaceans because in high concentrations it imparts putrid and musty odours (Sarnoski et al. 2010).

All the organisms were able to grow at 4–35˚C, however only strains SH 23-4 and SH 11-4(b), *C. balustinum*, *C. gleum* and *C. piscium* could grow at 42 °C which might be an indication that these strains have pathogenic characteristics since *C. gleum* is a recognised human pathogen (Vandamme et al. 1994). The ability of these strains to grow at lower temperatures also makes them possible agents of the destruction of food kept at low temperatures because members of the genus *Chryseobacterium* have been shown to grow and cause spoilage of refrigerated food products, especially milk and dairy products (Cousin 1982). Strain SH 11-4 (b) was also the only *Chryseobacterium* strain which could readily grow in 5% NaCl inclusion level and it shared this characteristic with *C. piscium.* The ability of these isolates to grow at 5% NaCl inclusion level therefore suggests that they might be able to spoil food preserved with salt.

In Chapter 4 the BIOLOG^{TM} Omnilog Gen III identification system was not only used for identification purposes, but was also used for spoilage estimation of the isolates through carbon source oxidation determination. Thirty-one carbon sources made it possible to differentiate between the species of the *Chryseobacterium* strains studied. Both strain SH 23-4 and SH 11-4(b) utilised most carbon sources (25/31), followed by *C. gleum* which utilised 21/31 which could be an indication that these species have the potential to cause more pronounced types of spoilage than the other species tested in this study. Strain SH 11-3(b) utilised the least carbon sources (7/31), together with strain IJ 3-2 which utilised only 9/31.

Most isolates were able to metabolise D-mannose, gentibiose, L-glutamic acid, acetic acid, dextrin and Tween 40. Bacteria will initially metabolise carbohydrates which serve as nutrients for their growth (Venugopal 1990). The degradation of amino acids and other metabolic substances only begins when carbohydrates have been depleted (Dainty 1996). The breakdown of amino acids results in metabolites such as ammonia, amines, dimethyl sulphide and hydrogen sulphide which are responsible for off-odour production (Dainty 1996). The oxidation/reduction of glutamic acid may yield acetic acid, butyric acid, carbon dioxide, ammonia and hydrogen and butyric acid and ammonia are associated with rancid odours in food (Banwart 1989). Acetic acid results in vinegar-like and pungent aromas to fish sauce

(Mohamed et al. 2012). Complex polysaccharides such as dextrin are normally broken-down to simple sugars before utilisation by microorganisms and this is a spoilage indicator because some of the metabolic products resulting from the oxidation of these polysaccharides include organic acids, alcohols and CO_2 which may produce off-odours, sourness and bitter defects (Banwart 1989). Hydrolysis of Tween 40 serves as a lipase and esterase indicator (Harrigan and McCance 1976). Lipolytic enzymes are responsible for both pleasant and unpleasant flavours occurring in dairy products, and they do this by hydrolysing milk triacylglycerols (Mielman 2006).

Sensory evaluation was performed on fish juice samples inoculated with the 11 *Chryseobacterium* test isolates and three reference strains incubated at 4 and 25 °C. There were no statistical significance differences between all the inoculated fish juice samples in this study at both 4 and 25 °C. Significant differences were only observed between inoculated samples and the controls. The most described odours were cabbage-like, smelly feet, rotten fruit, fruity, and sour. Organisms which are responsible for spoilage of marine fish are considered to be those which produce off-odours when cultivated in pure cultures on fresh fish or in fish press juice (Engelbrecht 1992). Most of the bacterial species isolated from fish and fish products are considered to be proteolytic in nature (Venugopal 1990). Proteolytic and lipolytic enzymes produced by *Chryseobacterium* are usually responsible for the production of off-odours and flavours (Tsôeu et al. 2016). It can therefore be concluded that all the strains in this study are potential spoilage organisms of marine fish because they were able to produce off-odours in FJB.

Volatile compounds produced by *Chryseobacterium* isolates were determined using headspace SPME-GC/MS analysis. A total of 87 volatile compounds were detected from the fish juice samples inoculated with the *Chryseobacterium* strains incubated at 4 and 25 °C. The composition of the compounds detected at 25 °C were slightly higher than the samples incubated at 4 °C. *Chryseobacterium gleum* and strain SH 30-1 had the most detected volatile compounds while strains SH 19-2(b) and SH 11-4(b) had the least detected compounds. The most produced compounds were 2-ethyl-1-hexanol, indole, dimethyl disulphide, 2-phenylethanol, S-methyl 3-methylbutanethioate, ethylene glycol mono-tert-butyl ether, 2-nonanone, 2,4-di-tert-butyl phenol, 3-methoxy-3-methylbutanol, 1,3-di-tert-butylbenzene, 2,4-dimethylheptan-1-ol, 2,4-dimethylheptan-2-one, methyl mercaptan, 3-hydroxy-2,2,4-trimethylpentyl isobutyrate, 4-methyl heptanone, 2-nonanol and trimethylamine.

The volatile compounds with disagreeable odours were most prevalent in strains SH 30-1, SH 28-3, SH 25-4 and *C. gleum*. For instance, 1-ethyl-2-hexanol which was the most detected compound is associated with odours such as, raw fish, green, and chalk (Tao et al. 2014;

Bartsch et al. 2016). Indole which originates from the degradation of the amino acid tryptophan, was the second most detected compound and it can be associated with faecal and nauseating odours (Schiffman et al. 2001). Dimethyl disulphide was also among the most detected compounds and was most prevalent in strains SH 25-4, SH 30-1 and SH 20-4. Odours such as cooked cabbage, foul, putrid and decayed vegetables were linked with sulphur containing compounds (Schiffman et al. 2001; Giri et al. 2010).

Although strains SH 30-1, SH 23-4 and SH 28-3 were classified as possible strains of *C. piscium* in terms of the 16S rRNA gene sequence analysis results and phylogenetic analysis, their differential characteristics in terms of the BIOLOG^{TM} Omnilog substrate oxidation, odour and volatile production by sensory and GC/MS analysis varied. Strains SH 28-3 and 23-4 were able to grow at 42 °C together with strain SH 11-4, and their ability to grow at high incubation temperatures makes them possible pathogens of fish and humans because *C. gleum*, which is a recognised pathogen, was also able to grow at 42 °C. Strain SH 23-4 was one of the isolates that metabolised most of the carbon sources when estimating in the BIOLOG^{TM} Omnilog system which may imply that this strain might be able to cause more pronounced spoilage than all the other strains evaluated in this study. For volatile compound detection, most volatile compounds with unpleasant odours were detected in FJB samples containing strains SH 30-1 and SH 28-3.

When comparing the seven strains that may be novel, SH 11-4(b) is the one that had exceptional characteristics. Strain SH 11-4(b) was amongst the three strains that were able to grow at 42 °C, together with *C. gleum.* The isolate also shared a characteristic with *C. piscium* by being able to grow at 5% NaCl inclusion levels. It was the only strain that was unable to grow on MacConkey agar and this is also linked with pathogenic potential. It also degraded 25/31 carbon sources together with SH 23-4 in the BIOLOG^{TM} Omnilog system. Overall, it can be concluded that strain SH 11-4(b) might have both pathogenic and spoilage potential.

When considering all the results obtained during this study, it can therefore be concluded that sensory analysis and GC/MS were therefore useful in estimating the spoilage potential of the *Chryseobacterium* species through odour production. The BIOLOG^{TM} system can be used as an effective screening method for identifying the carbon sources utilised by the *Chryseobacterium* species which could then be investigated further for their potential to produce food spoilage characteristics. *Chryseobacerium* species used in this study have the potential to spoil fish and/other fish products because of their psychrotolerant and proteolytic nature.

Future research may include:

- The use of a polyphasic approach to identify and describe the isolates identified in this study as possibly being novel species of the genus *Chryseobacterium*.
- Evaluate the novel *Chryseobacterium* strains for pathogenicity by determining a range of virulence factors as well as other characteristics such as resistance to antimicrobials and resistance to commercially available disinfectants.
- Further estimation of the spoilage potential by determining the effect of other parameters such as pH, water activity, oxygen concentration and nutrient content of the food on the growth kinetics of the *Chryseobacterium* strains.

CHAPTER 6

REFERENCES

Abiola C, Oyetayo VO (2016) Isolation and biochemical characterization of microorganisms associated with the fermentation of Kersting’s groundnut (*Macrotyloma geocarpum*). *Research Journal of Microbiology* 11: 47–55.

Aguilera-Arreola MG (2015) Identification and typing methods for the study of bacterial infections: a brief review and mycobacterial as case of study. *Archives in Clinical Microbiology* 7: 1. http:// www.acmicrob.com.

Amenduni A, Brattoli M, de Gennaro G, Massari F, Palmisani J, Tutino M (2016) Chemical characterization of odour active volatile organic compounds emitted from perfumes by GC/MS-O. *Environmental Engineering and Management Journal* 15(9): 1963–1969.

Amoore JE (1977) Specific anosmia and the concept of primary odors. *Chemical Senses* 2: 267–281.

Austin B Austin DA (1999) *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*, 3rd edition. Praxis Publishing, Chichester, UK, p 457.

Ayres JC, Mundt JO, Sandine WE (1980) *Microbiology of Foods*. WH Freeman and Company, San Francisco.

Badings HT, Van der Pol JJG, Neeter R (1981) Aroma compounds which contribute to the difference in flavour between pasteurized milk and UHT milk. In: *Flavour '81*, Schreier P (Ed.), Walter de Gruyter, New York, USA, pp 683–692.

Balraj M, Chaturvedi P, Sonam T (2011) Restriction Fragment Length Polymorphism. *Encyclopedia Elsevier*. Doi: 10.1016/B978-0-12-374984-0.01314-0.

Banwart GJ (1989) *Basic Food Microbiology*. Chapman and Hall: London.

Baron S (1996) Classification. In: *Medical Microbiology,* 4th edition. The University of Texas Medical Branch, Galveston.

Bartsch J, Uhde E, Salthammer T (2016) Analysis of odour compounds from scented consumer products using gas chromatography-mass spectrometry and gas chromatography-olfactometry. *Analytica Chimica Acta* 904: 98–106.

Beattie G (2006) Plant-associated bacteria: Survey, molecular phylogeny, genomics and recent advances. Doi: 10.1007/978-1-4020-4538-7_1.

Behrendt U, Ulrich A, Spröer C, Schumann P (2007) *Chryseobacterium luteum* sp. nov., associated with the phyllosphere of grasses. *Journal of Systematic and Applied Microbiology.* 57: 1881–1885.

Behrendt U, Ulrich A, Sproer C, Schumann P (2008) *Chryseobacterium gregarium* sp. nov., isolated from decaying plant material. *International Journal of Systematic and Evolutionary Microbiology* 58: 1069–1074.

Bekker A (2011) *Growth and Spoilage Characteristics of Chryseobacterium Species in Milk*. M.Sc. dissertation. University of the Free State: Bloemfontein, South Africa.

Bekker A, Steyn L, Charimba G, Jooste P, Hugo C (2015) Comparison of the growth kinetics and proteolytic activities of *Chryseobacterium* species and *Pseudomonas fluorescens. Canadian Journal of Microbiology* 61(12): 977–982.

Bekker A, Jooste P, Steyn L, Bothma C, Hugo A, Hugo C (2016) Lipid breakdown and sensory analysis of milk inoculated with *Chryseobacterium joostei* and *Pseudomonas fluorescens*. *International Dairy Journal* 52: 101–106.

Benmalek Y, Cayol J, Bouanane NA, Hacene H, Fauque G, Fardeau M (2010) *Chryseobacterium solincola* sp. nov, isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* 60: 1876–1880.

Bentz K, Wilber PG, Fitzgerald H, Peterson A (2018). Unit 7: Supportive, selective and differential media and streak isolation practice. In: *BIO 2110 Microbiology Course*, Central New Mexico Community College: Albuquerque, New Mexico. pp 1–15.

Bergey DH, Breed RS (1948) Genus III. *Flavobacterium*. In: *Bergey's Manual of Determinative Bacteriology*, 6th edition. Breed RS, Murray EGD, Hitchens AP (Eds). Bailliére, Tindall and Cox: London.

Bernardet J-F, Nakagawa Y (2006) An introduction to the Family Flavobacteriaceae. In: *The Prokaryotes*, 7th edition. Stackebrandt E, Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (Eds). Springer: Berlin, Germany, pp 455–480.

Bernardet J-F, Nakagawa Y, Holmes B (2002) Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Internation Journal of Systematic and Evolutionary Microbiology* 52: 1049–1070.

Bernardet J-F, Vancanneyt M, Matte-Tailliez O, Grizez L, Tailliez P, Bizet C, et al. (2005) Polyphasic study of *Chryseobacterium* strains isolated from diseased aquatic animals. *Journal of Systematic and Applied Microbiology* 28: 640–660.

Bernardet J-F, Hugo C.J, Bruun B (2006) The genera *Chryseobacterium* and *Elizabethkingia*. In: *The Prokaryotes,* 7th edition. Stackebrandt E, Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (Eds). Springer: Berlin, Germany, pp 638–676.

Bernardet J-F, Hugo CJ, Bruun B (2011) Genus X. *Chryseobacterium* Vandamme, Bernardet, Segers, Kersters and Holmes 1994, 829^{VP}. In: *Bergey's Manual of Systematic Bacteriology*, 2nd edition, Volume 4: The *Bacteroidetes*, *Spirochaetes*, *Tenericutes* (*Mollicutes*), *Acidobacteria*, *Fibrobacteres*, *Fusobacteria*, *Dictyoglomi*, *Gemmatimonadetes*, *Lentisphaerae*, *Verrucomicrobia*, *Chlamydiae*, and *Planctomycetes.* Krieg NR, Staley JT, Brown DR, Hedlund BP, Paster BJ, Ward NL, Ludwig W, Whitman WB (Eds). Springer: New York, pp 180–196.

Bertolino M, Dolci P, Giordano M, Rolle L, Zeppa G (2011) Evolution of chemico-physical characteristics during manufacture and ripening of Castelmagno PDO cheese in winter time. *Food Chemistry Journal* 129: 1001–1011.

Biolog (2008) Biolog User Guide http://www.biolog.com/pdf/Database_Listing_ver_6.01.pdf

Bisen PS (2014) Microbial taxonomy. In: *Microbes in Practise.* IK International Publishing House International: New Delhi, India.

Bisen PS, Debnath M, Prasad GBKS (2012) Identification and classification of microbes. In: *Microbes: Concepts and Applications,* 1st edition. John Wiley and Sons, Inc: London, pp 275–337. https://www.researchgate.net/publication/307107872

Bouchet V, Huot H, Goldstein R (2008) Molecular genetic basis of ribotyping. *Clinical Microbiology Reviews* 21(2): 262–273.

Braun P, Fehlhaber K, Kluga C, Kopp K (1999) Investigations into the activity of enzymes produced by spoilage-causing bacteria: a possible basis for improved shelf-life estimation. *Food Microbiology* 16: 531–540.

Busse HJ, Denner EBM, Lubitz W (1996) Classification and identification of bacteria: Current approaches to an old problem. Overview of methods used in bacterial systematics. *Journal of Biotechnology* 47: 3–7.

Campbell S, Harada RM, Li QX (2008) *Chryseobacterium arothri* sp. nov, isolated from the kidneys of a pufferfish. *International Journal of Systematic and Evolutionary Microbiology* 58: 290–293.

Campo M, Nute G, Wood J, Elmore S, Mottram D, Enser M (2003) Modelling the effect of fatty acids in odour development of cooked meat in vitro: Part I – Sensory perception. *Meat Science* 63: 367–375.

Cascio A, Stassi G, Costa GB, Crisafulli G, Rulli I, Ruggeri C, Iaria C (2005) *Chryseobacterium indologenes* bacteraemia in a diabetic child. *Journal of Medical Microbiology* 54: 677–680.

Chalupnicki M, Dittman D, Starliper CE, Iwanowicz DD (2015) Efficacy of iodine for disinfection of Lake Sturgeon eggs from the St. Lawrence River, New York. *North American Journal of Aquaculture* 77: 82–89.

Champagne CP, Laing RR, Roy D, Mafu AA, Griffiths MW (1994) Psychrotrophs in dairy products — their effects and their control. *Critical Reviews in Food Science and Nutrition* 34: 1–30.

Chandrasekaran M, Lakshmanaperumalsamy P, Chandramohan D (1985) Fish flesh agar medium - a suitable experimental medium for the detection of spoilage bacteria*. Antonie van Leeuwenhoek* 51: 219–225.

Charimba G, Jooste P, Albertyn J, Hugo C (2013*) Chryseobacterium carnipullorum* sp. nov., isolated from raw chicken. *International Journal of Systematic and Evolutionary Microbiology* 63: 3243–3249.

Chaudhary DK, Kim J (2017) *Chryseobacterium nepalense* sp. nov., isolated from oil-contaminated soil. *International Journal of Systematic and Evolutionary Microbiology* 67: 646–652.

Chen L, Daniel RM, Coolbear T (2003) Detection and impact of protease and lipase activities in milk and milk powders. *International Dairy Journal* 13(4): 255–275.

Chen L, Cai Y, Zhou G, Shi X, Su J (2014) Rapid Sanger Sequencing of the 16S rRNA gene for identification of some common pathogens. *PLoS ONE* 9(2): e88886. doi:10.1371/journal.pone.0088886.

Cheng J-H, Sun D-W (2014) Hyperspectral imaging as an effective tool for quality analysis and control of fish and other seafoods: Current research and potential application. *Trends in Food Science and Technology* 37: 78–91.

Cho J-C, Tiedje JM (2001) Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. *Applied and Environmental Microbiology* 67(8): 3677–3682.

Cho SH, Lee KS, Shin DS, Han JH, Park KS, Lee CH, et al. (2010) Four new species of *Chryseobacterium* from the rhizosphere of coastal sand dune plants, *Chryseobacterium elymi* sp. nov., *Chryseobacterium hagamense* sp. nov., *Chryseobacterium lathyri* sp. nov. and *Chryseobacterium rhizosphaerae* sp. nov. *Systematic and Applied Microbiology* **33**: 122–127.

Chojniak J, Wasilkowski D, Płaza G, Mrozik A, Brigmon R (2015) Application of BIOLOG microarrays techniques for characterization of functional diversity of microbial community in phenolic-contaminated water*. International Journal of Environmental Research* 9(3): 785–794.

Clarridge JE (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical and Microbiology Reviews* 17: 840–862.

Cousin MA (1982) Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. *Journal of Food Protection* 45: 172–207.

Cowan ST (1974) *Cowan and Steel's Manual for the Identification of Medical Bacteria* 2nd edition. University Press: Cambridge.

Czekajło KU, Giedrys-Kalemba S, Mędrala D (2006) Phenotypic and genotypic characteristics of *Pseudomonas aeruginosa* strains isolated from hospitals in the north-west region of Poland. *Polish Journal of Microbiology* 55: 103–112.

Dainty RH (1996) Chemical/biochemical detection of spoilage. *International Journal of Food Microbiology* 33: 19–33.

Dalgaard P (2000) Fresh and lightly preserved seafood. In: Shelf life evaluation of foods, 2nd edition, Man CD, Jones AA (Eds), Aspen Publishing Inc, Maryland, USA pp. 110–139.

De Beer H (2005) *A Taxonomic Study of Chryseobacterium Species in Meat*. Ph.D. thesis. University of the Free State: Bloemfontein, South Africa.

De Beer H, Hugo CJ, Jooste PJ, Willems A, Vancanneyt M, Coenye T, Vandamme PAR (2005) *Chryseobacterium vrystaatense* sp. nov., isolated from raw chicken in a chicken-processing plant. *International Journal of Systematic and Evolutionary Microbiology* 55: 2149–2153.

De Beer H, Hugo CJ, Jooste PJ, Vancanneyt M, Coenye T, Vandamme PAR (2006) *Chryseobacterium piscium* sp. nov., isolated from fish of the South Atlantic Ocean, South Africa. *International Journal of Systematic and Evolutionary Microbiology* 56: 1317–1322.

Demain AL, Jackson M, Trenner NR (1967) Thiamine-dependent accumulation of tetramethylpyrazine accompanying a mutation in the isoleucine-valine pathway. *Journal of Bacteriology* 94: 323–326.

Dilbaghi N, Sharma S (2007) Food spoilage, food infections and intoxications caused by microorganisms and methods for their detection. *Proceedings of Food and Industrial Microbiology,* pp 2–42.

Du J, Ngo HTT, Won K-H, Kim K-Y, Jin F-X, Yi T-H (2015) *Chryseobacterium solani* sp. nov., isolated from field-grown eggplant rhizosphere soil. *International Journal of Systematic and Evolutionary Microbiology* 65: 2372–2377.

Duflos G, Coin VM, Cornu M, Antinelli J-F, Malle P (2006) Determination of volatile compounds to characterize fish spoilage using headspace/mass spectrometry and solid-phase micro extraction/gas chromatography/mass spectrometry. *Journal of the Science of Food and Agriculture* 86: 600–611.

Engelbrecht K (1992) *Spoilage Bacteria Associated with Cape Hake (Merluccius capensins, Merluccius paradoxus) and Other Edible South Atlantic Species*. M.Sc. Dissertation. University of the Free State: Bloemfontein, South Africa.

Engelbrecht K, Jooste PJ, Prior BA (1996a) Quantitative and qualitative determination of the aerobic bacterial populations of Cape marine fish. *South African Journal of Food Science and Nutrition* 8: 60–65.

Engelbrecht K, Jooste PJ, Prior BA (1996b) Spoilage characteristics of Gram-negative genera and species isolated from Cape marine fish. *South African Journal of Food Science and Nutrition* 8, 66–71.

Euzéby JP (2019) *List if Prokaryotic Names with Standing in Nomenclature*. http://www.bacterio.net/chryseobacterium.html

Fan W, Qian MC (2006) Identification of aroma compounds in Chinese 'Yanghe Daqu' liquor by normal phase chromatography fractionation followed by gas chromatography/olfactometry. *Flavour and Fragrance Journal* 21: 333–342.

Felsenstein J (2004) Inferring phylogenies. Sunderland (MA): Sinauer Associates.

Forsythe SJ (2000). *The Microbiology of Safe Food*. Blackwell Science: London.

Fukami K, Ishiyama S, Yaguramaki H, Masuzawa T, Nabeta Y, Endo K, Shimoda M (2002) Identification of distinctive volatile compounds in fish sauce. *Journal of Agricultural and Food Chemistry* 50: 5412−5416.

Gallego V, García MT, Ventosa A (2006) *Chryseobacterium hispanicum* sp. nov., isolated from the drinking water distribution system of Sevilla, Spain. *International Journal of Systematic and Evolutionary Microbiology* 56: 1589–1592.

García-López M, Meier-Kolthoff JP, Tindall B, Gronow S, Woyke T, Kyrpides NC, Göker M (2019) Analysis of 1,000 type-strain genomes improves taxonomic classification of Bacteroidetes. *Frontiers in Microbiology* 10: 2083. DOI:10.3389/fmicb.2019.02083.

Gerhardt KO (1990) Gas chromatography-mass spectrometry. In: *Principles and Applications of Gas Chromatography in Food Analysis*. Ellis Horwood Series in Food Science and Technology. Gordon MH (Eds). Springer: Boston, MA. https://doi.org/10.1007/978-1-4613-0681-8_2.

German Agricultural Society (DLG) (2017) Panel training on odour and aroma perception for sensory analysis. *DLG Expert Report: Food Sensory Technology* 1: 1–24.

Ghaly AE, Dave D, Budge S, Brooks MS (2010) Fish spoilage mechanisms and preservation techniques: Review. *American Journal of Applied Sciences* 7: 859–877.

Gillis M, Vandamme P, De Vos P, Swings J, Kersters K (2001) Polyphasic taxonomy. In *Bergey's Manual of Systematic Bacteriology*, 2nd edition. Boone DR, Castenholz RW, Garrity GM. Springer: Berlin. pp. 43–48.

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. *Nature* 345: 60–63.

Giri A, Osako K, Ohshima T (2010) Identification and characterisation of headspace volatiles of fish miso, a Japanese fish meat based fermented paste, with special emphasis on effect of fish species and meat washing. *Food Chemistry* 120: 621–631.

González CJ, Santos JA, García-López ML, Otero A (2000) Psychrobacters and related bacteria in freshwater fish. *Journal of Food Protection* 63: 315–321.

Gram L, Huss HH (1996) Microbiological spoilage of fish and fish products. *International Journal of Food Microbiology* 33: 121–137.

Gram L, Dalgaard P (2002) Fish spoilage bacteria—problems and solutions. *Current Opinion in Biotechnology* 13: 262–266.

Gram L, Ravn L, Rasch M, Bruhn JB, Christensen AB, Givskov M (2002) Food spoilage – interactions between food spoilage bacteria. *International Journal of Food Microbiology* 78: 79–97.

Hahnke RL, Meier-Kolthoff JP, García-López M, Mukherjee S, Huntemann M, Ivanova NN, Woyke T, Kyrpides NC, Klenk H-P, Göker M (2016) Genome-based taxonomic classification of Bacteroidetes. *Frontiers in Microbiology* 7: 1–37.

Hall BG (2011) Phylogenetic trees made easy: a how-to manual. 4th edition. Sunderland (MA): Sinauer Associates.

Hall BG, Pikis A, Thompson J (2009) Evolution and biochemistry of family 4 glycosidases: implications for assigning enzyme function in sequence annotations. *Molecular Biology and Evolution* 26: 2487–2497.

Hantsis-Zacharov E, Halpern M (2007a) *Chryseobacterium haifense* sp. nov., a psychrotolerant bacterium isolated from raw milk. *International Journal of Systematic Evolutionary Microbiology* 57: 2344–2348.

Hantsis-Zacharov E, Halpern M (2007b). Culturable phsychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. *Applied and Environmental Microbiology*, 73: 7162–7168.

Hantsis-Zacharov E, Senderovich Y, Halpern M (2008a) *Chryseobacterium bovis* sp. nov., isolated from raw cow's milk. *International Journal of Systematic and Evolutionary Microbiology* 58: 1024–1028.

Hantsis-Zacharov E, Shaked T, Senderovich Y, Halpern M (2008b) *Chryseobacterium oranimense* sp. nov., a psychrotolerant, proteolytic and lipolytic bacterium isolated from raw cow's milk. *International Journal of Systematic and Evolutionary Microbiology* 58: 2635–2639.

Harrigan WF, McCance ME (1976) *Laboratory Methods in Food and Dairy Microbiology*. Academic Press Inc. Ltd.: London.

Harrison FC (1929) The discoloration of halibut. *Canadian Journal of Research* 1: 214–239.

Hassoun A, Karoui R (2017) Quality evaluation of fish and other seafood by traditional and non-destructive instrumental methods: Advantages and limitations. *Critical Reviews in Food Science and Nutrition* 57: 1976–1998. DOI: 10.1080/10408398.2015.1047926.

Hertzog P, Winkler I, Wolking D, Kämpfer P, Lipski A (2008) *Chryseobacterium ureilyticum* sp. nov., *Chryseobacterium gambrini* sp. nov., *Chryseobacterium pallidum* sp. nov. and *Chryseobacterium molle* sp. nov., isolated from beer-bottling plants. *International Journal of Systematic and Evolutionary Microbiology* 58: 26–33.

Holmes B and Owen RJ (1981) Emendation of the genus *Flavobacterium* and the status of the genus. Developments after the 8th edition of Bergey's Manual. In: *The Flavobacterium-*

Cytophaga Group, Gesellschaft für Biotechnologische Forschung Monograph Series No. 5. Reichenbach H and Weeks OB (Eds). Weinheim: Verlag Chemie. pp. 17–26.

Holmes B, Owen RJ, Steigerwalt AG, Brenner DJ (1984) *Flavobacterium gleum*, a new species found in human clinical specimens. *International Journal of Systematic Bacteriology* 34: 21–25.

Holmes B, Steigerwalt AG, Nicholson AC (2013) DNA–DNA hybridization study of strains of *Chryseobacterium, Elizabethkingia* and *Empedobacter* and of other usually indole producing non-fermenters of CDC groups IIc, IIe, IIh and IIi, mostly from human clinical sources, and proposals of *Chryseobacterium bernardetii* sp. nov., *Chryseobacterium carnis* sp. nov., *Chryseobacterium lactis* sp. nov., *Chryseobacterium nakagawai* sp. nov. and *Chryseobacterium taklimakanense* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 63: 4639–4662.

Hugo CJ, Jooste PJ (2003) Culture media for genera in the family *Flavobacteriaceae*. In: *Handbook of Culture Media for Food Microbiology*, 2nd edition. Corry JEL, Curtis GDW, Baird RM (Eds). Elsevier Science: Amsterdam. pp 355–367.

Hugo CJ, Jooste PJ, Segers P, Vancanneyt M, Kersters K (1999) A polyphasic taxonomic study of *Chryseobacterium* strains isolated from dairy sources. *Systematic and Applied Microbiology* 22: 586–595.

Hugo CJ, Segers P, Hoste B, Vancanneyt M, Kersters K (2003) *Chryseobacterium joostei* sp. nov., isolated from the dairy environment. *International Journal of Systematic and Evolutionary Microbiology* 53: 771–777.

Hugo C, Bernardet J-F, Nicholson A, Kämpfer P (2019) Genus *Chryseobacterium*. In: *Bergey's Manual of Systematics of Archaea and Bacteria*, Whitman WB, Bergeys Manual Trust. John Wiley & Sons, Inc., in association with Bergeys Manual Trust: Hoboken, New Jersey, pp 1–107. DOI: 10.1002/9781118960608.gbm00301.pub2.

Ilardi P, Avendaño-Herrera R (2008) Isolation of *Flavobacterium*-like bacteria from diseased salmonids cultured in Chile. *Bulletin of the European Association of Fish Pathology* 28: 176–185.

Ilardi P, Fernandez J, Avendano-Herrera R (2009) *Chryseobacterium piscicola* sp. nov., isolated from diseased salmonid fish. *International Journal of Systematic and Evolutionary Microbiology* 59: 3001–3005.

Ilardi P, Abad J, Rintamäki P, Bernardet J-F, Avendaño-Herrera R (2010) Phenotypic, serological and molecular evidence of *Chryseobacterium piscicola* in farmed Atlantic salmon, *Salmo salar* L., in Finland. *Journal of Fish Diseases* 33 (2): 179–181.

Jacobs A (2007) *Investigation and Comparison of Adherence- and Biofilm-Forming Capacities of Yellow-Pigmented Chryseobacterium, Elizabethkingia and Myroides spp. Isolated from South African Aquaculture Systems*. M.Sc. Dissertation. University of Stellenbosch: Stellenbosch, South Africa.

Jacoby GA (1964) The induction and repression of amino acid oxidation in *Pseudomonas fluorescens*. *The Biochemical Journal* 92(1): 1–8. doi:10.1042/bj0920001.

Jeong JJ, Lee DW, Park B, Sang MK, Choi IG, Kim KD (2017) *Chryseobacterium cucumeris* sp. nov., an endophyte isolated from cucumber (*Cucumis sativus* L.) root, and emended description of *Chryseobacterium arthrosphaerae*. *International Journal of Systematic and Evolutionary Microbiology* 67: 610–616.

Jesumirhewe C, Ogunlowo PO, Olley M, Springer B, Allerberger F, Ruppitsch W (2016) Accuracy of conventional identification methods used for Enterobacteriaceae isolates in three Nigerian hospitals. *Peer Journal* 4: e2511. doi:10.7717/peerj.2511.

Johnson JL (1991) DNA reassociation experiments. In: *Nucleic Acid Techniques in Bacterial Systematics*. Stackebrandt E and Goodfellow M (Eds). Chichester: Wiley. pp. 21–44.

Jooste PJ (1985) *The Taxonomy and Significance of Flavobacterium-Cytophaga Strains from Dairy Sources*. Ph.D. thesis, Bloemfontein: University of the Orange Free State.

Jooste PJ, Britz TJ and Lategan PM (1986a) The prevalence and significance of *Flavobacterium* strains in commercial salted butter. *Milchwissenschaft* 41: 69–73.

Jooste PJ, Hugo CJ (1999) The taxonomy, ecology and cultivation of bacterial genera belonging to the family *Flavobacteriaceae*. *International Journal of Food Microbiology* 53: 81–94.

Joshi M, Deshpande JD (2011) Polymerase chain reaction: Methods, principles and application. *International Journal of Biomedical Research* 1: 81–97.

Joung Y, Joh K (2011) *Chryseobacterium yonginense* sp. nov., isolated from mesotrophic artificial lake. *International Journal of Systematic and Evolutionary Microbiology*, 61: 1413–1417.

Kämpfer P (2010) The characterization of prokaryote strains for taxonomic purposes. *International Journal of Systematic and Evolutionary Microbiology* 60: 1–7.

Kämpfer P, Dreyer U, Neef A, Dott W, Busse HJ (2003) *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *International Journal of Systematic and Evolutionary Microbiology* 53: 93–97.

Kämpfer P, Vaneechoutte M, Lodders N, De Baere T, Avesani V, Janssens M, Busse H-J, Wauters G (2009) Description of *Chryseobacterium anthropi* sp. nov., to accommodate clinical isolates biochemically similar to *Kaistella koreensis* and *Chryseobacterium haifense*, proposal to reclassify *Kaistella koreensis* as *Chryseobacterium koreense* comb. nov. and emended description of the genus *Chryseobacterium*. *International Journal of Systematic and Evolutionary Microbiology* 59: 2421–2428.

Kämpfer P, Fallschissel K, Avenaño-Herrera R (2011) *Chryseobacterium chaponense* sp. nov., isolated from farmed Atlantic salmon (*Salmo salar*). *International Journal of Systematic and Evolutionary Microbiology* 61: 497–501.

Kämpfer P, McInroy JA, Glaeser SP (2014a) *Chryseobacterium zeae* sp. nov., *Chryseobacterium arachidis* sp. nov., and *Chryseobacterium geocarposphaerae* sp. nov., isolated from the rhizosphere environment. *Antonie van Leeuwenhoek* 105: 491–500.

Kämpfer P, Poppel MT, Wilharm G, Busse H-J, McInroy JA, Glaeser SP (2014b) *Chryseobacterium gallinarum* sp. nov., isolated from a chicken, and *Chryseobacterium contaminans* sp. nov., isolated as a contaminant from a rhizosphere sample. *International Journal of Systematic and Evolutionary Microbiology* 64: 1419–1427.

Kämpfer P, Busse H-J, McInroy JA, Glaeser SP (2015a) *Chryseobacterium arachidiradicis* sp. nov., isolated from the geocarposphere (soil around the peanut) of very immature peanuts (*Arachis hypogaea*). *International Journal of Systematic Evolutionary Microbiology* 65: 2179–2186.

Kämpfer P, Busse H-J, McInroy JA, Glaeser SP (2015b) *Chryseobacterium sediminis* sp. nov., isolated from a river sediment. *International Journal of Systematic Evolutionary Microbiology* 65: 4019–4024.

Kämpfer P, Mcinroy JA and Glaeser SP (2015c). *Chryseobacterium rhizoplanae* sp. nov., isolated from the rhizoplane environment. *Antonie van Leeuwenhoek* 107, 533-538.

Kämpfer P, Trček J, Skok B, Šorgo A, Glaeser SP (2015d) *Chryseobacterium limigenitum* sp. nov., isolated from a dehydrated sludge. *Antonie van Leeuwenhoek* 107: 1633–1638.

Kan H, Zhao F, Zhang XX, Ren H, Gao S (2015) Correlations of gut microbial community shift with hepatic damage and growth inhibition of *Carassius auratus* induced by pentachlorophenol exposure. *Environmental Science and Technology* 49: 11894–11902.

Kawai T (1996) Fish flavor*. Critical Reviews: Food Science and Nutrition* 36: 257–298.

Khatib RM, Veena, Konanavar N (2014) Modern identification methods of bacteria. *Research and Reviews: Journal of Agriculture and Allied Sciences* 3(3): 32–38.

Kim KK, Bae H-S, Schumann P, Lee S-T (2005a). *Chryseobacterium daecheongense* sp. nov., isolated from freshwater lake sediment. *International Journal of Systematic and Evolutionary Microbiology* 55: 133–138.

Kim KK, Kim MK, Lim JH, Park HY, Lee ST (2005b) Transfer of *Chryseobacterium meningosepticum* and *Chryseobacterium miricola* to *Elizabethkingia* gen. nov. as *Elizabethkingia meningoseptica* comb. nov. and *Elizabethkingia miricola* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 55: 1287–1293.

Kim KK, Lee KC, Oh HM, Lee JS (2008) *Chryseobacterium aquaticum* sp. nov., isolated from a water reservoir. *International Journal of Systematic and Evolutionary Microbiology* 58: 533–537.

Kim T, Kim M, Kang O, Jiang F, Chang X, Liu P, Zhang Y, Da X, Zheng CF, Ang C, Peng F (2016) *Chryseobacterium frigidum* sp. nov., isolated from high-Arctic tundra soil, and emended descriptions of *Chryseobacterium bernardetii* and *Chryseobacterium taklimakanense*. *International Journal of Systematic Evolutionary Microbiology* 66: 609–615.

Kirk KE, Hoffman JA, Smith KA, Strahan BL, Failor KC, Krebs KE, Gale AN, Do TD, Sontag TC, Batties AM, Mistiszyn K, Newman JD (2013) *Chryseobacterium angstadtii* sp. nov., isolated from a newt tank. *International Journal of Systematic Evolutionary Microbiology* 63: 4777–4783.

Koeleman JGM, Stoof J, Biesmans DJ, Savelkoul PHM, Vandenbroucke-Grauls CJE (1998) Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii. Journal of Clinical Microbiology* 36(9): 2522–2529.

Kook M, Son H-M, Ngo HTT, YI T-H (2014) *Chryseobacterium camelliae* sp. nov., isolated from green tea. *International Journal of Systematic Evolutionary Microbiology* 64: 851–857.

Krechel A, Faupel A, Hallmann J, Ulrich A, Berg G (2002) Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode Meloidogyne incognita (Kofoid & White) Chitwood. *Canadian Journal of Microbiology* 48: 772–786.

Krüsemann EMJ, Wenng FM, Pennings JLA, de Graaf KT, Alhout R, Boesveldt S (2019) Sensory evaluation of e-liquid flavours by smelling and vaping yields similar results. *Nicotine and Tobacco Research* 2019: 1–8.

Kuley E, Durmus M, Balikci E, Ucar Y, Joe M, Özoğul F, Joe M (2017) Fish spoilage bacterial growth and their biogenic amine accumulation: Inhibitory effects of olive by-products. *International Journal of Food Properties* 20(5): 1029–1043. https://doi.org/10.1080/10942912.2016.1193516

Kumar S, Stecher G, Tamura K (2016) Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870–1874.

Land M, Hauser L, Jun S-R, Nookaew I, Leuze MR, Ahn T-H, Karpinets T, Lund O, Kora G, Wassenaar T, Poudel S, Ussery DW (2015) Insights from 20 years of bacterial genome sequencing. *Functional and Integrative Genomics* 15: 141–161. DOI: 10.1007/s10142-015-0433-4.

Lane DJ, Pace BG, Olsen J, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proceedings of the National Academy of Sciences of the United States of America* 82: 6955–6959.

Leduc F, Tournayre P, Kondjoyan N, Mercier F, Malle P, Kol O, Berdagué JL, Duflos G (2012) Evolution of volatile odorous compounds during the storage of European seabass (*Dicentrarchus labrax*). *Food Chemistry* 131: 1304–1311.

Lee K, Lee HK, Choi TH, Cho JC (2007) *Sejongia marina* sp. nov., isolated from Antarctic seawater. *International Journal of Systematic and Evolutionary Microbiology* 57: 2917–2921.

Li Z, Zhu H (2012) *Chryseobacterium vietnamense* sp. nov., isolated from forest soil. *International Journal of Systematic and Evolutionary Microbiology* 62: 827–831.

Li C, Wu J, Li Y, Dai Z (2013) Identification of the aroma compounds in stinky mandarin fish (*Siniperca chuatsi*) and comparison of volatiles during fermentation and storage. *International Journal of Food Science and Technology* 48: 2429–2437.

Li Z, Dong L, Jeon J, Kwon SY, Zhao C, Baek HH (2019) Characterization and evaluation of aroma quality in doubanjiang, a chinese traditional fermented red pepper paste, using aroma extract dilution analysis and a sensory profile. *Molecules* 24(17): 3107. doi:10.3390/molecules24173107.

Lin SY, Hameed A, Liu YC, Hsu YH, Hsieh YT, Lai WA, Young CC (2017) *Chryseobacterium endophyticum* sp. nov., isolated from a maize leaf. *International Journal of Systematic Evolutionary Microbiology* 67: 570–575.

Lo CI, Sankar SA, Mediannikov O, Ehounoud CB, Labas N, Faye N, Raoult D, Fournier P-E, Fenollar F (2016) High-quality genome sequence and description of *Chryseobacterium senegalense* sp. nov. *New Microbe and New Infections* 10: 93–100.

Loch TP, Faisal M (2014) *Chryseobacterium aahli* sp. nov., isolated from lake trout (*Salvelinus namaycush*) and brown trout (*Salmo trutta*), and emended descriptions of *Chryseobacterium ginsenosidimutans* and *Chryseobacterium gregarium*. *International Journal of Systematic and Evolutionary Microbiology* 64: 1573–1579.

Longo MA, Sanromán MA (2006) Production of food aroma compounds: Microbial and enzymatic methodologies. *Food Technology and Biotechnology* 44(3): 335–353.

Luo T, Liu Y, Chen C, Luo Q, Rao Q, Huang M, Weng B (2018) *Chryseobacterium aurantiacum* sp. nov., isolated from a freshwater pond used for Murray cod (*Maccullochella peelii peelii*) culture. *International Journal of Systematic and Evolutionary Microbiology* 68: 3397–3403.

Macé S, Cardinal M, Jaffrès E, Cornet J, Lalanne V, Frédérique Chevalier F, Sérot T, Pilet M-F, Dousset X, Joffraud J-J (2014) Evaluation of the spoilage potential of bacteria isolated from spoiled cooked whole tropical shrimp (*Penaeus vannamei*) stored under modified atmosphere packaging. *Food Microbiology* 40: 9–17.

MacFaddin JF (1980) *Biochemical Tests for Identification of Medical Bacteria*, 2nd edition. Williams and Wilkins Company: Baltimore.

Madigan MT, Martinko JM, Parker J (2000) *Brock Biology of Microorganisms*, 9th edition. Prentice Hall International: New Jersey

Marsili RT (1999) SPME-MS-MVA as an electronic nose for the study of off-flavors in milk. *Journal of Agricultural and Food Chemistry* 47: 648–654.

Maslow JN, Mulligan ME, Arbeit RD (1993) Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. *Clinical Infections and Diseases* 17: 153–164.

Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 14: 60 (2013). DOI:10.1186/1471-2105-14-60.

Mielmann A (2006) *Food Spoilage Characteristics of Chryseobacterium Species*. M.Sc. Dissertation. University of the Free State: Bloemfontein, South Africa.

Miller MJ, Biddle JW, Quenzer VK, McLaughlin JC (1993) Evaluation of Biolog for identification of members of the family Micrococcaceae. *Journal of Clinical Microbiology* 31(12): 3170–3173.

Mohamed HN, Man YC, Mustafa S, Manap YA (2012) Tentative identification of volatile flavor compounds in commercial Budu, a malaysian fish sauce, using GC-MS. *Molecules* 17: 5062–5080.

Montero-Calasanz MC, Göker M, Rohde M, Spröer C, Schumann P, Busse H-J, et al. (2013) *Chryseobacterium hispalense* sp. nov., a plant-growth-promoting bacterium isolated from a rainwater pond in an olive plant nursery, and emended descriptions of *Chryseobacterium defluvii*, *Chryseobacterium indologenes*, *Chryseobacterium wanjuense* and *Chryseobacterium gregarium*. *International Journal of Systematic and Evolutionary Microbiology* 63: 4386–4395.

Montero-Calasanz MC, Göker M, Rohde M, Spröer C, Schumann P, Busse H-J, Schmid M, Klenk H-P, Tindall BJ, Camacho M (2014) *Chryseobacterium oleae* sp. nov., an efficient plant growth promoting bacterium in the rooting induction of olive tree (*Olea europaea* L.) cuttings and emended descriptions of the genus *Chryseobacterium*, *C. daecheongense*, *C. gambrini*, *C. gleum*, *C. joostei*, *C. jejuense*, *C. luteum*, *C. shigense*, *C. taiwanense*, *C. ureilyticum* and *C. vrystaatense*. *Systematic and Applied Microbiology* 37: 342–350.

Morgan M, Boyette M, Goforth C, Sperry K, Greene S (2009) Comparison of the Biolog OmniLog Identification System and 16S ribosomal RNA gene sequencing for accuracy in identification of atypical bacteria of clinical origin. *Journal of Microbiological Methods* 79(3): 336–343.

Mossell DAA (1983) Essentials and perspectives of the microbial ecology of foods In: *Food Microbiology*: *Advances and Prospects*, Roberts TA, Skinner FA (Eds). Academic Press: New York. pp.1–25.

Mudarris M, Austin B, Segers P, Vancanneyt M, Hoste B, Bernardet J-F (1994) *Flavobacterium scophthalmum* sp. nov., a pathogen of turbot (*Scophthalmus maximus* L.). *International Journal of Systematic Bacteriology* 44(3): 447–453.

Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press: New York.

Nguyen NL, Kim YJ, Hoang VA, Yang DC (2013) *Chryseobacterium ginsengisoli* sp. nov., isolated from the rhizosphere of ginseng, and an emended description of *C. gleum*. *International Journal of Systematic and Evolutionary Microbiology* 63: 2975–2980.

Niswati A, Murase J, Kimura M (2005) Comparison of bacterial communities associated with microcrustaceans from the floodwater of a paddy field microcosm: estimation based on DGGE pattern and sequence analyses. *Soil Science and Plant Nutrition* 51: 289–290.

Nychas GJE, Drosinos EH, Board RG (1998) Chemical changes in stored meat. In *The Microbiology of Meat and Poultry*, Davies A, Board R (Eds). Blackie Academic and Professional: London. pp. 288–326.

Nychas GE, Skandamis PN, Tassou CC, Koutsoumanis KP (2008) Meat spoilage during distribution. *Meat Science* 78: 77–89.

ORA Laboratory Manual (FDA) (2013) Sensory analysis*. Orientation and Training* 4: 1–15.

Sharma-Kuinkel BK, Rude TH, Fowler VG Jr (2016) Pulse Field Gel Electrophoresis. *Methods in Molecular Biology* 1373: 117–130.

Park MS, Jung SR, Lee KH, Lee MS, Do JO, Kim SB, Bae KS (2006) *Chryseobacterium soldanellicola* sp. nov. and *Chryseobacterium taeanense* sp. nov., isolated from roots of sand-dune plants. *International Journal of Systematic and Evolutionary Microbiology* 56: 433–438.

Park SC, Kim MS, Baik KS, Kim EM, Rhee MS, Seong CN (2008) *Chryseobacterium aquifrigidense* sp. nov., isolated from a water-cooling system. *International Journal of Systematic and Evolutionary Microbiology* 58: 607–611.

Park SJ, Choi JH, Cha CJ (2013) *Chryseobacterium rigui* sp. nov., isolated from an estuarine wetland. *International Journal of Systematic and Evolutionary Microbiology* 63: 1062–1067.

Parlapani FF, Mallouchos A, Haroutounian SA, Boziaris LS (2014) Microbiological spoilage and investigation of volatile profile during storage of sea bream fillets under various conditions. *International Journal of Food Microbiology* 189: 153–163.

Pires C, Carvalho MF, De Marco P, Magan N, Castro PML (2010) *Chryseobacterium palustre* sp. nov., and *Chryseobacterium humi* sp. nov., isolated from industrially contaminated sediments. *International Journal of Systematic and Evolutionary Microbiology* 60: 402–407.

Pot B, Vandamme P, Kersters K (1994) Analysis of electrophoretic whole-organism protein fingerprints. In: *Chemical Methods in Prokaryotic Systematics*. Goodfellow M, O’Donnell AG (Eds). John Wiley and Sons: Chichester, U.K. pp 494–521.

Prakash O, Verma M, Sharma P, Kumar M, Kumari K, Singh A, Kumari H, Jit S, Gupta SK, Khanna M, Lal R (2007) Polyphasic approach of bacterial classification – An overview of recent advances. *Indian Journal of Microbiology* 47: 98–108.

Pridgeon JW, Klesius PH, Garcia JC (2012) Identification and virulence of *Chryseobacterium indologenes* isolated from diseased yellow perch (*Perca flavescens*). *Journal of Applied Microbiology* 114: 636–643.

Quan ZX, Kim KK, Kim MK, Jin L, Lee ST (2007) *Chryseobacterium caeni* sp. nov., isolated from bioreactor sludge. *International Journal of Systematic and Evolutionary Microbiology* 57: 141–145.

Queiroga RRE, Neta MTS, Sandes RDD, Narain N, Galvão M, Madruga MS, Costa RG (2019) An insight in key volatile compounds in goat milk based on their odor active values. *Journal of Food Science and Nutrition Research* 2(1): 49–60.

Raveendran S, Parameswaran B, Ummalyma SB, Abraham A, Mathew AK, Madhavan A, Pandey A (2018) Applications of microbial enzymes in food industry. *Food Technology and Biotechnology* 56(1): 16–30. doi:10.17113/ftb.56.01.18.5491.

Rawat S (2015) Food Spoilage: Microorganisms and their prevention. *Asian Journal of Plant Science and Research* 5(4): 47–56.

Regnault B, Grimont F, Grimont PA (1997) Universal ribotyping method using a chemically labelled oligonucleotide probe mixture. *Research in Microbiology* 148: 649–659.

Reichenbach H (1992) *Flavobacteriaceae* fam. nov. In *Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the IJSB*, List no. 41. *International Journal of Systematic Bacteriology* 42: 327–329.

Richard C, Kiredjian M (1995) *Laboratory Methods for the Identification of Strictly Aerobic Bacilli*. Intstitut Pasteur: Paris.

Ringø E, Sperstad S, Myklebust R, Refstie S, Krogdahl Å (2006) Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.): The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture* 261: 829–834.

Rochat T, Pérez-Pascual D, Nilsen H, Carpentier M, Bridel S, Bernardet J-F, Duchaud E (2019) Identification of a novel elastin-degrading enzyme from the fish pathogen *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology* 85(6): e02535-18. DOI**:** 10.1128/AEM.02535-18.

Roy K, Dey S, Uddin K, Barua R, Hossain T (2018) Extracellular pectinase from a novel bacterium *Chryseobacterium indologenes* strain SD and its application in fruit juice clarification*. Enzyme Research* pp 1–7.

Sandle T, Leavy C, Jindal H, Rhodes R (2014) Application of rapid microbiological methods for the risk assessment of controlled biopharmaceutical environments. *Journal of Applied Microbiology* 116: 1495–1505.

Sang MK, Kim HS, Myung IS, Ryu CM, Kim BS, Kim KD (2013) *Chryseobacterium kwangjuense* sp. nov., isolated from pepper (*Capsicum annuum* L.) root. *International journal of Systematic Evolutionary Microbiology* 63: 2835–2840.

Sarnoski P, O'Keefe S, Jahncke M, Mallikarjunan P, Flick G (2010) Analysis of crab meat volatiles as possible spoilage indicators for blue crab (*Callinectes sapidus*) meat by gas chromatography–mass spectrometry. *Food Chemistry* 122: 930–935.

Schiffman SS, Bennett JL, Raymer JH (2001) Quantification of odors and odorants from swine operations in North Carolina. *Agricultural and Forest Meteorology* 108: 213–240.

Senthilraj R, Ganduri P, Kunchithapatham J (2016) Sequence-based identification of microbial contaminants in non-parenteral products. *Brazilian Journal of Pharmaceutical Sciences* 52: 329–336.

Shen FT, Kämpfer P, Young CC, Lai WA, Arun AB (2005) *Chryseobacterium taichungense* sp. nov., isolated from contaminated soil. *International Journal of Systematic and Evolutionary Microbiology* 55: 1301–1304.

Shimomura K, Kaji S, Hiraishi A (2005) *Chryseobacterium shigense* sp. nov., a yellow-pigmented aerobic bacterium isolated from a lactic acid beverage. *International Journal of Systematic and Evolutionary Microbiology* 55: 1903–1906.

Sneath PHA, Sokal RR (1973) *Numerical Taxonomy*. Freeman WH: San Francisco.

Sneddon J, Masuram S, Richert JC (2007) Gas chromatography-mass spectrometry-basic principles, instrumentation and selected applications for detection of organic compounds. *Analytical Letters* 40(6): 1003–1012. DOI: 10.1080/00032710701300648.

Stackebrandt E, Ebers J (2006) Taxonomic parameters revisited: tarnished gold standards. *Microbiology Today* 33: 152–155.

Stackebrandt E, Goebel BM (1994) Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA gene sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology* 44: 846–849.

Stoeckel M, Lidolt M, Achberger V (2016). Growth of *Pseudomonas weihenstephanensis*, *Pseudomonas proteolytica* and *Pseudomonas* sp. in raw milk: impact of residual heat-stable enzyme activity on stability of UHT milk during shelf-life. *International Dairy Journal* 59: 20–28.

Strahan BL, Failor KC, Batties AM, Hayes PS, Cicconi KM, Mason CT, Newman JD (2011) *Chryseobacterium piperi* sp. nov., isolated from a fresh water creek. *International Journal of Systematic and Evolutionary Microbiology* 61: 2162–2166.

Suzina NE, Duda VI, Esikova TZ, Shorokhova AP, Gafarov AB, Olěinikov RR et al. (2011) Novel ultramicro bacteria, strains NF4 and NF5, of the genus *Chryseobacterium*: Facultative epibionts of *Bacillus subtilis*. *Mikrobiologiia* 80: 535–548.

Szoboszlay S, Atzél B, Kukolya, J, Tóth EM, Márialigeti K, Schumann P, Kritszt B (2008) *Chryseobacterium hungaricum* sp. nov., isolated from hydrocarbon-contaminated soil. *International Journal of Systematic and Evolutionary Microbiology* 58: 2748–2754.

Tai CJ, Kuo HP, Lee FL, Chen HK, Yokota A, Lo CC (2006) *Chryseobacterium taiwanense* sp. nov., isolated from soil in Taiwan. *International Journal of Systematic and Evolutionary Microbiology* 56: 1771–1776.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596–1599.

Tao N-P, Wu R, Zhou P-G, Gu S-Q, Wu W (2014) Characterization of odor-active compounds in cooked meat of farmed obscure puffer (*Takifugu obscurus*) using gas chromatography-mass-spectrometry olfactometry. *Journal of Food and Drug Analysis* 22: 431–438.

Tindall BJ, Rossello-Mora R, Busse H-J, Ludwig W, Kämpfer P (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. *International Journal of Systematic and Evolutionary Microbiology* 60: 249–266.

Truu J, Talpsep E, Heinaru E, Stottmeister U, Wand H, Heinaru A (1999) Comparison of API 20NE and Biolog GN identification systems assessed by techniques of multivariate analyses. *Journal of Microbiological Methods* 36: 193–201.

Tsôeu LI, Jooste PJ, Charimba G, Hugo CJ (2016) Spoilage potential of a novel group of bacteria isolated from dairy products. *South African Journal of Science* 112(1/2): 8 pages. http://dx.doi.org/10.17159/sajs.2016/20150227

Vandamme P, Bernardet J-F, Segers P, Kersters K, Holmes B (1994) New perspectives in the classification of the flavobacteria: Description of *Chryseobacterium* gen. nov., *Bergeyella* gen.

nov., and *Empedobacter* nom. rev. *International Journal of Systematic and Evolutionary Microbiology* 44(4): 827–831.

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiology Reviews* 60: 407–438.

Vaneechoutte M, Kämpfer P, de Baere T, Avesani V, Janssens M, Wauters G (2007) *Chryseobacterium hominis* sp. nov., to accommodate clinical isolates biochemically similar to CDC groups II-h and II-c. *International Journal of Systematic and Evolutionary Microbiology*, 57: 2623–2628.

Variet V, Knockaert C, Prost C, Serot T (2006). Comparison of odor-active volatile compounds of fresh and smoked salmon. *Journal of Agricultural and Food Chemistry* 54: 3391–3401.

Veciana-Nogués MT, Mariné-Font A, Vidal-Carou MC (1997) Biogenic amines as hygienic quality indicators of tuna, relationships with microbial counts, ATP-related compounds, volatile amines, and organoleptic changes. *Journal of Agricultural and Food Chemistry* 45: 2036–2041.

Vences M, Dohrmann AB, Künzel S, Granzow S, Baines JF, Tebbe CC (2015) Composition and variation of the skin microbiota in sympatric species of European newts (*Salamandridae*). *Amphibia-Reptilia* 36: 5–12.

Venil CK, Nordin N, Zakaria ZA, Ahmad WA (2014) *Chryseobacterium artocarpi* sp. nov., isolated from the rhizosphere soil of *Artocarpus integer*. *International Journal of Systematic and Evolutionary Microbiology* 64:3153–3159.

Venter H, Osthoff G, Litthauer D (1999) Purification and characterization of a metalloprotease from *Chryseobacterium indologenes* Ix9a and determination of the amino acid specificity with electrospray mass spectrometry. *Protein Expression and Purification* 15: 282–295.

Venugopal V (1990) Extracellular proteases of contaminant bacteria in fish spoilage: A review. *Journal of Food Protection* 53(4): 341–350.

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Methods in Molecular and Cell Biology* 5, 25–40.

Vithanage NR, Dissanayake M, Bolge G (2016) Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential. *International Dairy Journal* 57: 80–90.

Wang B, Xu S (2009) Effects of different commercial lipases on the volatile profile of lipolyzed milk fat. *Flavour and Fragrance Journal* 24: 335–340.

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky L, Moore LH, Moore WC, Murray RGE et al. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology* 37: 463–464.

Wekell JC, Barnett H (1991) New method for analysis of trimethylamine oxide using ferrous sulphate and EDTA. *Journal of Food Science* 56: 132–138.

Weon HY, Kim BY, Yoo SH, Kwon SW, Cho YH, Go SJ, Stackebrandt E (2006) *Chryseobacterium wanjuense* sp. nov., isolated from greenhouse soil in Korea. *International Journal of Systematic and Evolutionary Microbiology* 56: 1501–1504.

Weon HY, Kim BY, Yoo SH, Kwon SW, Stackebrandt E, Go SJ (2008) *Chryseobacterium soli* sp. nov., and *Chryseobacterium jejuense* sp. nov., isolated from soil samples from Jeju, Korea. *International Journal of Systematic and Evolutionary Microbiology* 58: 470–473.

Winters AD, Marsh TL, Brenden TO, Faisal M (2015) Analysis of bacterial communities associated with the benthic amphipod Diporeiain the Laurentian Great Lakes Basin. *Canadian Journal of Microbiology* 61: 72–81.

Williams R (1989). Restriction fragment length polymorphism (RFLP). *American Journal of Physical Anthropology* 32: 159–184. 10.1002/ajpa.1330320508

Wu Y-F, Wu Q-L, Liu S-J (2013) *Chryseobacterium taihuense* sp. nov., isolated from a eutrophic lake, and emended descriptions of the genus *Chryseobacterium*, *Chryseobacterium taiwanense, Chryseobacterium jejuense* and *Chryseobacterium indoltheticum. International Journal of Systematic and Evolutionary Microbiology* 63: 913–919.

Yabuuchi E, Kaneko T, Yano I, Moss CW, Miyoshi N (1983) *Sphingobacterium* gen. nov., *Sphingobacterium spiritivorum* comb. nov., *Sphingobacterium multivorum* comb. nov., *Sphingobacterium mizutae* sp. nov., and *Flavobacterium indologenes* sp. nov.: glucose-nonfermenting gram-negative rods in CDC groups IIk-2 and IIb. *International Journal of Systematic Bacteriology* 33: 580–598.

Yamaguchi S, Yokoe M (2000) A novel protein-deamidating enzyme from *Chryseobacterium proteolyticum* sp. nov., a newly isolated bacterium from soil. *Applied and Environmental Microbiology* 66: 3337–3343.

Yang F, Liu H, Zhang R, Chen D, Wang X, Li S, Hong Q (2015) *Chryseobacterium shandongense* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* 65: 1860–1865.

Yassin AF, Hupfer H, Siering C, Busse HJ (2010) *Chryseobacterium treverense* sp. nov., isolated from a human clinical source. *International Journal of Systematic and Evolutionary Microbiology* 60: 1993–1998.

Yasushura A, Shibamoto T (1995) Quantitative analysis of volatile aldehydes formed from various kinds offishflesh during heat treatment. *Journal of Agriculture and Food Chemistry* 43: 94–97.

Yoon JH, Kang SJ, Oh TK (2007) *Chryseobacterium daeguense* sp. nov., isolated from wastewater of a textile dye works. *International Journal of Systematic and Evolutionary Microbiology* 57: 1355–1359.

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J (2017) Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology* 67: 1613–1617.

Young CC, Kämpfer P, Shen FT, Lai WA, Arun AB (2005) *Chryseobacterium formosense* sp. nov., isolated from the rhizosphere of *Lactuca sativa* L. (garden lettuce). *International Journal of Systematic and Evolutionary Microbiology* 55: 423–426.

Yuan L, Sadiq FA, Liu T J, Li Y, Gu J S, Yang HY, He GQ (2018). Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes. *Journal of Zhejiang University. Science B*, *19*(8): 630–642.

Yu J, Ward O (1996) Investigation of the biodegradation of pentachlorophenol by the predominant bacterial strains in a mixed culture. *International Biodeterioration and Biodegradation* 5: 181–187.

Zamora L, Fernández-Garayzábal JF, Palacios MA, Sánchez-Porro C, Svensson-Stadler LA, DomÍnguez L, Moore ERB, Ventosa A, Vela AI (2012a) *Chryseobacterium oncorhynchi* sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*). *Systematic and Applied Microbiology* 35: 24–29.

Zamora L, Vela AI, Palacios MA, Domínguez L, Fernández-Garayzábal JF (2012b) First isolation and characterization of *Chryseobacterium shigense* from rainbow trout. *BMC Veterinary Research* 8(1): 77.

Zamora L, Vela AI, Palacios MA, Sánchez-Porro C, Moore ERB, Domínguez, L, Ventosa A, Fernández-garayzábal JF (2012c) *Chryseobacterium tructae* sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*). *Systematic and Applied Microbiology* 35: 315–319.

Zamora L, Vela AI, Palacios MA, Sánchez-Porro C, Svensson-Stadler LA, Domínguez L (2012d) *Chryseobacterium viscerum* sp. nov., isolated from diseased fish. *International Journal of Systematic and Evolutionary Microbiology* 62(12): 2934–2940.

Zhang YX, Zou AH, Manchu RG, Zhou YC, Wang SF (2008) Purification and antimicrobial activity of antimicrobial protein from brown-spott grouper, *Epinephelus fario*. *Zoological Research* 29: 627–632.

Zhao R, Chen XY, Li XD, Chen ZN, Li YH (2015) *Chryseobacterium takakiae* sp. nov., a member of the phylum Bacteroidetes isolated from *Takakia iepidozioides*. *International Journal of Systematic and Evolutionary Microbiology* 65: 71–76.

Zhao Z, Tu Y-Q, Shen X, Han S-B, Zhang C-Y, Sun C, Wu M (2017) *Chryseobacterium lineare* sp. nov., isolated from limpid stream. *International Journal of Systematic and Evolutionary Microbiology* 67: 800–805.

Zhou Y, Dong J, Wang X, Huan, X, Zhang KY, Zhang YQ, Guo YF, Lai R, Li WJ (2007) *Chryseobacterium flavum* sp. nov., isolated from polluted soil. *International Journal of Systematic and Evolutionary Microbiology* 57: 1765–1769.