UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in

Scienze Molecolari e Biotecnologie Agrarie, Alimentari ed Ambientali

Molecular Sciences and Plant, Food and Environmental Biotechnology

Dottorato di Ricerca in

Chimica, Biochimica ed Ecologia degli Antiparassitari (XXVIII ciclo)

Chemistry, Biochemistry and Ecology of Pesticides

Exploring Bacterial Molecular Factors in the Symbiotic Interactions with Insects

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Academic Year 2014/2015

Contents

Abstract

Many of the cellular mechanisms underlying host responses to pathogens have been well conserved during evolution. Homeostatic interactions between insects and commensal microbes are widespread in nature. Commensal microbes have many roles in the biology and lifecycle of most insect species, affecting different aspects of their life. The recently recognized acetic acid bacteria (AAB) are the most abundant microorganisms in the insects and also metabolically linked to one another. These symbionts establish close interactions with their animal host, including insects and mosquitoes. In particular, gut microbiota establishes strict interactions with its host and, indeed, close interactions are established among the different microbial partners. Fine regulation of the immunity in the host gut is required for homeostasis of gut microbiota. A recent study highlighted the role of bacterium-secreted uracil as a signal molecule controlling immunity in the gut of flies**.** Moreover, diversity of microbial symbionts suggests that synergistic activity in their role is favored. This gut bacterial symbionts have a role in the fitness of insects that are vectors of the most severe diseases in plant, animal and human affecting the agricultural production and the environment. The mosquitoes *Anopheles* are vectors of *Plasmodium* parasites, the causative agents of malaria. *Asaia* is a useful model for the study of promising tools in the control of disease-transmitting mosquitoes like *Anopheles* and AAB symbionts.

The aim of my Ph.D. research work was to explore the molecular factors of acetic acid bacteria (AAB) involved in the interaction with the host in order to unveil targets addressable for novel strategies of pathogen biocontrol. The purpose was challenged by a comparative proteomic approach considering *Asaia* SF2.1 as a model AAB-symbiont. Since this approach is based on the analysis of two-dimensional electrophoresis (2D-PAGE) protein profiles, it requires a consistent amount of bacterial-specific protein that cannot be achieved in the gut environment. To this end, hypoxia was applied as a model condition eliciting or suppressing pathways possibly involved in the symbiotic

interaction. Hypoxia condition was applied to mimic the insect gut condition, where the oxygen concentration is usually low from 2% to 8%.

Microbial population of model host insects (*Drosophyla suzukii* and *Anopheles stephensi*) was characterized by using 16S rRNA and ITS-PCR. The analysis showed that *Asaia* SF2.1 is among the most abundant acetic acid bacteria (AAB) species. Most abundant AAB species were selected from each genus (i.e. *Gluconobacter*, *Acetobacter, Komagataeibacter* and *Asaia*) and the biomass production was defined. Furthermore, for all strains, conditions for protein extraction methods were optimized followed by protein quality assessment and quantification. *Asaia* SF2.1 was selected for comparative proteomic approach based on the availability of genome sequence, protein quality and quantity. Different sample preparation methods were used for the setup of protein extraction. The best method for protein extraction was selected and was followed by optimization of the 2D-PAGE run conditions.

Using bioreactor, hypoxia (6%) and normal (20%) condition (control) of *Asaia SF2.1* were setup and growth data evaluated. 2D-PAGE protein profiles of more than 220 spots were obtained by applying the optimized protocol. Total 12 protein spots with altered expression differences were observed and the corresponding proteins were identified by mass spectrometry technique. MS analysis identified different proteins from the selected spots and functional categorization of proteins was carried out according to the annotated information. Protein spots were mostly identified as being involved in transcription, cellular respiratory function, cell wall biogenesis, protein biosynthesis, pentose-phosphate shunt. Gene ontology analysis suggested interesting putative candidate pathways involved in endosymbiosis, thus paving targets for biocontrol strategies.

Aim of the work

Homeostatic interactions between insects and commensal microbes are widespread in nature. Commensal microbes have many roles in the biology and lifecycle of most insect species, affecting different aspects of their life. Recently recognized prokaryotes of acetic acid bacteria (AAB), genera *Gluconobacter*, *Acetobacter, Komagataeibacter* and *Asaia*, the most abundant microorganisms in the insects and also metabolically linked to one another. In this last recent years, the microbiota associated to insects, and the mechanisms determining these associations, are under investigation. In particular, gut microbiota symbionts have a role in the fitness of insects that are vectors of the most severe diseases in plant, animal and human affecting the agricultural production and the environment.

The gut microbiota establishes strict interactions with its host and, indeed, close interactions are established among the different microbial partners. Fine regulation of the immunity in the host gut is required for homeostasis of gut microbiota. A recent study highlighted the role of bacterium-secreted uracil as a signal molecule controlling immunity in the gut of flies**.** Moreover, diversity of microbial symbionts suggests that synergistic activity in their role is favored. The mosquitoes *Anopheles* are vectors of *Plasmodium* parasites, the causative agents of malaria. Promising tools in the control of disease-transmitting mosquitoes like *Anopheles* and AAB symbionts, '*Asaia'* is a useful model. The aim of my Ph.D. research work was to explore the molecular factors of acetic acid bacteria (AAB) involved in the interaction with the host in order to unveil targets addressable for novel strategies of pathogen biocontrol. The purpose was challenged by a comparative proteomic approach considering *Asaia* SF2.1 as a model AAB-symbiont. Since this approach requires a consistent amount of bacterial-specific protein, hypoxia was applied as a model condition eliciting or suppressing pathways possibly involved in the symbiotic interaction. Hypoxia condition was applied to mimic the insect gut condition, where the oxygen concentration is usually low from 2% to 8%.

The aim of work was to evaluate the endosymbionts of Acetic Acid Bacteria (AAB) biomass and optimization of protein extraction method for 2D-PAGE setup. The endo symbionts of AAB biomass isolated from *Anopheles stephensi, D. suzukii,* which belongs to four genera, i.e. *Komagataeibacter*, *Gluconobacter*, *Asaia* and *Acetobacter*. Each genus with most abundant species of isolates were selected and evaluated the growth curve and harvested the bacterial biomass in the late exponential phase.

Further review on the deepen knowledge of experiments were discussed to evaluate which protein extraction method is more appropriate and suitable in order to perform the proteomic profiles by two-dimensional gel electrophoresis (2DE) of selected acetic acid bacterial (AAB) symbionts. The following steps were involved: The condition for protein extraction methods were optimized and obtained the good quality of protein extraction of all strains of AAB using SDS-PAGE. Further quantification of protein were performed by Bradford method and measured the amount of protein concentration of all strains. The approximate amount of protein required for 2D-PAGE setup were confirmed by Bradford method. In comparison with all AAB strains, *Asaia* SF2.1 were selected for further 2D-PAGE setup based on the protein quality, quantity and availability of genome sequence.

To setup 2D-PAGE of *Asaia* SF2.1, different trials of sample preparation methods were followed such as Urea buffer extract, NaOH and TCA-acetone and extracted the protein followed by 2D-PAGE setup and focused the clear round spots. The protein were extracted from different methods followed by Iso-electric focusing (IEF) program were optimized and in second dimension the proteins were separated according to the molecular weight. Finally, the results from gel images concludes, that TCA-acetone were the best method, which showed the good quality of protein and obtained the clear spots of protein profile of *Asaia* SF2.1 compared to other methods.

In order to mimic the insect gut condition (hypoxia condition) growth of *Asaia* SF2.1. The aim was to setup different growth condition (i.e., hypoxia 6%

and normal 20%) of *Asaia* SF2.1 and to perform the comparative proteomic analysis by 2D-PAGE followed by mass spectrometry analysis.

To set up the hypoxia condition (6% oxygen) and normal condition (20% oxygen) of *Asaia* SF2.1 were grown in bioreactor and successfully setup the different percentage of dissolved oxygen, and evaluated the growth curve and mass cultivation of *Asaia* SF2.1. Five replicates of 2DE gels were obtained from both conditions by using TCA-acetone protein extraction method. The 2DE gel image analysis were done using ImageMaster 2D Platinum 6.0 program and analyzed the *Asaia* SF2.1 protein expression profile. Based on the protein expression profile of two different condition (6% and 20% oxygen), protein spots were selected for mass spectrometry (MS) analysis for identification of proteins with the expression altered by the “interaction” condition.

From MS analysis results, among different proteins, we obtained interesting candidates such as 6-phosphogluconate dehydrogenase, Transaldolase, and Aconitate hydratase and in particularly, it’s interesting to confirm further about 6-phosphogluconate dehydrogenase role in insect gut function, which is an important enzyme of oxidative phase of pentose phosphate pathway (PPP).

In conclusion, further studies required to confirm the role of 6-phosphogluconate dehydrogenase in cellular function and thereby it may be the good strategy for biocontrol of pathogen transmission in insects.

Chapter I

Acetic Acid Bacteria, an Endo-symbionts bacteria and its growth

Abstract

Bacterial population growth require inoculation of viable cells into a sterile broth medium and incubation of the culture under optimum temperature, pH, and gaseous conditions. The aim of work was the evaluation of different phases of Acetic Acid Bacteria (AAB) growth and analyse the characterization of each species and compare the biomass production of all species. Initially evaluated the growth of AAB isolated from *D.suzukii* and *Anopheles stephensi*, which belongs to four genera i.e. *Gluconobacter*, *Acetobacter, Komagataeibacter* and *Asaia*. Most abundant species were selected from each genus and growth was measured. During the growth culture, some of the species produced biomass aggregates while others were not able to do it. The bacterial growth was monitored based on the absorbance (OD) and quantification of pellet. Depends on the growth, OD and collection time (h), each species conditions were optimized and biomass was collected. In conclusion, all strains of acetic acid bacteria were showing considerable (or) applicable biomass yield. In particular, aggregating strains were showing higher biomass yield (mg/ml of culture) at late exponential phase compared to non-aggregates.

Introduction

Insects are the most diverse and successful animal group on earth, which is reflected in the variety of habitats they live in, their abundance and species richness. The successful occupation of a large variety of ecological niches is often facilitated by symbiotic microorganisms (Ratzka, et al., 2012 and Basset et al., 2012). Many insect lifestyles were found to be associated with endo-

symbiotic microorganisms. The diversification and evolutionary success of insects have depended in part on their myriad relationships with their beneficial microorganisms. Insects are the most species-rich group of organisms, and it has been estimated that at least 15–20% of all insects live in symbiotic relationships with their bacteria. The term symbiosis (living together) was first introduced by Anton de Bary in 1879 as the permanent association between two or more specifically distinct organisms, at least during a part of the life cycle (Gil et al., 2004; and Dominguez-Bello et al., 2010). Symbiosis often refers to the long-term and mutually beneficial interactions among different species. Symbiotic microbes living inside the host species are referred to as endosymbionts. Generally, gut microbes in host, to regulate the fine immunity is required for host fitness and host survival rate.

In insect, the gut bacteria gave rise to endosymbiotic bacteria (Husník et al., 2011), which is based on the similarities of gut bacteria and insect endosymbionts (Fukatsu and Hosokawa 2002). The diversity and composition of the gut microbiota within and between individuals of a host species is influenced by topographical and temporal variation in the microbial communities, with particular bacterial species occupying specific niches in the body habitat or being associated with particular growth or maturation phases of the host (Tremblay, et al., 1989; Thao et al., 2002). Acetic acid bacteria (AAB) not only colonizes in the insect gut, but also localized in the other body part of insect body like salivary glands and the male and female reproductive systems, which are crucial sites for the bacterial transmission by horizontal and vertical routes (Damiani et al., 2008; Crotti et al., 2009; Gonella et al., 2012).

Interestingly, the endosymbionts of AAB have been associated with insects that rely on sugar-based diets, in particular those belonging to the orders Diptera, Hymenoptera and Hemiptera (Table 1.1). These endosymbiotic relationships have also been described for many insect families are including weevils, mealybugs, whiteflies, tsetse flies, psyllids, leafhoppers (Moran et al., 2003; Fukatsu & Hosokawa, 2002; Baumann, 2005; Hosokawa et al., 2006) carpenter ants and cockroaches (Panizzi et al., 2000). The arthropods, such as Wolbachia are facultative endosymbionts and obligate intracellular bacterial

that exhibit a parasitic association to their host. In contrast, Wolbachia of filarial nematodes are obligate, mutualistic endosymbionts that are also dependent on metabolic compounds produced by the host, but they in turn provide other metabolic products that are essential for worm survival, e.g. the nematode host is not able to generate purine, pyrimidine, flavin adenine dinucleotide (FAD) and riboflavin and thus needs to obtain these compounds by external sources or from their endosymbiont that is still able to synthesize them. However, symbiosis microbes plays an important roles for wide range of plants, animals and also mainly involved in the biology and life cycle of most insects species affecting different aspect of their life (Villegas and Pimenta, 2014). These

AAB and insect host(s)
Commensalibacter intestini ***D. melanogaster* (Diptera: Drosophilidae)**
***Acetobacter* sp.** ***D. melanogaster* (Diptera: Drosophilidae), *A. mellifera* (Hymenoptera: Apidae), *B. oleae* (Diptera: Tephritidae), *A. tabida* (Hymenoptera: Braconidae), *S. sacchari* (Homoptera: Pseudococcidae)**
***Gluconobacter* sp.** ***A.mellifera* (Hymenoptera:Apidae), *D.melanogaster* (Diptera: Drosophilidae), *S. sacchari* (Homoptera: Pseudococcidae)**
***Gluconacetobacter* sp.** ***D.melanogaster* (Diptera: Drosophilidae), *A.mellifera* (Hymenoptera:Apidae), *S. sacchari* (Homoptera: Pseudococcidae)**
***Asaia* sp.** ***Anopheles* sp. (Diptera: Culicidae), *A. aegypti* (Diptera: Culicidae), *S. titanus* (Hemiptera: Cicadellidae), *M. leopardiana* (Hymenoptera: Aphelinidae), *P. rapae* (Lepidoptera: Pieridae)**
Saccharibacter floricola ***A. mellifera* (Hymenoptera: Apidae)**

Table 1.1 Shows that AAB inhabit several insect hosts belonging to different orders like Diptera, Hymenopera, Hemiptera, and Homoptera (Crotti et al., 2010).

microbes symbionts has been involving insects in various functions such as nutritional upgrading of the diet, reproduction, immunity, providing defense against pathogens (or) parasites (or) natural enemies, contribute to inter and intraspecific communication, regulate mating and reproductive systems and detoxifying the ingested food material. Metazoans have partly faced this challenge by forming symbiosis with microorganisms that both synthesize essential nutritional compounds and increase the efficiency of nutrient digestion and absorption (Fraune and Bosch, 2010; Moran, 2007). However, many insects are inhabited by complex microbial community, but functional analyses of complete microbe communities are scarce (Crotti et al., 2009). Symbionts can serve a range of nutritional functions, from mobilizing stored nitrogen to contributing essential amino acids (Brune and Ohkuma, 2011; Douglas 2009; Feldhaar, 2011; Kaufman and Klug, 1991), and hosts often rely on symbiotic microorganisms to supply nutrients required for viability and fertility (Dillon and Dillon, 2004; Douglas et al., 2010; and Moran and Baumann, 2000).

General characteristic of Acidic acid bacteria (AAB)

The group of Acetic acid bacteria (AAB) is formed by gram-negative bacteria, aerobic, non-spore forming and attending to their morphology, they can be ellipsoidal to rod shaped bacteria. Their size ranges from 0.4-1μm wide and 0.8-4.5 long. Under the microscope, they are observed as single and pairs (or) chains and can present peritrichous (or) polar flagella which makes them motile microorganisms (Yamada and Yuphkan, 2008). They are able to vary their aggregation and even their morphology depending on the culture age and the environmental conditions (Park et al., 2003; Trcek et al., 2007). They have a strict aerobic metabolism with oxygen as the terminal electron acceptor, and characterized by catalase positive and oxidase negative.

The general feature of Acetic acid bacteria (AAB) is to derive their energy from the oxidation of ethanol to acetic acid during fermentation, as ethanol is a good carbon source for AAB. After oxidation process, the strains *of Acetobacter* and *Gluconoacetobacter* can further oxidize acetic acid to CO_2 and H_2O that is called acetate overoxidation, this process carried out by tricarboxylic acid (TCA

cycle). But, strains of *Gluconobacter* that can only oxidise ethanol to acetic acid, these *Gluconobacter* are not able to over oxidize acetic acid because of non-functional α-ketoglutarate dehydrogenase and succinate dehydrogenase of tricarboxylic acid (Gullo and Giudici, 2008). This is one of the most common difference between these strains and also distinguish between these strains, the biochemical and physiological tests may be considered (Du Toit and Pretorius, 2002). Most of the AAB can grow between pH 5 and pH 6.3 (Holt et al., 1994), but in some cases, they could grow at a pH lower than 4 (Du Toit and Pretorius, 2002) reported that in aerated culture containing acetate, AAB were isolated at pH values of 2.0 – 2.3. Their optimal growth temperature is 25°C – 30°C (Ndoye et al., 2006; Saeki et al., 1997).

AAB belongs to the family *Acetobacteraceae* are ubiquitous and adapted to various sugars and ethanol rich environments like flowers, or palm sap, herbs, fermented foods and beverages (Crotti et al., 2010). In recent times, the interactions between insects and microorganisms have received more importance. These endosymbiotic bacteria are largely present in the insect taxa, where they are given supplementary components of nutrients that is essential for the survival of insects. For example, Aphids are plant phloem-feeding insects, a diet that is rich in sugars, but poor in nitrogenous compounds. Therefore, the bacterium *Buchnera aphidicola* genomes (Tamas et al., 2002) revealed the presence of genes coding for the biosynthesis of essential amino acids that are lacking in the aphids diet, given support to the nutritional role for this symbiosis.

AAB produce different types of polysaccharides such as cellulose (De Ley et al., 1984). The exopolysaccharide (EPS) of cellulose is the main constituent of the extracellular matrix around the AAB. This matrix that are involved in tight contact between the microbial cells and the host epithelium, indicate a role in microbial interaction with host surfaces. (Romling, 2002). One of the bacterium *A. xylinum* that produced cellulose by metabolic activity. The substrate for cellulose synthesis is glucose, which is major constituent of the medium by cultivation of *A. xylinum* (Scott Williams and Robert, 1989). AAB are involved in some important industrial process (De Vero, 2006; Raspor & Goranovic, 2008).

AAB are well known for the ability to oxidize the sugars and alcohols, resulting an accumulation of organic acids as final products. Acetic acid bacteria can oxidize alcohols into sugars; mannitol into fructose; sorbitol into sorbose or erythritol into erythrulose (Gonzales, 2005). For example, *Gluconobacter* is an important genus for the industrial production of L-sorbose from D-sorbitol; D-gluconic acid, 5-keto- and 2-keto-D-gluconate from D-glucose; and dihydroxyacetone from glycerol (Gupta et al., 2001). Moreover, AAB also present in the plant rhizosphere (Fuentes-Ramirez et al., 2001) and also involved in production of food and fermented beverages like (vinegar, cocoa powder, palm wine kombucha tea etc.,) (Nielsen et al., 2007; Yamada and Yukphan, 2008). In vinegar industry, AAB can produce high concentrations of acetic acid from ethanol and also AAB is involved in the production of sorbose and cellulose (Gonzales, 2005). (Bartowsky & Henschke, 2008). However, these microorganisms not only play the positive roles and also spoilers fermented beverages such as wine, cider and beer, fruits (Mamlouk and Gullo, 2013).

Taxonomic Aspects of Acetic Acid Bacteria

AAB belongs to the family Acetobacteriace under the subclass of α-proteobacteria (De Ley et al., 1984; Sievers et al., 1994). In the beginning, AAB taxonomy was subjected to study and classification based on morphological, biochemical, and physiological criteria. In the earliest, AAB were classified into two main genera that is *acetobacter, gluconobacter*. The classification of this bacteria group has been substantially changed several times, including additions of new genera and species (Malimas et al., 2009; Yukphan et al., 2010; Yamada et al., 2012) and reclassifications of species and strains (Malimas et al., 2008). Nowadays, AAB are recognized as 14 genera: *Acetobacter* (A), *Gluconobacter* (G), *Acidomonas* (AC), *Asaia* (AS), *Gluconoacetobacter* (Ga), *Kozakia* (K), *Swaminathania* (Sw), *Saccharibacter* (S), *Neoasaia* (N), *Granulibacter* (Gr), *Tanticharoenia* (T), *Ameyamaea* (Am), *Neokomagataea* (Ne), and *Komagataeibacter* (Ko) (Sengun, Karabiyikil, 2011 and Yamada et al., 2012). Among the great variety of microorganisms that were identified and isolated, *Gluconobacter*, *Komagataeibacter*, and *Acetobacter* are

some of the predominant bacterial groups of the bee microbiota (Yamada and Yukphan, 2008 and Crotti et al., 2009). However, recently, proposed the new genus *Komagataeibacter*, which includes 12 species that previously belonged to the *Gluconacetobacter* genus, and with KO (Yamada et al., 2012). Xylinus as the species type of this new genus. Therefore, nowadays, *Acetobacter* continues to be the most diverse genus with 24 species, but now followed by *Komagataeibacter* and *Gluconobacter* with 14 species and *Gluconacetobacter* with 11 species.

Insect gut and role of Acetic acid bacteria:

The gut microbiome consists of microorganisms that live in the digestive tracts of animals, and is an important component of the gut of an organism.

Host Cell (Bacteriocytes)

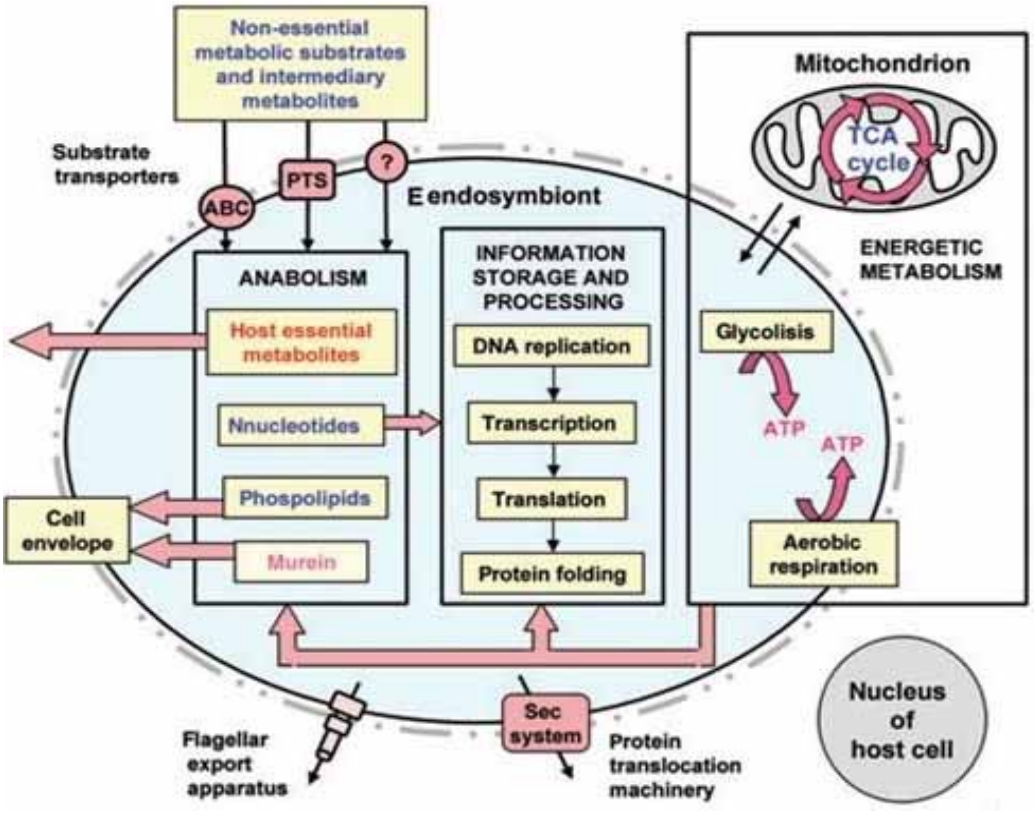


Figure 1.1 A General overview of the functions of endosymbionts of bacteria, and their metabolic interactions with the host cell (Gil et al., 2004).

Diverse populations of symbiotic associations of bacteria have developed within the insect gut (Hooper and Gordon, 2001). The composition of gut microbiome varies greatly from species to species. Even within the same species, variation in gut microbiome occurs among different individuals, which causes phenotypic differences among these individuals (Holmes and Nicholson, 2005). The dynamic variation in insect gut microbiota can be determined by gut morphology and physicochemical conditions such as pH and oxygen availability in the insect gut (Dillon and Dillon, 2004; Engel and Moran, 2013).

Generally, Bacterial endosymbionts of insects can be classified into two groups that is obligate or primary symbionts and facultative or secondary symbionts. Primary endosymbionts have an obligate and ancient association with their insect host (Ratzka, et al., 2002). These endosymbionts live in a very close environment, inside specialized host cells that is called *bacteriocytes*, which may form an organ-like structure (bacteriome) in the body cavity of the insects. These bacteria cannot be cultured outside the host, whereas the host needs the bacteria essential for normal growth and reproduction (Fig. 1.1) (Gil et al., 2004).

They are vertically transmitted to mother and offspring at early stages of embryogenesis. Their vertical transmission suggests parallels between symbionts and insect genes. Secondary endosymbionts are usually facultative symbionts that are not restricted to bactericides, but may also be found extra or intracellularly in other host tissues as well as in the haemocoel. The vertical transmission also common to the secondary symbionts like maternally transmitted, but horizontal transmission is also possible (Baumann et al., 2005).

These secondary symbionts are more sporadic and distribution among species, and even among individuals of the same species, which indicates that they are not essential for host survival. Therefore, they do not share a long evolutionary history with their hosts. Besides, primary symbionts are mutualists that protect to have a nutritional function and typically occur in insects that feed on imbalanced diets such as plant saps or cellulose. So these symbionts are provided nutrients to the insect diet with amino acids or vitamins that are rare or

absent in the food source (Ferrari and Vavre, 2011). For example, bacterium *Buchnera aphidicola*, is a the primary symbiont of aphids, it's synthesizes several amino acids that are required for the aphids metabolism, and aphids in which the symbiont has been artificially removed have extremely affect low survival and fertility (Tamas et al., 2002). Primary symbionts not only have nutritional effects, but might also affect their host's interactions with natural enemies. One more example, removing Wigglesworthia symbionts in tsetse flies makes them more susceptible to trypanosome infection is a vectors of tsetse flies-Diptera: Glossinidae (Pais et al., 2008). Hence a Comprehensive understanding of the biology of insects cannot do without the characterization of its microbiota (Russell et al., 2003), as an essential component of system.

The important role of AAB is to interact with insect host is by colonizing in the host tissue, interaction with the innate immune system and the pathways of the host (Ryu et al., 2008; Gross et al., 2009; Douglas et al., 2011; Shin et al., 2011; Lee et al., 2013; Login and Heddi 2013). The AAB, *Asaia* is involved in the development role of mosquito larvae (Chouaia et al. 2012; Mitraka et al., 2013) and thus affects the expression of genes related to cuticle formation (Mitraka et al., 2013). Likewise, AAB promotes are involved in the modulation of innate immunity in *Drosophila*, which keeps pathogenic strains under control (Ryu et al., 2008). Furthermore, *Acetobacter pomorum* in *Drosophila* modulates the insulin signaling, a pathway involved in the regulation of development, body size, energy metabolism, and intestinal stem cell activity of the host (Shin et al., 2011). The aim of work is to focus on the evaluation of AAB growth and towards understanding the in depth analysis of different stages and comparison of their biomass production.

Results and Discussion

Selection of Acetic Acid Bacteria (AAB)

Acetic acid bacteria (AAB) strains were selected from *D. suzukii* and *Anopheles stephensi* (Table 1.2) based on a previous extensive characterization of isolates (Vacchini *et al*., submitted), with the aim to phylogenetically define the

Table 1.2. Identification of cultivable bacteria associated to *D. suzukii*. All the isolates showed a percentage of identity >97% in relation to the indicated species.

Isolates	No. isolates	LP	PP	AP fly	AF fly
Acetobacter tropicalis	1	0	0	0	1
Acetobacter orleanensis/malorum/cerevisiae	4	0	0	0	4
Acetobacter peroxydans	1	0	0	0	1
Acetobacter indonesiensis	10	0	1	1	8
Acetobacter persici	10	0	1	1	8
Acetobacter orientalis	1	0	0	0	1
Acetobacter cibinongensis	1	0	0	0	1
Gluconacetobacter liquefaciens	4	1	3	0	0
Komagataeibacter sp	118	0	0	0	118
Gluconacetobacter/Komagataeibacter sp.	1	0	0	0	1
Gluconobacter kondonii	1	0	0	0	1
Gluconobacter oxydans	12	0	0	0	12
Gluconobacter kanchanaburiensis	5	3	1	1	0
Pseudomonas geniculata	1	0	0	1	0
Serratia sp.	8	2	6	0	0
Micrococcus sp.	5	0	0	0	5
Microbacterium foliorum	2	0	0	0	2
Streptococcus salivarius	1	0	0	1	0
Staphylococcus sp.	12	0	0	0	12
Paenibacillus sp.	2	0	0	0	2
Lactococcus lactis	1	0	0	0	1
Lactobacillus plantarum	1	0	1	0	0
Total	202	6	13	5	178

LP: larvae fed with artificial diet; PP: pupae fed with artificial diet; AP: Adults fed with artificial diet; AF: Adults fed with fruit diet

selected strains, internally transcribed spacer (ITS)-PCR fingerprinting was performed with primers ITSF (5'-GCC AAG GCA TCC AAC-3') and ITSR (5'–GTC GTA ACA AGG TAG CCG TA-3'). ITS-PCR amplification patterns of all the isolates were visually compared to cluster the isolates into ITS groups or profiles. At least 2 candidates for each ITS profile were selected and 16S rRNA gene was amplified with universal bacterial 16S rRNA gene primers 27F (5'-TCG ACA TCG TTT ACG GCG TG-3') and 1495R (5'-CTA CGG CTA CCT TGT TAC GA-3'). Near full-length sequencing of 16S rRNA has been performed by macrogen (South Korea) and consensus sequences were compared to the public databases at the National Centre for Biotechnology Information (NCBI) using BLASTn (Altshul *et al*., 1990). The most abundant 10 isolates (i.e., *A. persicus, K. saccharivorans, K. hansenii, K .intermedius, A. indonesiensis, A. tropicalis, Asaia* SF2.1, *G. kanchanaburiensis, G. oxydans, and G. kondonii*), were selected from (Table 1.2) each genus (i.e *Komagataeibacter, Gluconobacter, Asaia and Acetobacter*) were grown at lab condition in order to understand the bacterial growth characterization and comparison of biomass yield of all species.

Evaluation of the AAB growth condition

To understand the strains of AAB growth, required cells were inoculated in a liquid medium in which the nutrients and environmental conditions were controlled. The medium supplies all nutrients required for growth and

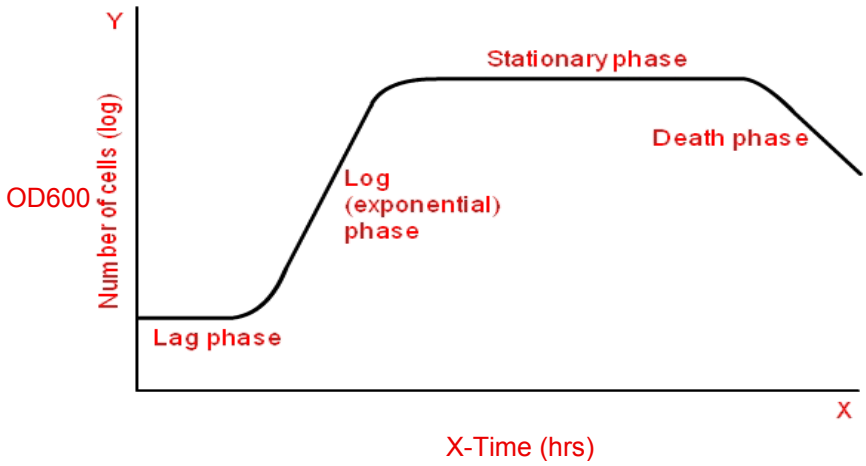


Figure 1.2 Shows the growth of all acetic acid bacteria (AAB) of isolates species (i.e. *Komagataeibacter*, *Gluconobacter*, *Acetobacter,* and *Asaia*) and achieved good biomass production of each species at 600 nm optical density (OD) at late exponential phase.

environmental parameters were optimal, the increase in numbers or bacterial bio mass measured as a function of time to obtain a growth curve (Baranyi and Pin., 1999). Several distinct growth phases were observed during the growth culture (Fig.1.2). These include the lag phase, the exponential or log phase, the stationary phase, and the death phase. Each of these phases represents a distinct period of growth that is associated with typical physiological changes in the cell culture.

Lag phase

During this phase, the AAB bacteria were introduced into fresh Gly-medium, and cells were adjusted to their new environment (lag phase). During this stage, cellular metabolism accelerated, so cells increased in size, but the bacteria were not able to replicate, therefore no increase in cell mass. In this phase, the bacterium started synthesizing the necessary proteins, co-enzymes and vitamins needed for their growth. As a result, initiation of cell growth, division and the transition to exponential phase were slowed down.

Exponential or Logarithmic (log) phase

During exponential growth the rate of increase of cells in the culture is proportional to the number of cells present at any particular time. During this phase, the metabolic activity increased and the bacteria grown rapidly and divided. The bacteria begins the DNA replication by binary fission at a constant rate. The growth medium were exploited by bacteria at maximal level and the culture reached the maximum growth rate. Finally the single cell divide into two, which replicate into four, eight, sixteen, thirty two and so on (i.e., 2^0, 2^1, 2^2, 2^3.....2^n, n is the number of generations). The time taken by the bacteria to double in number during a specified that is called generation time. The generation time variation based on the organisms. Generally, *E.coli* divides in every 20 minutes, hence its generation time is 20 minutes, and for our case we checked the generation time of AAB divides every 30 minutes.

Stationary phase

In this phase, the bacterial growth were very slow because of the growth-limiting factor such as depletion of essential nutrients, the accumulation of toxic

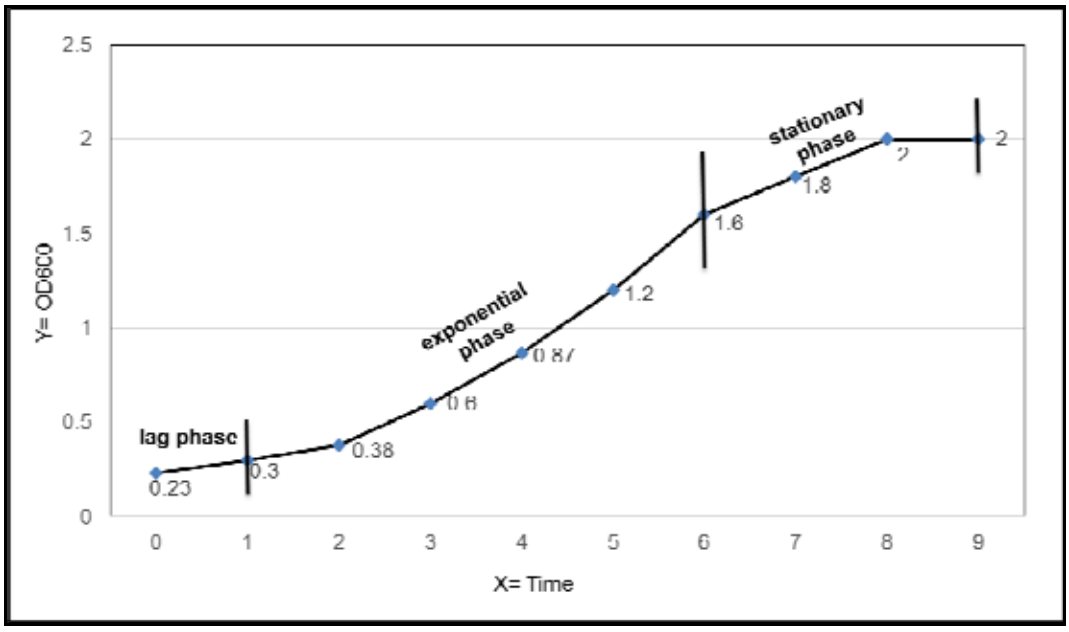


Figure 1.3 Shows the growth curve of *Asaia* SF 2.1 biomass were collected at late exponential phase.

materials, metabolic waste products and inhibitory compounds such as antibiotics in the medium. The cells undergoing division equals to the number of cell death, and finally bacterial division were completely stop (Fig. 1.3). As a result, “smooth,” horizontal linear part of the curve during the stationary phase were obtained.

Decline or Death phase

The depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in the media, facilitates the bacterium to move on to the death phase. During this stage, the bacterium completely lost its ability to reproduce. Individual bacteria begin to die due to the unfavorable conditions and the death was rapid and at uniform rate. The number of dead cells exceeds the number of live cells. Some organisms which can resist this condition can survive in the environment by producing endospores. Finally our

results indicates, the characterization of each species growth were optimized. Results shows that some species were aggregating while others non-aggregates (Fig 1.4a, 1.4b). During growth, obtained length of log phase varies, depending on the strains, although the average were estimated to last 8 to 13 hours of all AAB species. Among all strains, in some cases for example *K. hansenii* (24hrs), *K. intermedius* (21hrs), *K. saccharivorans* (20hrs), takes long time length of log or exponential phase.

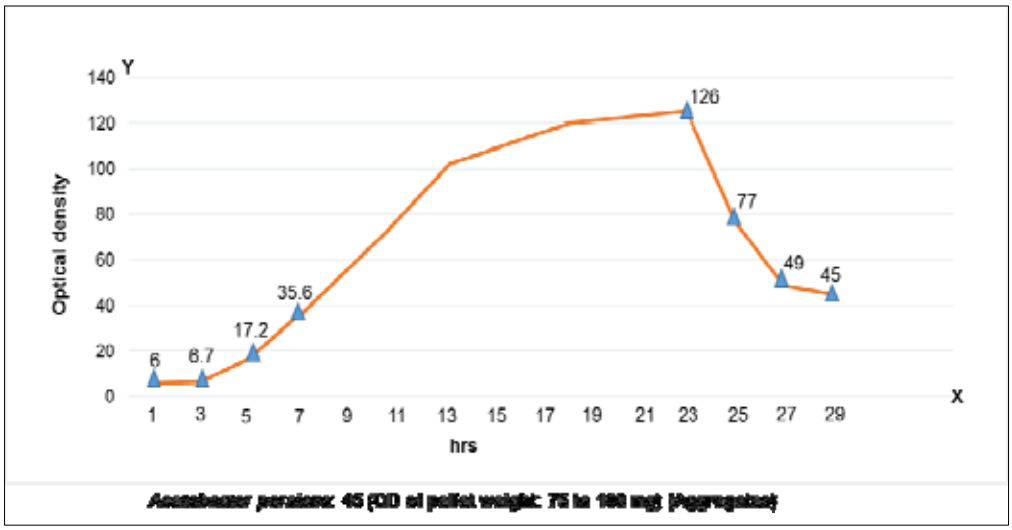


Figure 1.4a Shows aggregates formation of *Acetobacter persicus*

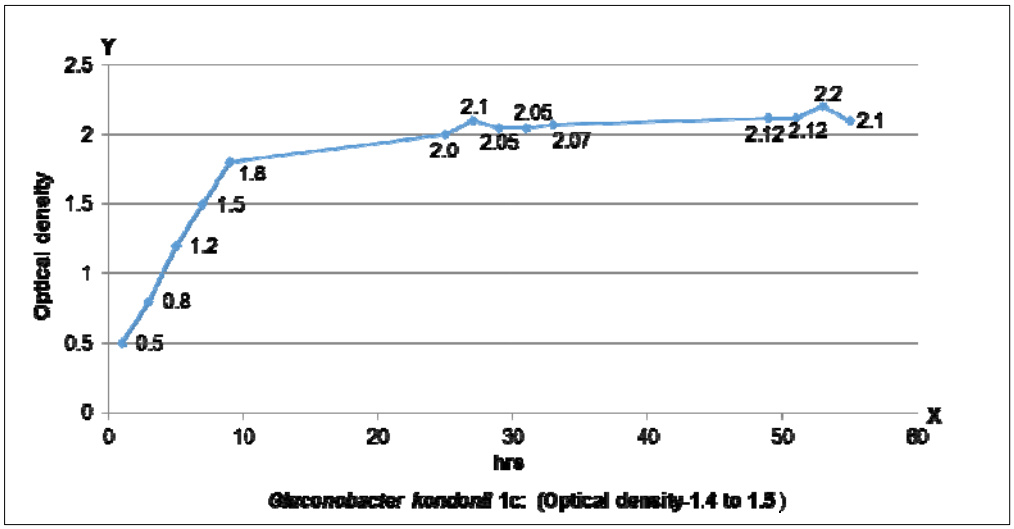


Figure 1.4b Shows non-aggregates of *Gluconobacter kondonii* 1c.

Harvested the bacterial (AAB) biomass yield at late exponential phase

The production of bacteria biomass was measured by using the spectrophotometer. The degree of turbidity in the broth culture is directly related to the number of microorganism present, either viable or dead cells, thus a convenient and rapid method of measuring cell growth rate of an organism. Hence, the increasing turbidity of the broth medium indicates increase of the microbial cell mass. As a result, depends on the growth culture, some of the species were aggregates like beads formation and turbidity, so all the cells were sticky to each other and some were normal in growth i.e., non-aggregates. Two methods were applied and monitored the growth of pellet

		Average and sdev of exponential and stationary phase and depends on growth hrs biomass yield were optimized						
					Growth curve data		biomass collection	
Strain name	Aggregates	pre-inoculum	Exponential phase		Stationary phase		Collection	Biomass yield
and		amount	% cell culture	% aggregates	% cell culture	% aggregates	time (h)	(mg/ml of culture)
Assigned species		(%) / (v/v)	culture OD (h)	culture time (h)	culture OD (h)	culture time (h)		
44 (Acetobacter indonesiensis)	_	2.90%	1.1 ± 0.16	_	2 ± .06	_	9	5.9
			(29 ± 1.4)	_	(28.3 ± .5)	_		
45 (Acetobacter persicus)	Yes	4.80%	_	61 ± 1	_	127 ± 1	6	5.7
			_	(8 ± 2)	_	(23.5 ± .5)		
23 (Acetobacter tropicalis)	_	2%	1.3 ± .1	_	1.7 ± .08	_	6	3.4
			(22 ± 1.4)	_	(28 ± .5)	_		
65A (Komagataeibacter saccharivorans)	_	20%	0.52 ± .2	_	1.5 ± .03	_	20	2.4
			(6 ± 2)	_	24.5 ± .5	_		
114 (Komagataeibacter hansenii)	yes	4.80%	_	188 ± 38	_	339 ± 28	24	19.5
			_	(16 ± 0)	_	(44 ± 0)		
59B (Komagataeibacter *intermedius)*	yes	7%	_	55 ± 5	_	148 ± 10	21	6.9
			_	(13.5 ± .5)	_	(26 ± 2)		
15 (Gluconobacter kanchanaburiensis)	_	9.10%	0.8 ± .15	_	1.7 ± .05	_	5.15	2
			(3 ± 1)	_	(25 ± 1)	_		
9A (Gluconobacter oxydans)	_	4.80%	0.7 ± .05	_	1.3 ± .03	_	15	2.2
			(6 ± 0)	_	(24 ± 1)	_		
1C (Gluconobacter kondonii)	_	2.30%	1 ± 1.2	_	2.05 ± .05	_	6	1.9
			(3 ± 1)	_	(25 ± 1)	_		
Asaia SF2.1	_	4.80%	1 ± 0.2	_	2. ± 0.15			
			5 ± 1	_	8 ± 1	_	7	5.5

Table 1.2 Our results shows the overall average and standard deviation data of exponential and stationary phase of each species. Biomass collection (hrs and OD) were optimized for each species. In between the two stage were collected the biomass at late exponential phase. Finally all species were shows applicable biomass yield (mg/ml of culture).

weight. 1. Based on OD at 600 nm at Spectrophotometer; 2. Quantification of the pellet. **Based on OD at 600nm –** The standard procedure (Baranyi and Pin 1999) were followed for normal growth of all species. This method is based on

the optical density measurement of number of viable cells/per ml. The cell number of biomass (Table 1.2) were indirectly observed using spectrophotometry for every one hour at 600nm. **Quantification of the pellet–** This common procedure (Zwietering et al., 1990) were followed for those isolates, which showed aggregates, and the pellet were quantified. Every one hour, the sample were collected from the culture by centrifugation and quantified the pellet as grams of wet weight per ml of sample. Once optical density reached in all strains then immediately stopped the bacterial growth and cells were harvested and obtained only the pellet or cells weight. Finally obtained, all strains have a good biomass yield (mg/ml of culture) at late exponential phase (Fig. 1.5).

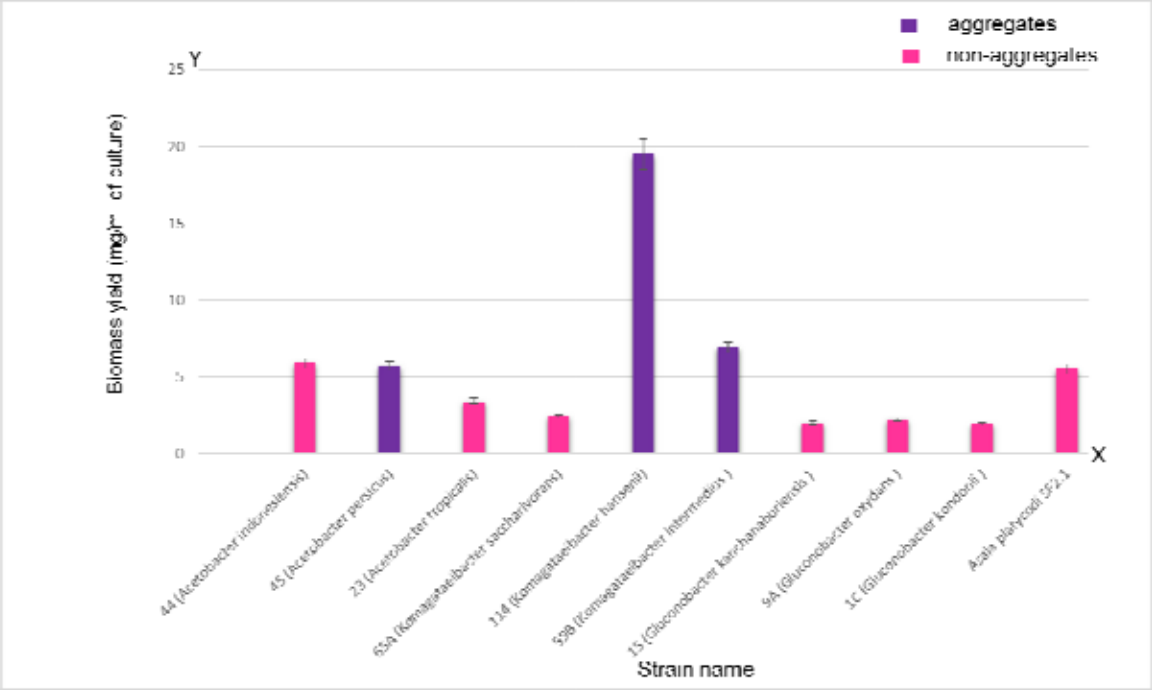


Figure 1.5 All strains of acetic acid bacteria showing applicable good biomass yield (mg/ml of culture) at late exponential phase. In particular, aggregates showed higher biomass than non-aggregates.

Conclusions

In conclusion, growth curve of AAB isolates species were evaluated based on the optical density and quantification of the pellet. Finally our results indicates that all strains of acetic acid bacteria were showing good and considerable biomass yield (mg/ml of culture) at late exponential phase as follows: *A. indonesiensis* (5.9mg/ml), *A. tropicalis* (3.4mg/ml), *K. saccharivorans* (2.4mg/ml), *Asaia* SF2.1 (5.5mg/ml), *G. kanchanaburiensis* (2mg/ml), *G. oxydans* (2.2mg/ml), *A. persicus* (5.7mg/ml), *K. hansenii* (19.5mg/ml), *K. intermedius* (6.9mg/ml), and *G. kondonii* (1.9mg/ml).

Materials and method

Insects

Wild specimens of *D. suzukii* were field collected as adults/larvae in Trentino Alto Agide region (Italy) and reared in laboratory condition both on fruits and on a sugar-based artificial diet (composed with 71 g of corn flour, 10 g of soy flour, 5.6 g of agar, 15 g of sucrose, 17 g of brewer's yeast, 4.7 ml of propionic acid, 2.5 g of vitamins mix for each Kg of the preparation) at the Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Turin. Insects were kept in cages at 25 °C with a 14:10 h light-dark photoperiod.

Isolation of AAB

Insects (5 males, 6 females, a pool of 3 males and a pool of 3 females), reared on fruits, were surface sterilized by rinsing once with ethanol 70% and twice with 0.9% NaCl under sterile conditions, before being homogenized by grinding in 200 µl of 0.9% NaCl. Forty µl of each insect homogenate were inoculated in different enrichment liquid and solid media, selected for AAB growth: enrichment medium I (hereafter indicated as TA1, Yamada *et al*. 1999; Kounatidis *et al.*, 2009), enrichment medium II (here after indicated as TA2, Yamada *et al*. 2000), a basal medium (here after indicated as TA4, Kadere *et al.* 2008), Hoyer-Frateur medium (De Ley and Frateur, 1974), acid YE medium (yeast extract 2%, ethanol 2%, acetic acid 1%, pH 6). One hundred µl of serial dilutions of the insect homogenate were spread on plates containing mannitol agar medium (mannitol 2.5%, peptone 0.3%, yeast extract 0.5%, pH 7, agar 15 g/L) or R2A agar (Reasoner *et al.,* 1979), both supplemented with 0.7% $CaCO_3$ and 0.01% cycloheximide. Other 6 insect adults reared on fruit diet, 6 adults, 3 pupae and 3 larvae reared on the artificial diet, were washed three times with deionized water and the washing water of the last step was plated on MA solid medium. Pupae and larvae were smashed, as previously described, and inoculated in TA1 and TA2 enrichment media. All the enrichment liquid media were incubated at 30°C, in aerobic condition with shaking, until turbidity of the liquid media was reached. Serially dilutions were plated on MA medium,

supplemented with $CaCO_3$ (1% D-glucose, 1% glycerol, 1% bactopeptone, 0.5% yeast extract, 0.7% $CaCO_3$, 1% ethanol, 1.5% agar, pH 6.8) and incubated at 30 °C, in aerobic conditions. For the solid media, colonies were picked up and streaked on MA solid medium, with $CaCO_3$. Colonies capable of clearing the calcium carbonate were purified on agarized MA medium, and pure strains were conserved in 15% glycerol at -80 °C. Total DNA was extracted from the isolates by boiling protocol and stored at – 20 °C.

16S rRNA gene-based identification and fingerprinting analysis of the isolates

Internally transcribed spacer (ITS)-PCR fingerprinting was performed with primers ITSF (5'-GCC AAG GCA TCC AAC-3') and ITSR (5'–GTC GTA ACA AGG TAG CCG TA-3') as previously described (Daffonchio *et al*., 1998). ITS-PCR amplification patterns of all the isolates were visually compared to cluster the isolates into ITS groups or profiles. At least 2 candidates for each ITS profile were selected and 16S rRNA gene was amplified and sequenced for identification by Macrogen (South Korea). 16S rRNA gene was amplified with universal bacteria 16S rRNA gene primers 27F (5'-TCG ACA TCG TTT ACG GCG TG-3') and 1492R (5'-CTA CGG CTA CCT TGT TAC GA-3'). Reaction mixture for 16S amplification was carried out in a final volume of 50 µL , using 1 unit of Taq DNA polymerase, 1X PCR Buffer, 0.12 mM of each dNTP, 0.3 µM of each primer, 1.5 mM $MgCl_2$ and 2 µL of DNA. Reaction was run for 4 min at 94°C, followed by 35 cycles of 1 min at 90°C, 1 min at 55°C, 2 min at 72°C and then a final extension of 10 min at 72°. 16S rRNA gene sequences were compared to the databases at the National Centre for Biotechnology Information (NCBI) using BLASTn (Altshul *et al*., 1990) and aligned with their closest type strain relatives using Clustal W (http://align.genome.jp/).

AAB bacteria biomass: The common procedure were followed (Kadere, etal., 2008) the Gly agar (glycerol 2.5%, yeast extract 1%, agar 2%, pH 5) medium for growing of AAB at lab condition, incubated at 30^{o}C for two days in order to isolate the pure strain. Then followed by the preparation of the pre-

inoculum of 25 ml of GLY liquid medium and Inoculated this pure strain and incubated in 30^{o}C at overnight. Following day, 5 ml of this pre-inoculated culture was transferred into fresh GLY medium (100 ml or 200 ml), Incubated at 30°C and monitored the growth based on OD 600 nm (Jenway spectrophotometer 6705). Once reached optical density in all strains at late exponential phase then immediately the growth were arrested and cells were harvested. Further removed cell debris by centrifugation at 3000rpm for 15min at 4 ^{o}C and washed 3 times with 1X PBS (137Mm NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4) buffer and quantified the pellet and stored at -80 ^{o}C.

References

Baumann P. (2005). Biology bacteriocytes-associated endosymbionts of plant sap-sucking insects. Annual Review of Microbiology. 59, 155–189.

Baranyi J. and Pin C. (1999). Estimating Bacterial Growth Parameters by Means of Detection

Bartowsky EJ, Henschke PA. (2008). Acetic acid bacteria spoilage of bottled red wine - a review. Int J Food Microbiol. 2008 Jun 30; 125 (1):60-70.

Basset Y, Cizek L, Cuénoud P, Didham RK, Guilhaumon F, Missa O, Novotny V, Ødegaard F, Roslin T, Schmidl J, Tishechkin AK, Winchester NN, Roubik DW, Aberlenc HP, Bail J, Barrios H, Bridle JR, Castaño-Meneses G, Corbara B, Curletti G, Duarte da Rocha W, De Bakker D, Delabie JH, Dejean A, Fagan LL, Floren A, Kitching RL, Medianero E, Miller SE, Gama de Oliveira E, Orivel J, Pollet M, Rapp M, Ribeiro SP, Roisin Y, Schmidt JB, Sørensen L, Leponce M. (2012). Science.14; 338 (6113):1481-4.

Brune A. & Ohkuma M. (2011). Role of the termite gut microbiota in symbiotic digestion. In: Biology of termites: a modern synthesis (D.E. Bignell, Y. Roisin, N. Lo, eds.). Springer, Dordrecht, pp 439-475.

Chouaia B. (2012). Delayed larval development in Anopheles mosquitoes deprived of Asaia bacterial symbionts. BMCMicrobiol. 12 (Suppl 1) : S2.

Crotti, E., C. Damiani, M. Pajoro, E. Gonella, A. Rizzi, I. Ricci, I. Negri, P. Scuppa, P. Rossi, P. Ballarini, N. Raddadi, M. Marzorati, L. Sacchi, E. Clementi, M. Genchi, M. Mandrioli, C. Bandi, G. Favia, A. Alma, and D. Daffonchio (2009). Asaia, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically-distant genera and orders. Environmental Microbiology 11, 3252–3264.

Crotti E., Rizzi A., Chouaia B., Ricci I., Favia G., Alma A., Sacchi L., Bourtzis K., Mandrioli M., Cherif A., Bandi C., Daffonchio D. (2010). Acetic acid bacteria, newly emerging symbionts of insects. Applied and Environmental Microbiology 76, 6963–6970.

Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, Esposito F, Bandi C, Daffonchio D & Favia G. (2008). Paternal transmission of symbiotic bacteria in malariavectors. Curr Biol 18: R1087–R1088.

De Ley J., Gillis M., Swings J. (1984). Family VI. Acetobacteraceae. Bergey's Manual of Systematic Bacteriology 1, 267–278.

Dillon RJ, Dillon VM. (2004). The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. 49:71–92

Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. (2010). Proc Natl Acad Sci U S A. Jun 29; 107 (26):11971-5.

Douglas AE., (2009). The microbial dimension in insect nutritional ecology. Functional Ecology 23, 38-47

Douglas, A.E., (2010). The Symbiotic Habit. Princeton University Press, New Jersey.

Douglas AE, Bouvaine S, Russell RR. (2011). How the insect immune system interacts with an obligate symbiotic bacterium. Proc R Soc Lond B Biol Sci. 278:333–338.

De Vero, L., Gala, E., Gullo, M., Solieri, L., Landi, S., Giudici, P., (2006). Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. Food Microbiology 23, 809–813.

Du Toit, W.J and Pretorius, I.S., (2002). The occurrence, control and esoteric effect of acetic acid bacteria and winemaking. Annals of microbiology, 52, 155-179

Engel P, Moran NA. (2013). The gut microbiota of insects-diversity in structure and function. FEMS Microbiol. Rev. 37:699–735

Feldhaar, H. (2011). Bacterial symbionts as mediators of ecologically important traits of insect hosts. DOI: 10.1111/j.1365-2311.2011.01318.

Ferrari, J., Vavre, F. (2011). Bacterial symbionts in insects or the story of communities affecting communities. Phil. Trans. R. Soc. B 366, 1389–1400.

Fuentes-Ramírez, L.E., Bustillos-Cristales, R., Tapia-Hernández, A., Jiménez-Salgado, T., Wang, E.T., Martínez-Romero, E., Caballero-Mellado, J. (2001). Novel nitrogen-fixing acetic acid bacteria, Gluconacetobacter johannae sp. nov. and Gluconacetobacter azotocaptans sp. nov., associated with coffee plants. International Journal of Systematic and Evolutionary Microbiology 51, 1305–1314.

Fukatsu T, Hosokawa T. (2002). Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, Megacopta punctatissima. Appl Environ Microbiol. Jan; 68 (1):389-96.

Fraune S, Bosch TC. (2010). Why bacteria matter in animal development and evolution. Bioessays. 32(7):571-80. doi: 10.1002/bies.200900192. Review.

Gil, R., Latorre, A., Moya, A. (2004). Bacterial endosymbionts of insects: insights from comparative genomics Environmental Microbiology, Vol 6, 1109–1122.

Gonella E. (2012). Horizontal transmission of the symbiotic bacterium Asaia sp. in the leafhopper Scaphoideus titanus Ball (Hemiptera: Cicadellidae). BMC Microbiol. 12 (Suppl 1): S4.

Gonzales, A. (2005). Application of molecular techniques for identification of acetic acid bacteria. PhD thesis, Universitat Rovira I Virgili, Tarragona, Spain.

Gupta A, Singh VK, Qazi GN, Kumar A. (2001). Gluconobacter oxydans: its biotechnological applications. J Mol Microbiol Biotechnol. 2001 Jul; 3 (3):445-56.

Gross R. (2009). Immunity and symbiosis. Mol Microbiol. 73:751–759.

Husník F, Chrudimský T, Hypša V. (2011). Multiple origins of endosymbiosis within the Enterobacteriaceae (γ-Proteobacteria): convergence of complex phylogenetic approaches. BMC Biol. 28;9:87. doi: 10.1186/1741-7007-9-87.

Holmes, E.; Nicholson, J. (2005). Variation in gut microbiota strongly influences individual rodent phenotypes. *Toxicological Sciences*, *87*, 1–2.

Hooper LV, Gordon JI. (2001). Commensal host-bacterial relationships in the gut. Science 292:1115–1118.

Hosokawa T, Kikuchi Y, Nikoh N, Shimada M, Fukatsu T. (2006). Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biol. Oct; 4(10):e337.

Kaufman, M. G. and M. J. Klug. (1991). The contribution of hindgut bacteria to dietary carbohydrate utilization by crickets (Orthoptera: Gryllidae). Comp. Biochem. Physiol. 98A:117-123.

Kadere,T.T., Miyamoto,T., R. K. Oniang`o.R.K., Kutima, P.M., Njoroge,S.M., (2008). Isolation and identification of the genera Acetobacter and Gluconobacter in coconut toddy (mnazi). African Journal of Biotechnology Vol. 7, pp. 2963-2971.

Lee KA. (2013). Bacterial-derived uracil as a modulator of mucosal immunity and gut-microbe homeostasis in Drosophila. Cell 153: 797–781.

Login FH, Heddi A. (2013). Insect immune system maintains long-term resident bacteria through a local response. J Insect Physiol. 59: 232–239.

Maria Gullo and paolo Giudici, (2008). Acetic acid bacteria in traditional balsamic vinegar: phenotypic traits relevant for starter cultures selection.International journal of food microbiology 125 :46-53.

Malimas, T., Yukphan, P., Takahashi, M., Muramatsu, Y., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M. (2008). Gluconobacter roseus (ex Asai 1935) sp., nom. rev., a pinkcolored acetic acid bacterium in the Alphaproteobacteria 54, 119–125.

Malimas, T., Yukphan, P., Takahashi, M., Muramatsu, Y., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M., Yamada, Y. (2009). Gluconobacter japonicus sp. nov., an acetic acid bacterium in the Alphaproteobacteria. International Journal of Systematic and Evolutionary Microbiology 59, 466–471.

Mamlouk, D., Gullo, M. (2013). Acetic Acid Bacteria: Physiology and Carbon Sources Oxidation. Indian Journal of Microbiology 53, 377–384.

Mitraka E. (2013). Asaia accelerates larval development of Anopheles gambiae. Pathog Glob Health. 107(6):305–11.

Moran, N.A., Baumann, P. (2000). Bacterial endosymbionts in animals. Curr. Opin. Microbiol. 3, 270–275.

Moran, N.A., Dale, C., Dunbar, H., Smith, W.A., and Ochman, H. (2003). Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome. Environmental Microbiology 5: 116–126.

Moran, N.A. (2007). Symbiosis as an adaptive process and source of phenotypic complexity. Proc Natl Acad Sci U S A. 104 Suppl 1:8627-33.

Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel, W.H. (2007). The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. International Journal of Food Microbiology 114, 168–186.

Ndoye, B., Lebecque, S., Dubois-Dauphin, R., Tounkara, L., Guiro, A.T., Kere, C., Diawara, B., Thonart, P. (2006). Thermoresistant properties of acetic acids bacteria isolated from tropical products of SubSaharan Africa and destined to industrial vinegar. Enzyme Microbiology and Technology 39, 916–923.

Pais, R., Lohs, C., Wu, Y. N., Wang, J. W., Aksoy, S. (2008). The obligate mutualist Wigglesworthia glossinidia influences reproduction, digestion, and immunity processes of its host, the tsetse fly. Applied and Environmental Microbiology. 74, 5965–5974.

Park, J.K., Jung, J.Y., Park, Y.H. (2003). Cellulose production by Gluconacetobacter hansenii in a medium containing ethanol. Biotechnology Letters 25, 2055–2059.

Panizzi L, Catalano S, Miarelli C, Cioni PL, Campeol E. (2000). In vitro antimicrobial activity of extracts and isolated constituents of Geum rivale. Phytother Res.; 14(7):561-3.

Raspor PP, Goranovicˇ D. (2008). Biotechnological applications of acetic acid bacteria. Crit. Rev. Biotechnol. 28:101–124.

Ratzka,C., Gross,R., Feldhaar.H. (2012). Endosymbiont Tolerance and Control within Insect Hosts. Insects 3, 553-572.

Romling, U. (2002). Molecular biology of cellulose production in bacteria. Research in Microbiology 153, 205–212

Russell, J.A., Latorre, A., Sabater-Munoz, B., Moya, A., Moran, N.A. (2003). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Molecular Ecology 12, 1061–1075.

Ryu JH. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. Science 319: 777–782.

Sievers, M., Ludwig, W., Teuber, M. (1994). Phylogenetic positioning of Acetobacter, Gluconobacter, Rhodopila and Acidiphilium species as a branch of acidophilic bacteria in the α-subclass of proteobacteria based on 16S ribosomal DNA sequences. Systematic and Applied Microbiology 17, 189–196.

Saeki, A., Theeragool, G., Matsushita, K., Toyama, H., Lotong, N., Adachi, O. (1997). Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. Biosciences Biotechnology and Biochemistry 61, 138–145

Scott williams, W., Robert. E. (1989). Alternative Environmental Roles for Cellulose Produced by Acetobacter xylinum. Applied and environmental microbiology, 55, 2448-2452

Sengun I.Y., Karabiyikil S. (2011). Importance of acetic acid bacteria in food industry. Food control 22, 647-656

Shin SC. (2011). Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334: 670–674.

Tamas, I., Klasson, L., Canback, B., Naslund, A.K., Eriksson, A.S., Wernegreen, J.J. (2002). 50 million years of genomic stasis in endosymbiotic bacteria. Science 296: 2376–2379.

Thao ML, Gullan PJ, Baumann P. (2002). Secondary *(γ-Proteobacteria)* endosymbionts infect the primary *(β-Proteobacteria)* endosymbionts of mealybugs multiple times and coevolve with their hosts. Appl Environ Microbiol 68: 3190–3197.

Trcek, J., Jernejc, K., Matsushita, K. (2007). The highly tolerant acetic acid bacterium Gluconacetobacter europaeus adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH expression. Extremophiles 11, 627–635.

Tremblay, E. (1989). Coccoidea endosymbiosis. In: Insect Endocytobiosis: Morphology, Physiology, Genetics, Evolution (W. Schwemmler & G. Gassner, eds), pp. 145–174. CRC Press, Boca Raton, FL.

Villegas, L.M., Pimenta, P. (2014). Meta genomics paratransgenesis and the Anopheles microbiome a portrait of the geographical distribution of the Anophilne microbiota based on a meta- analysis of reported taxa. Mem Inst Oswaldo cruz, Ri de jenerio, Vol 109(5), 672-684.

Yamada, Y., Yukphan, P., Lan Vu, H.T., Muramatsu, Y., Ochaikul, D., Tanasupawat, S., Nakagawa, Y. (2012). Description of Komagataeibacter gen. nov., with proposals of new combinations (Acetobacteraceae). Journal of Genetics and Applied Microbiology 58, 397–404.

Yukphan, P., Malimas, T., Muramatsu, Y., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M., Yamada, Y. (2010). Neokomagataea gen. nov., with descriptions of Neokomagataea thailandica sp. nov. and

Neokomagataea tanensis sp. nov., osmotolerant acetic acid bacteria of the Alphaproteobacteria. Bioscience Biotechnology and Biochemistry 75, 419–426.

Yamada, Y., Yukphan, P. (2008). Genera and species in acetic acid bacteria. International Journal of Food Microbiology 125, 15–24.

Zwietering, M. H., Jongenburger,I., Rombouts, F. M., (1990). Modeling of the Bacterial Growth Curve. Applied and environmental microbiology. p. 1875-1881.

Chapter II

Evaluation of Acetic Acid Bacteria (AAB) proteome

Abstract:

Aim: In order to highlight pathways involved in the interaction with host a proteomic-based approach was used. In particular the proteomic approach was addressing in evidencing differential expression of specific proteins by comparing bacterial protein profiles grown under different conditions. The growth under hypoxia (6% oxygen) was chosen as the condition emulating the insect gut environment. Acetic Acid Bacteria (AAB) strains were selected from *D. suzukii* and *Anopheles stephensi* based on a previous extensive characterization of 202 isolates were identified by ITS-PCR and 16S rRNA. Most abundant 10 isolates were selected for proteomic analysis by qualitative (SDS-PAGE) and quantitative (Bradford) method. The approximate amount of protein required for 2D-PAGE setup were confirmed by Bradford method. Further, different procedure of sample preparation methods such as Urea buffer extract, NaOH and TCA-Acetone were performed for 2D-PAGE setup in order to focus the clear spots. As a result, TCA-Acetone is the best sample precipitation method compared to other methods. Based on the availability of genome sequence, protein quality and quantity, *Asaia* SF2.1 were selected for 2D-PAGE setup. First (1^{st}) dimension program were optimized with 350-400µg protein of *Asaia* SF2.1 and followed by Second (2^{nd}) dimension, approximately 220 clear round spots were obtained.

Introduction:

Proteomics insights in the study of endo-symbiosis

Proteomics is a field that promises to bridge the gap between genome sequence and cellular behaviour. It aims to study the dynamic protein products of the genome and their interactions. Together, genomic and proteomic approaches promise to reveal the multidimensional view of a biological system (Alain et al., 2007). The cell process is normally dependent upon a multitude of protein-mediated metabolic and regulatory pathways for its survival. There is no strict linear relationship between genes and the protein complement or 'proteome' of a cell. Proteomics is complementary to genomics because it focuses on the gene products, which are the active agents in cells. For this reason, for example, proteomics directly contributes to drug development as almost all drugs are directed against proteins. Any biological conclusion that is drawn from a proteomic study is only as strong as data come from reproducible protein extracts and rich in protein diversity (Lilley et al 2002; Pandey and Mann, 2000). For example, Cilia et al. (2009) discussed about *Schizaphis graminum* (Sg), an aphid species of an agricultural important pest. These aphids are plant-feeding insects and vectors of numerous viruses that infect crop plants and affect the grain sorghum in North America. Proteomics approaches were claimed in understanding the molecular mechanisms of virus transmission in order to reveal targets for new approaches for disease management that specifically disrupt specific-aphid protein functions or protein functions involved in aphid-virus interactions (Hesler et al., 2006; Hogenhout et al., 2008; Snihur et al., 2005 and Zwiener et al., 2005).

The cellular mechanisms responsible for vector specificity are regulated by distinct interactions between virus structural proteins and unknown proteins in the aphid. Furthermore, aphids harbor maternally derived endosymbionts bacterium, including *Buchnera aphidicola*, which are necessary for aphid survival and have been implicated in virus transmission. Besides the obvious damage done to the plant by feeding, aphids are vectors of numerous viruses that infect crop plants. Proteomics approaches were claimed in understanding

the molecular mechanisms of virus transmission in order to reveal targets for new approaches for disease management that specifically disrupt specific-aphid protein functions involved in aphid-virus interactions (Yang, et al.,2008; Seddas et al.,2004; Papura et al., 2002). The cellular mechanisms responsible for vector specificity are regulated by distinct interactions between virus structural proteins and unknown proteins in the aphid. Furthermore, aphids harbor maternally derived endosymbionts bacterium, including *Buchnera aphidicola*, which are necessary for aphid survival and have been implicated in virus transmission (Yang et al., 2008). Furthermore, multidimensionality of symbionts–host interactions has been performed on the Hawaiian squid Euprymna scolopes and the luminous bacterium *Vibrio fisheri* (Verma, SC., and Miyashiro, T. 2013).

The AAB microbiome of the spotted wing fly, *Drosophila suzukii*

Symbiotic relationships between insects and microorganisms are widespread in nature. In recent years, the microbiota associated to insects, and the mechanisms determining these associations, are under investigation, because of the possibility that these microorganisms interfere with the capacity of insect pathogen transmission (Chung and Kasper, 2010)**.** Many insects are, in fact, vectors of most severe diseases in plant, animal and human affecting the agricultural production and the environment (Cini et al., 2012). *Drosophila suzukii* (Diptera: Drosophilidae) is an emerging pest in the Western countries. It is a high invasive species that has been introduced from Southern Asia, also found in European countries including (Italy, Spain, and France). This pest (*D. suzukii*) is able to attack healthy soft fruits, differently from the other *Drosophila* species, which attacks rotten and overripe fruits are considered secondary parasites. *D. suzukii* eggs are laid inside the fruits and the hatched larvae grow causing severe damages (Walsh et al., 2011). *D. suzukii* is known to infest wide host plants ranging from blueberries, strawberries, cherries, raspberries, blackberries, grapes, figs, apricots, peaches, and plums. The adult's flies commonly referred as Spotted Winged Drosophila (SWD) are 2-3 mm in length, red eyes resembling vinegar flies *D. melanogaster.* A promising, but sometimes

neglected method of reducing pest populations is the exploitation of the intimate association of the pest species with endosymbionts. For instance, arthropods are frequently infected with one or more microorganisms which can have beneficial, neutral or detrimental effects on their hosts. These interactions may directly or indirectly influence the population dynamics of many pest species and could thus potentially be of great interest for agricultural pest management (Zindel et al., 2011). For insect vectors of disease, symbiotic microorganisms can influence vectoring efficiency (McMeniman et al., 2009; Ricci et al., 2012) or developmental time and thus providing targets for potential disease control.

The *Anopheles stephensi* bacterial endosymbionts: *Asaia* as a model system:

Asaia is an AAB genus belonging to the family Acetobacteraceae, which is traditionally isolated from fermented foods and plant materials. In recent years, bacteria of this genus have been observed to infect insects belonging to different orders, including Diptera, Hemiptera, Hymenoptera and Lepidoptera. Many of the species known to be stably associated with *Asaia* are important vectors of human interest (e.g. *Anopheles* and *Aedes* mosquitoes). Besides mosquitoes are vectors of a variety of infectious diseases which have considerable effect on public health, like malaria, yellow fever, dengue and chikungunya. Although the common knowledge that these diseases are caused by microorganisms, the interactions between mosquitoes and their microbial community have not been deeply investigated.

The *Anopheles* mosquitoes are vectors of *Plasmodium* parasites, the causative agents of malaria. Promising tools in the control of disease-transmitting mosquitoes like *Anopheles* and AAB symbionts, '*Asaia'* is a useful model (Dillon et al., 2004; Favia et al., 2007; Crotti et al., 2010). It was recently shown that, the main component of genus *Asaia,* is stably associated with larvae and adults of the malaria mosquito vectors such us *An. stephensi, An. maculipennis* and *An. gambiae* and promotes the development of

mosquitoes (Crotti et al., 2010; Favia et al., 2007). The distribution of *Asaia* in the body of *An. stephensi* has been investigated by the use of a green fluorescent protein containing *Asaia* strain through which, it was possible to track the behaviour of the symbiont with in the different parts of the host body such us gut, salivary glands and male and female reproductive organs, which are essential for bacterial transmission by horizontal and vertical routes in insects for the development of the cycle of malaria *plasmodium* parasites(Crotti *et al*., 2009). Moreover, some features of *Asaia* traits for making a useful system to understand in insect : (i) the efficient capacity of *Asaia* of colonizing adults and larvae of *An. stephensi* by the way of multiple transmission routes (horizontal acquisition through feeding, venereal transmission from male to female and maternal transmission to offspring and vertical transmitted from the mother to offspring by egg smearing (Damiani *et al*., 2008); (ii) the ability to efficiently spread through insect populations supported by the capacity of the bacterium to colonize and cross-colonize phylogenetically related or distant hosts. By using suitable heterologous secretion signals several anti-plasmodium effector proteins could be efficiently secreted by the strain without apparently affecting the growth rate of Plasmodium in the mosquito midgut in order to block the diffusion of a pathogen (Bisi and Lampe, 2011). These symbionts are recently focusing attention because of their recognition as strong and effective immunomodulators of insects**.** In *Anopheles gambiae*, it has been shown that the *Asaia* titer in the host body is kept under control of the innate immune system and it massively proliferates in the hemolymph when the hypervariable immunoglobulin domain-encoding gene (AgDscam) component of the immune response is silenced (Dong et al., 2006). In another example, *Asaia* sp. colonize in gut epithelial cells of mosquito *Anopheles* sp. through a specific association between the insect epithelial cells and extracellular polysaccharide matrix that surrounds bacterial cells (Crotti *et al*., 2009, Favia et al., 2007). Because our *A. pomorum* strain produced large quantities of extra cellular polysaccharides, which contact between *Drosophila* gut epithelial cells and *A. pomorum* may be similar to that observed between *Asaia* and *Anopheles* gut cells (Shin et al., 2011). However little information is known about the effect of *Asaia* on the host. In *Drosophila melanogaster* AAB have

been shown to regulate the microbiota homeostasis, by keeping under control pathogenic species following a fine tuning of the host immune response (Roh SW et al 2008).

Molecular pathways of endo-symbionts favoring the mid gut colonization

Insect guts present distinctive environments for microbial colonization, and presence of bacteria in the gut potentially provide many benefits to their hosts. Insects display a wide range in degree of dependence on gut bacteria for basic functions. Therefore, further research has to be conducted to clarify the molecular mechanisms at the base of the symbiosis. Fine regulation of the immunity in the host gut is required for homeostasis of gut microbiota. Several research teams have investigated the relationship between AAB and their symbionts (Engel and Moran, 2013). Recent progress toward understanding gut-microbe interactions using Drosophila revealed that a fine regulation of gut immunity is required for the preservation of a healthy commensal community structure for promoting the host fitness and for ensuring normal host survival rates. Also gut microbial community is involved in several aspects of the host life, ranging from the nutritional contribution to the energy salvage through fermentation, protect from predators, parasites, and pathogens; contribute to inter and intraspecific communication affect efficiency as disease vectors and govern mating and reproductive system (Sharon et al., 2010) (Dale and Moran, 2006).

Generally, gut microbiota regulates the fine immunity, host fitness and host survival rate. The gut microbiota controls the gut epithelial cells by regulating stem cell activity and it is also involved in energy balance and metabolic homeostasis of host. Recent studies shown that the drosophila gut controls bacteria populations by producing antimicrobial molecules at the intestinal epithelial cell layer, such as: anti-microbial peptides (AMPs) and reactive oxygen species (ROS), (Liehl et al., 2006; Nehme et al., 2007; and Buchon et al., 2009b). The peptidoglycan mediated generation of (AMPs) only activate immune deficiency (IMD) pathway (Engel P and Moran NA. 2013). A

recent study highlighted the role of bacterium-secreted uracil as a signal molecule controlling immunity in the gut of flies. Only uracil-producing bacteria induce a dual oxidase DUOX, mediated generation of Reactive Oxygen Species (ROS) having bactericidal effect (Ha et al., 2009) . ROS also act as key messengers that modulate the protein degradation machinery of several signaling components of signalling pathway, leading to gut development and reduce the gut inflammation. In conclusion, the 'fine-tuned' regulation of two synergistically acting immune responses, consisting of AMP production and ROS synthesis, seems to contribute to homeostasis in the midgut of *D. melanogaster* by tolerating the commensal microbiota and combating deleterious pathogens. Recent publications shows that in different Drosophila strains two taxonomically different bacteria, i.e. *Acetobacter pomorum* and *Lactobacillus plantarum*, modulate the insulin signaling pathway and target of rapamycin (TOR) pathway, this TOR pathway has central role in organizing the cell growth respectively, through different bacterial products (Douglas, 2011; Storelli et al., 2012).

In *Acetobacter pomorum*, the acetic acid produced by the activity of the pyrroloquinoline quinone dependent alcohol dehydrogenase (PQQADH) modulates the insulin signaling in Drosophila, which in turn controls several host homeostatic programs, including the developmental rate, the body size, the energy metabolism and the intestinal stem cell activity (Shin et al., 2011). PQQ-ADH is the primary dehydrogenase in the ethanol oxidative respiratory chain of *Acetobacter pomorum.* This PQQ-ADH activity of a *Acetobacter pomorum*, modulates insulin/insulin-like growth factor signaling (IIS) in Drosophila to regulate host homeostatic programs controlling developmental rate, body size, energy metabolism, and intestinal stem cell activity (Shin et al., 2011). Finally that, the *Drosophila-Acetobacter* interaction system is a useful genetic model for understanding the mechanisms links between microbiome-modulated host signaling pathways and host physiology. Furthermore, another study highlighting, *Lactobacillus plantarum,* a strain isolated in *Drosophila* gut, it seems to enhance protein assimilation from the diet, resulting in the regulation of diet derived branched chain amino acid (BCAA) levels in the

hemolymp. This in turn stimulates TOR kinase activity both in the fat body and the prothoracic gland. In the fat body, TOR activity optimizes systemic InR signaling and promotes growth rate, while in the prothoracic gland, TOR potentiates (Ecd) ecdysone steroid hormone production during late larval stage to reduce the length of the growth phase. In *Drosophila*, genetic studies have implicated the product of the slimfast gene in the regulation of TOR kinase activity in the fat body (Colombani et al., 2003). In fat bodies, TOR pathway normally acts stimulating the systemic production of insulin like peptides and thus promoting the growth. It has been hypothesized that (1) the stimulation of the insulin signaling in the presence of commensals could be the result of the evolution conflict between the host and its microbiota; (2) bacterial metabolites are cues for the host to be informed on the environmental nutritional availability for the host development (Hamdi et al., 2011). Thus according to this second hypothesis the host would exploit its microbiota to sense the environment. Bacteria are known to communicate through quorum sensing, which allows the regulation of their activity and physiological processes. This process depends on the synthesis and diffusion of signaling molecules, called autoinducers, into the surrounding environment. Quorum sensing outcomes are important advantages for bacteria, i.e., host colonization, defense against pathogens, bioluminescence production and adaptation to changing environments. This kind of interaction here hypothesized implies a higher level of interaction between symbionts and host. The molecular mechanisms that regulate the host microbe crosstalk are still poorly understood. However, all these studies highlight the key role of microbial partners in influencing the systemic growth of the host and in preserving its health. The work of (Ryu et al., 2008), indicates that AAB have an important role in regulating the immune system of homeostasis of the regulation of the immune response in insects and suggests that AAB could be tools for interfering with the immune response and hence the survival of the host (Li et al., 2012).

Finally, all mentioned studies shows that there is a strict and constant association between *Drosophila* and AAB, within the particular genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*. All these investigations

shows that AAB are among the dominant microorganisms in the bacterial community associated to these insects and can compete with the pathogen along the host epithelia, physically occupying the available niches and nutritionally competing with the pathogens (Kommanee et al., 2008). Moreover, acid and exopolysaccharide production may contribute to successful AAB colonization of the insect gut (Kounatidis et al., 2009; Cox and Gilmore, 2007).

Application of two-dimensional gel electrophoresis (2DE) to microbial system

The introduction of 2D-PAGE in 1975 by O'Farrell for separating cellular proteins under denaturing conditions enabled the resolution of hundreds of proteins. The 2DE is unique not only for its ability to simultaneous separation of proteins but also for detecting post and co-translational modifications, which cannot be predicted from genome sequences (Lopez 2007). The term *proteome* refers to the proteins expressed by a genome at a particular point in time. The proteome is like a photographic snapshot of the protein expression at a particular moment and under specific conditions. The genome provides only static information, while the proteome provides an overall information of the cell machinery, which can be studied under various conditions and could provide information regarding dynamic processes. The clarification of the expressed part of the genome is required to link genomic data to biological functions (Gallo et al., 2010). The principle of 2D-PAGE separation based on two independent properties that is IEF in the first dimension, followed by SDS-PAGE in the second dimension. Proteins are separated on a strip using isoelectric focusing (IEF) (Fig. 2.1), which separates proteins according to their isoelectric point, followed by electrophoresis in a second dimension in the presence of sodium dodecyl sulfate (SDS), which separates proteins according to their molecular mass (Issaq and Veenstra 2008). Each spot on the resulting two-dimensional gel potentially corresponds to a single protein species of the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein can be obtained. However, one of the key areas where 2DE gels should deliver in the future will be the study of modification landscapes, i.e. how

protein modifications combine to modulate protein activity in cells. The fundamental goal of molecular biological research is to determine the function of genes, the role of proteins in metabolic pathways and networks and finally provide a detailed understanding of how these molecules interact and collaborate to work in a biological system under different conditions. This is achieved by considering biological systems as a whole and not individual part, which is referred to as system biology. Applications of 2-DE include proteome analysis, cell differentiation, and detection of disease markers, therapy monitoring, drug discovery, cancer research, purity checks, and microscale protein purification.

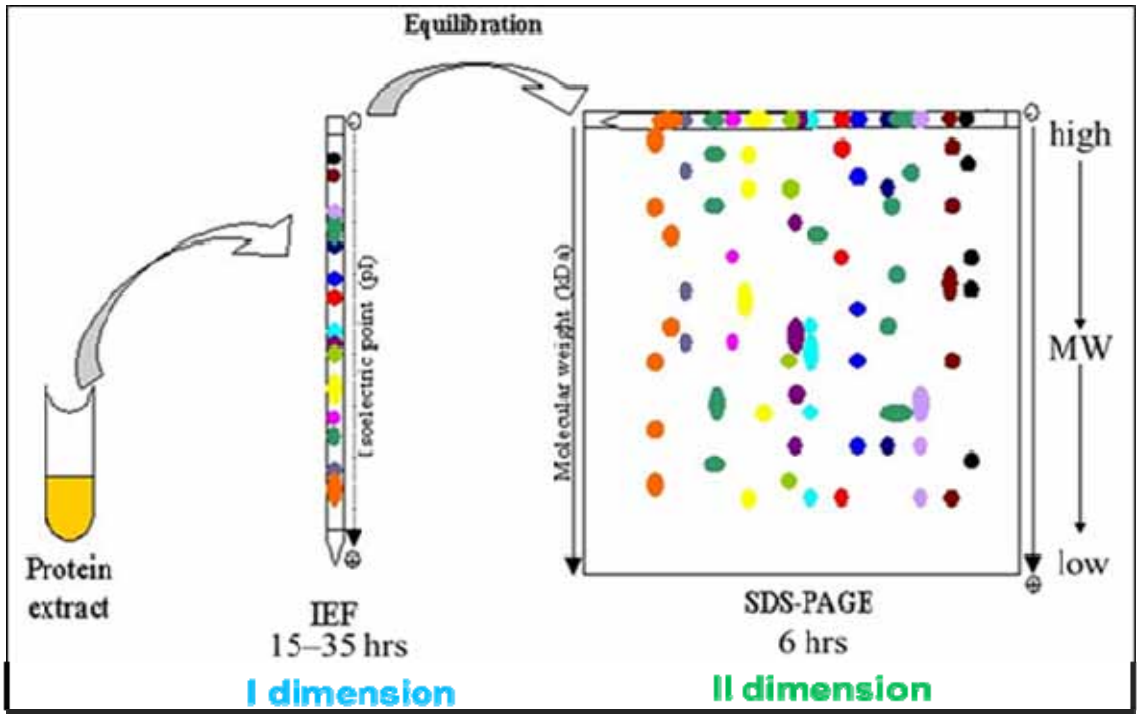


Figure 2.1 General principles of 2D-PAGE: Mixtures of proteins are separated by two independent properties: First dimension (Protein separation by charge- pI); Second dimension (Protein separation by size- MW). Initially extraction of proteins from the biological sample to get an IEF-compatible sample. The sample is then loaded onto a pH gradient oriented with the acidic side at the anode and the basic side at the cathode. After the IEF step, the proteins have reached their pI and thus have no remaining electrical charge. The strip is then equilibrated in a SDS-containing buffer, so that all proteins becomes strongly negatively charged. The IEF gel is then loaded on top of a SDS PAGE gel, and the proteins are separated according to their molecular masses. After this step, the proteins are detected directly on the gel.

Results and Discussion

Setting protein extraction for 2DE of *Asaia* SF2.1

From total 202 isolates, most abundant 10 isolates (Table 2.1) of AAB were selected from *D. suzukii* and *Anopheles stephensi*, belongs to four genera i.e. *Gluconobacter*, *Acetobacter, Komagataeibacter* and *Asaia.* The protein profiles of isolates extracts were determined using Bradford assay and Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE). In qualitative method, quality of protein extraction were purified by denaturing gel electrophoresis according to Laemmli protocol (Laemmli, et al 1970). Results from SDS-PAGE analysis (Fig. 2.2) shows the separated protein bands after dilutions (2x and 2.9x) of same sample and clear protein bands were observed, where each band had the same relative intensities as their corresponding bands in the mixture lanes. Quantification of protein were performed by Bradford method followed by SDS-PAGE (Fig 2.3). According to the information from Bradford method, the approximate amount of protein (350-400µg) used for 2D-PAGE setup. Based on the protein quality and quantity, *Asaia* SF2.1 (available genome sequence) were selected for 2D-PAGE setup.

Strain name	Isolate number	Biomass yield (mg/ml)
Acetobacter indonesiensis	44	5.9
Acetobacter persicus	45	5.7
Acetobacter tropicalis	23	3.4
Asaia SF2.1	-	5.5
G. kanchanaburiensis	15	2.0
Gluconobacter oxydans	9A	2.2
Gluconobacter kondonii	1C	1.9
K. saccharivorans	65A	2.4
K. hansenii	114	19.5
K .intermedius	59B	6.9

Table 2.1 Shows the list of most abundant 10 isolates for protein analysis.

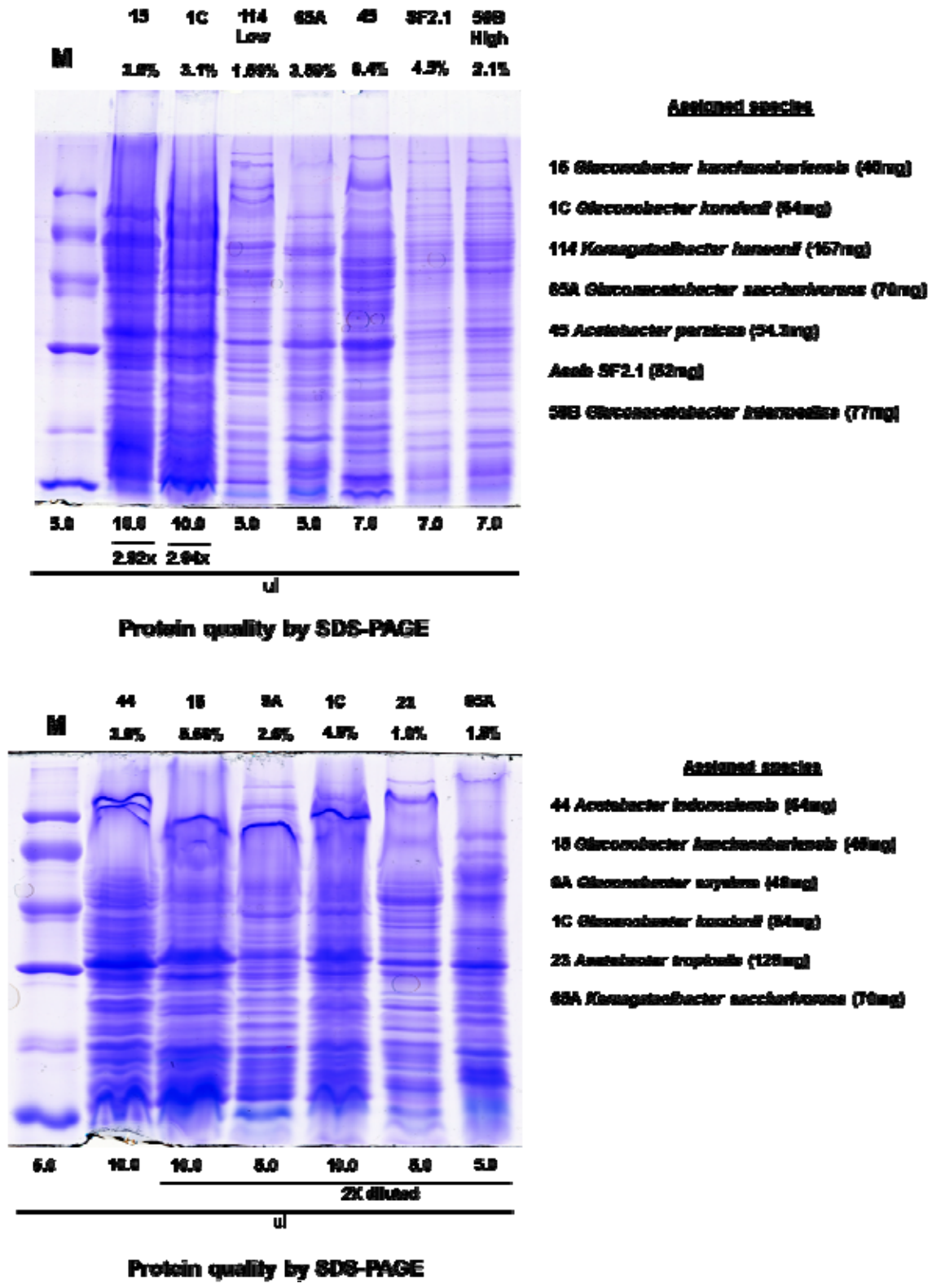


Figure 2.2 SDS-PAGE qualitative analysis of different protein extracts of AAB isolates. Each lane represents different percentage of protein sample of AAB isolates (i.e., the total pellet weight of protein concentration of isolates species).

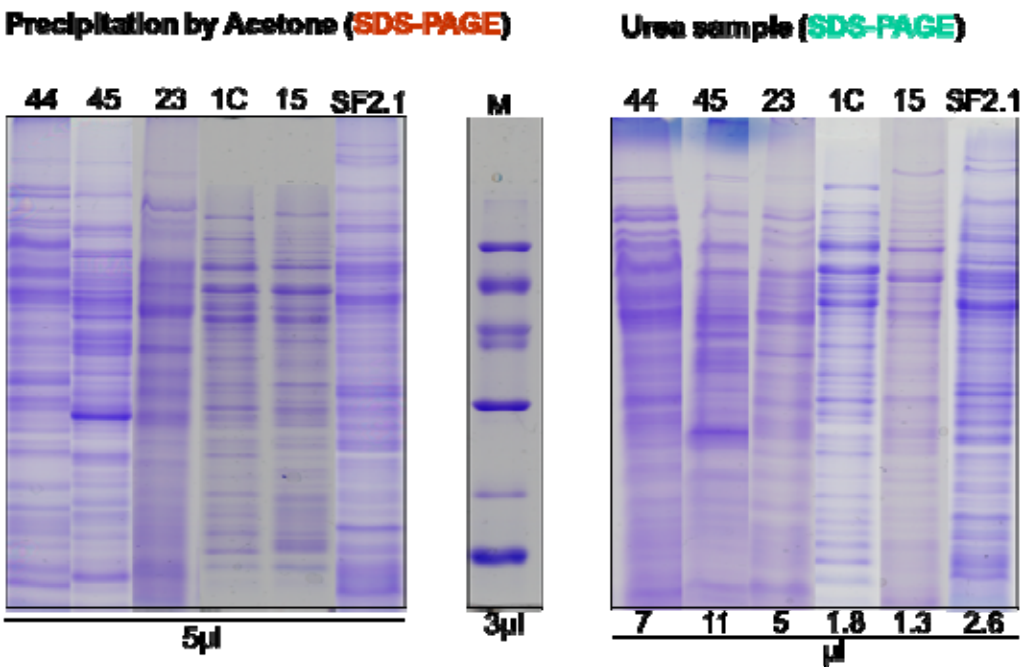


Figure 2.3 SDS-PAGE analysis comparison between urea extract sample (5µg/µl) and acetone precipitation (25µg/(concentration/2) of AAB isolates. Each lane represents proteins from urea sample (right) and acetone sample (left) based on the protein concentration obtained by Bradford assay (µg/µl).

2DE PAGE setup of *Asaia* SF2.1

Initially, protein extraction methods were followed for sample preparation in order to focus the clear spots of *Asaia* SF2.1 protein profile. Generally, the sample preparation (Fig. 2.4) step is absolutely essential for good focusing of 2D-PAGE results. During this process, cells must be disrupted completely in order to focus clear proteins spot and it's important to minimize protein modification or degradation, thus avoiding a quantitative loss of high molecular weight proteins, membrane proteins, and/or nuclear proteins. The first-dimension IEF step of 2DE electrophoresis is particularly sensitive to low molecular weight ionic impurities. Non protein impurities in the sample can interfere with separation and cause horizontal streaks and vertical streaks result from less protein separation in the SDS-PAGE. The three important goals in sample preparation were followed: (i) complete dissociation of protein/protein interactions, (ii) removal of non-protein sample components (e.g., ions, lipids,

nucleic acids, and salts), and other contaminants may affect focusing of 2DE results. and (iii) significant reduction or elimination of protease activity (Nandakumar et al 2003)**.** During the sample preparation any contaminant with a net ionic charge will affect IEF and lead to horizontal streaking (Rabilloud 1998). Focusing of the proteins will not occur until the ions have moved to the ends of the strips, prolonging the time required for IEF. To alleviate these problems, salts can be removed and maintained at lower than 10mM in the sample preparation steps, in order to reduce the ionic strength of protein samples. TCA-acetone precipitation is particularly effective for removing salts and other contaminants, but also result in protein losses. Nucleic acid contamination samples contribute to horizontal streaking (Gorg., et al., 1997).

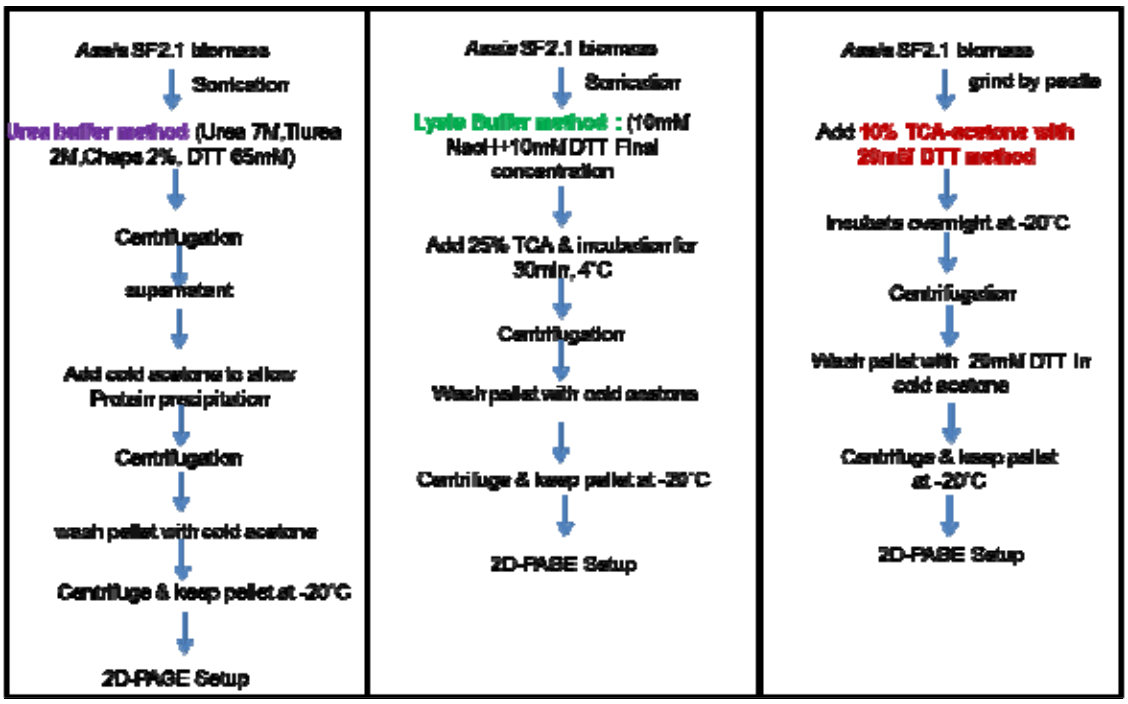


Figure 2.4 Sample preparation of *Asaia* SF2.1 by protein extraction methods (i.e., Urea buffer method, Lysis buffer method and TCA-acetone method)

Many proteins bind nucleic acids, which can generate a mixed population of protein-nucleic acid species, each with distinct isoelectric points. Nucleic acids removed from samples by treating the sample with nucleases prior to IEF. Ultracentrifugation used to remove large nucleic acids. However, this

technique may also remove high molecular weight proteins from sample. Also protein samples always prepared using high purity urea and not heated above 30°C. Polysaccharides can clog gel pores, preventing sample entry into the IPG strip, through affect precipitation, focusing times, resulting in horizontal streaking. Ultracentrifugation often sufficient to remove carbohydrates. Many proteins, particularly membrane proteins, are complexed with lipids. This reduces their solubility and can affect both the pI and the molecular weight. Lipids form complexes with detergents, reducing the effectiveness of the detergent as a protein-solubilizing agent. When we prepared the samples all these points were considered according to our setup. During the sample preparation, different protocols were performed to understand the suitable method for protein extraction in order to focus clear spots such as urea buffer extract method, NaOH method and TCA-acetone method. The samples prepared by the mentioned protocols were submitted to 2D-PAGE. The gel images results (Fig. 2.4) concludes, TCA–acetone method, showed the good quality of protein and obtained the clear spots of protein profile of *Asaia* SF2.1. TCA–acetone, which is an organic solvent commonly used to precipitate the protein, more effective to remove the residual part of salts and TCA plays the role in removing polysaccharide clogs from the gel pore. The gel image results (Fig. 2.5) concludes, urea buffer extract and NaOH methods shows the poor quality of protein spots of *Asaia* SF2.1, and not a suitable sample precipitation methods as the samples were not solubilized completely due to the presence of salts and ions, which affects the horizontal streaking and as a result the proteins were not clearly focused.

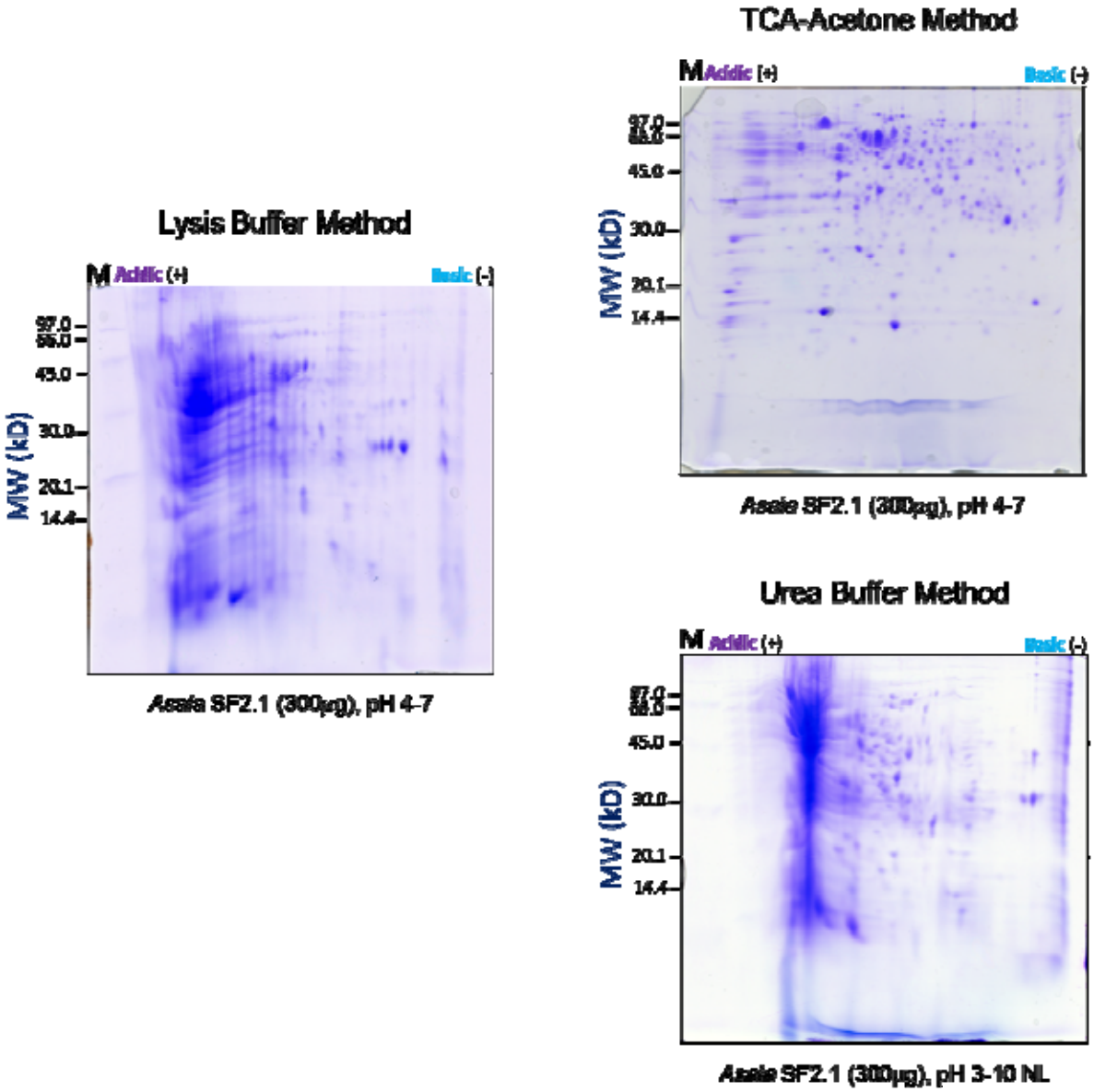


Figure 2.5 Comparison of 2DE protein profile between different protocol of sample preparation method such as Urea Buffer extract, NaOH method and, TCA-Acetone method followed by 2D-PAGE setup.

Conclusion

The condition for protein extraction methods were optimized and obtained the good quality of protein extraction of all strains of AAB using SDS-PAGE. Further quantification of protein were performed by Bradford method to measure the amount of protein concentration of all strains. The amount of protein (350-400ug) required for 2D-PAGE setup were confirmed by Bradford method. In comparison with all AAB strains, *Asaia* SF2.1 were selected for further 2D-PAGE setup based on the availability of genome sequence, protein quality and quantity. IEF (Iso electric focusing) program were successfully optimized for 1st dimension. The different trials of sample precipitation method were carried out followed by 2D-PAGE setup. Our results indicates that, TCA-acetone were the best sample precipitation method and TCA-acetone showed the clear round spots of proteins as the precipitates were solubilized completely. Further, comparative proteomic analysis of the selected bacterial strains (*Asaia* SF2.1) in different growth conditions (6% and 20% oxygen) in bioreactor were performed.

Materials and method

SDS-PAGE: Isolates strains of biomass (approx. 60-200 mg, wet weight), was taken from -80°C and cell pellet was re-suspended in 2 volume (v/w) of extraction mixture (i.e., 1 volume of phosphate buffer saline and 1 volume of denaturing buffer). The final extraction mixture was obtained by adding to the cell pellet. At first, 1 volume of phosphate buffer saline (PBS 1X; 15 mM KH_2PO_4, 150 mM NaCl, pH 7.4), 5-10 min vortexed, and then 1 volume of 2X SDS-PAGE sample buffer (0.5M Tris-HCl, pH 6.8, 0.4% SDS-150mg, Bromophenol blue solution-1 ml, β-mercapto ethanol-100 µl adjusted to 4 ml with glycerin) was added. After that 5-10 min vortexing, extracted samples were heated for 5 min by immersion in boiling water. Then loaded the sample 10 µl and 5 µl marker on SDS-PAGE. 12% poly acrylamide separating gel and 4% stacking gel were used for SDS-PAGE and pre-stained protein marker-brand range (Bio-Labs) was used as a molecular weight standard. SDS-PAGE gels were run at 80 V for 15 min and then, at 120 V for 2 h (Laemmli, 1970) and proteins were separated by based on molecular weight. At the end of electrophoresis, gels were visualized by staining with Coomassie Brilliant Blue according to (Holtzhauer 2006) and destained for 2 h in a solution containing 50% methanol and 10% glacial acetic acid. Gels were either directly acquired under trans UV light. The SDS-PAGE result showed that some strains had migration problems because some particulates of solid were present in the sample, So further dilutions (2x diluted and 2.9x diluted) of same sample were made and observed clear protein band of each strain.

Determination of the protein concentration by (Bradford assay): Protein concentration of complex samples was determined by the Bradford assay (Bradford et al., 1976) using bovine serum albumin (BSA) as standard assay (2mg BSA /ml of buffer) by absorbance 595nm at spectrophotometer (T80 UV-VIS spectrophotometry - PG Instrument ltd) (Colnaghi et al., 1996). This technique is a simple and accurate procedure for determining the concentration of protein in solution. This assay involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford et al,

1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form. However, when the dye binds to protein, it is converted to a stable unprotonated blue form. This blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer.

Sample preparation methods for protein extraction: Sample preparation is possibly the most important step of 2-DE because the quality of the 2-DE pattern largely depends on it. It has been necessary for the efficient solubilization of proteins and removal of the contaminants present in the sample often conflict with the constraints imposed by IEF with regard to sample buffer composition. During the sample preparation, 3 different trials were performed to understand the suitable method for sample in order to focus clear spots such as Urea Buffer extract method (Trial 1), NaOH method (Trial 2) and TCA-Acetone method (Trial 3).

Trial 1. Urea Buffer extract (Duche et al. 2002; Roy et al. 2014). The cell pellet of *Asaia* SF2.1 were used for protein extraction using urea extract buffer (Urea 7M, Tiurea 2M, Chaps 2%, DTT 65mM). Stored (-80°C) cell pellet of *Asaia* SF2.1 aliquots were used for protein extraction using urea extract. Normally urea is a one of the strong denaturing buffer and standardized procedure for extracts sample preparation. Initially, depends on the cell pellet weight (proposition: 125mg cell/ml of urea), pellet were re-suspended with urea buffer. Cells were broken by sonication (5 cycle of sonication at 30% power) followed by collection of urea supernatant by centrifugation and protein were quantified by Bradford method. The BSA were used for standard assay (2mg BSA /ml of buffer) and determined the protein concentration of *Asaia* SF2.1 cell pellet. According to the protein concentration, 2D-PAGE Setup were performed.

Trial 2. NaOH method (Mehmeti et al. 2011). The cell pellet of *Asaia* SF2.1 were re-suspended in lysis buffer instead of Urea buffer according to the propositions (800 µl of lysis solution/100mg of cell). After sonication process, 25% of TCA were added and incubated for 30 min at 4˚C followed by centrifugation process, after certification, protein were quantified by Bradford

method. Finally washed the pellet with cold acetone in order to remove the acetone odour in the pellet and stored at -20˚C until to start with 2D-PAGE Setup.

Trial 3. TCA-Acetone method (Valcu and Schlink K. et al., 2006). The TCA and acetone is commonly used to precipitate proteins during sample preparation of 2DE. Because it's effectively removing the salts, other contaminants and also solubilize the protein completely. Generally, acetone is an organic solvent and TCA plays the role in removing the polysaccharide clogs from gel pores otherwise it will affects horizontal streaking during focusing and also DTT (always freshly prepared) is used as protease Inhibitors. The cell biomass of *Asaia* SF2.1 were grinded using pestle instead of sonicator and 10% cold TCA-acetone with DTT were added instead of urea buffer and lysis buffer, and incubated overnight at -20˚C. After centrifugation, the pellet were washed with 20mM DTT in cold acetone instead of room temperature (RT) acetone followed by centrifugation and the pellet were stored at -20˚C until to start with 2D-PAGE Setup.

2-Dimensional gel electrophoresis (2D-PAGE): In any proteomic experiment, the most important step as a reliable and comprehensive protein extraction is the closest proteomic equivalent to a fully sequenced and annotated genome. 2DE gel electrophoresis was performed according to the method of (Mehmeti, et al., 2011) with some modifications. After sample preparation of each trial, the sample was re-suspended in rehydration buffer in order to focus IEF for 1st dimension and further followed SDS-PSGE. The protocol was common for all trials. Briefly, crude protein extracts were solubilized in 250µl (350-400µg) in a rehydration buffer (Urea 7M,Tiurea 2M, Chaps 2%, 0.05% 4-7 IPG buffer (Amersham), DTT 10 mg/ml and applied on 13cm IPG strips (Amersham Bioscience, pH 4-7 NL) rehydration with sample on re-swelling tray. The IPG strips with solubilized sample were rehydrated overnight at 20˚C under mineral oil for the first dimension. The strips were focused on Ettan IPGphor II (GE Healthcare). IEF was performed for a total of 50 kVh at 20 °C with the following program steps: 17V for 3.5h, 25V for 3.5h, and 100V for 1.5h, 500V for 3.5h, 1000V for 4h, and 6500V for 3.5h. After IEF,

strips were equilibrated prior to the second dimension twice for 15 min under gentle shaking at room temperature in 5 ml equilibration solution (1.87M Tris HCl pH 8.8, 6 M urea, 2% SDS, 20% w/v glycerol, 0.002% bromophenol blue, 1% DTT) and then for an additional 15 min in the same solution, except 1% DTT which was replaced with 5% iodoacetamide respectively. Equilibrated strips were positioned and sealed on top of the second dimension gel with a 0.5% low melting agarose solution. The focused proteins were separated in a vertical SDS-PAGE in 12% (w/v) polyacrylamide gel. Separation was performed in a Bio-Rad system using constant amperage (20 mA per gel). Following electrophoresis, the gels were stained with coomassie blue standard protocol at overnight; 10% acetic acid / 50% ethanol and 0.05% R-250 brilliant blue overnight; followed by destaining with 10% acetic acid / 30% ethanol and Gels were digitized using VersaDoc MP Image System(Bio Rad).

References

Alain, M.P., Christophe,M., Severine,S., Houria,A., Philippe,I., Lionel,R., (2007). Protein Extraction and Fingerprinting Optimization of Bacterial Communities in Natural Environment Volume 53, 426–434.

Bradford, M.M., (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of proteindye binding. Anal. Biochem. 72: 248-254.

Bisi, D.C., Lampe, D.J., (2011). Secretion of anti- Plasmodium effector proteins from a natural *Pantoea agglomerans* isolate by using PelB and HlyA secretion signals. Applied Environmental Microbiology; 77: 4669–4675.

Buchon, N., Broderick, NA., Poidevin, M., Pradervand, S., Lemaitre, B., (2009b). Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe 5: 200–211.

Chouaia B, Rossi P, Montagna M, Ricci I, Crotti E, Damiani C, Epis S, Faye I, Sagnon N, Alma A, Favia G, Daffonchio D, Bandi C, 2010. Molecular evidence for multiple infections as revealed by typing of Asaia bacterial symbionts of four mosquito species. Appl Environ Microbiol. 2010 Nov; 76(22):7444-50.

Chung, H., Kasper, D.L., (2010). Microbiota-stimulated immune mechanisms to maintain gut homeostasis. Current opinion in immunology 22,455-460.

Cini, A.C., Oriatti, C.I., Anfora, G., (2012). A review of the invasion of Drosophila suzukii in Europe and a draft research agenda for integrated pest management. Bulletin of Insectology 65, 149–160.

Cilia, M., Fish, T., Yang, X., Mclaughlin, M., Thannhauser, T.W., Gray, S., (2009). A Comparison of Protein Extraction Methods Suitable for Gel-Based Proteomic Studies of Aphid Proteins. Journal of Biomolecular Techniques 20, 201–215.

Colnaghi, R., Pagani, S., Kennedy, C., Drummond, M., (1996). Cloning, sequence analysis and overexpression of the rhodanese gene of *Azotobacter vinelandii*. *European Journal of Biochemistry* 236: 240-248.

Cox, C.R., Gilmore, M.S. (2007). Native microbial colonization of Drosophila melanogaster and its use as a model of Enterococcus faecalis pathogenesis. Infect Immun 75:1565–1576.

Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A., Ricci, I., 2009. *Asaia,* a versatile acetic acid bacteria symbiont, capable of cross colonizing insects of phylogenetically distant genera and orders. Environmental Microbiology;11,3252–3264.

Crotti E, Rizzi A, Chouaia B, Ricci I, Favia G, Alma A, Sacchi L, Bourtzis K, Mandrioli M, Cherif A, Bandi C, Daffonchio D, 2010. Appl Environ Microbiol. 2010 Nov; 76(21):6963-70.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., and Le opold, P. (2003). A nutrient sensor mechanism controls Drosophila growth. Cell 114, 739–749.

Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, et al. (2008). Paternal transmission of symbiotic bacteria in malaria vectors. *Curr Biol* 18: R1087–R1088.

Dale C. and Moran, NA. Molecular interactions between bacterial symbionts and their hosts. 2006. Cell. 126:453–465.

Duche,O., Tremoulet, F., Glaser, P., Labadie, J., (2002). Salt stress proteins induced in Listeria monocytogenes. Appl. Environ. Microbiol. 68(4): 1491-1498.

Douglas, A. (2011). Is the regulation of insulin signaling multiorganismal? Science Signaling .4. pe46.

Dong, Y., Taylor, HE. Dimopoulos, G., (2006). AgDscam, a hypervariable immunoglobulin domain-containing receptor of the Anopheles gambiae innate immune *system. PLOS Biology 4, 229.*

Dillon R.J. and Dillon V.M. (2004). The gut bacteria of insects, non-pathogenic interactions. *Annu Rev Entomol* 49, 71-92.

Engel P, Moran NA. 2013. The gut microbiota of insects-diversity in structure and function. FEMS Microbiology Review. 37:699–735.

Favia G., Ricci I., Damiani C., Raddadi N., Crotti E., Marzorati M,.(2007). Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. Proceedings of the National Academy of Science USA 104:9047–9051.

Gallo, G., Alduin, R., Renzone, G., Thykaer, J., Bianco, L., Eliasson-Lantz, A., Scaloni, A., Puglia, A.M. (2010). Differential proteomic analysis highlights metabolic strategies associated with balhimycin production in *Amycolatopsis balhimycina* chemostat cultivations. *Microbial Cell Factories, Vol. 9*, pp. 95-109.

Gorg., A., Obermaier, C., Boguth., G., Csordas, A., Diaz, J., Madjar, J., (1997). Very alkaline immobilized pH gradients for two dimensional electrophoresis of ribosomal and nuclear proteins, Electrophoresis 18, 328–327.

Hamdi C., Balloi A., Essanaa J., Gonella. E., Raddadi N., Ricci I., (2011). Gut microbiome dysbiosis and honeybee health. Journal Applied Entomology 135:524–533.

Ha, E.M., Lee, K.A., Seo, Y.Y., Kim, S.H., Lim, J.H., Kim, J., Lee, W.J., (2009). Coordination of multiple dual oxidase regulatory pathways in responses to commensal and infectious microbes in drosophila gut. Natural Immunology 10: 949–957.

Hesler LS, Riedell WE, Langham MA, Osborne SL. 2005. Insect infestations, incidence of viral plant diseases, and yield of winter wheat in relation to planting date in the Northern Great Plains. J Econ Entomol;98:2020 –2027.

Hogenhout SA, Ammar el-D, Whitfield AE, Redinbaugh MG. 2008. Insect vector interactions with persistently transmitted viruses. Annu Rev Phytopathol ;46:327–359.

Holtzhauer M. (2006). Basic methods for the biochemical lab: First English edition. *Springer Berlin Heidelberg,* New York.

Issaq, H.J., Veenstra, T.D., (2008). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives. BioTechniques 25th Anniversary 44:697-700.

J.L. Lopez., (2007). Two-dimensional electrophoresis in proteome expression analysis. Journal of Chromatography B, 849, 190–202.

Kommanee J, Akaracharanya A, Tanasupawat S, Malimas T, Yukphan P, Nakagawa Y, 2008. Identification of *Gluconobacter* strains isolated in Thailandbased on 16S–23S rRNA gene ITS restriction and 16S rRNA gene sequence analyses. Annals of Microbiology. 58:741–7.

Kounatidis I, Crotti E, Sapountzis P, Sacchi L, Rizzi A, Chouaia B, et al. *Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*) (2009). Applied and Environmental Microbiology;75:3281–328.

Li, G., Liu, B., Shang, Y., Yu, Z., Zhang, R., 2012. Novel activity evaluation and subsequent partial purification of antimicrobial peptides produced by Bacillus subtilis LFB112. Annals of Microbiology 62:667–74.

Lilley, KS, Razzaq, A., Dupree, P., (2002). Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation. Current opinion in chemical biology 6:46 –50.

Liehl, P., Blight, M., Vodovar, N., Boccard, F., Lemaitre, B., (2006). Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog 2: e56.

Laemmli, UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Mehmeti, I., Kiran1, F., Osmanagaoglu, O., (2011). Comparison of three methods for determination of protein concentration in lactic acid bacteria for proteomics studies. African Journal of Biotechnology Vol. 10(11), pp. 2178-2185.

McMeniman, CJ, Lane. RV., Cass, BN., Fong AWC, Sidhu M., Wang, Y-F & O'Neill SL., (2009). Stable introduction of a life shortening Wolbachia infection into the mosquito Aedes aegypti. Science 323: 141–144.

Nandakumar, M.P., Jie Shen, Babu Raman, and Mark R. Marten (2003). Solubilization of Trichloroacetic Acid (TCA) Precipitated Microbial Proteins via NaOH for Two-Dimensional Electrophoresis. Journal of Proteome Research 2, 89-93.

Nehme, NT, Liegeois, S., Kele B., Giammarinaro, P., Pradel, E., Hoffmann, JA., Ewbank JJ., Ferrandon, D., (2007). A model of bacterial intestinal infections in Drosophila melanogaster. PLoS Pathog 3: e173.

Pandey, A., and Mann, M., (2000). Proteomics to study genes and genomes. NATURE. vol 405, 15.

Papura D., Jacquot E., Dedryver CA., (2002). Two-dimensional electrophoresis of proteins discriminates aphid clones of Sitobion avenae differing in BYDV-PAV transmission. Arch Virology, 147:1881–1898.

Rabilloud T, (1998). Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis, Electrophoresis 19, 758–760.

Ryu, J.H., Kim, S.H., Lee, H.Y., Bai, J.Y., Nam, Y.D, Bae, J.W., (2008). Innate immune homeostasis by the homeo box gene caudal and commensal gut mutualism in Drosophila. Science 319:777–82.

Roh, SW., Nam, YD., Chang, HW., Kim, KH., Kim, MS., Ryu, JH., Kim, SH., Lee, WJ., Bae JW.,(2008) Phylogenetic characterization of two novel

commensal bacteria involved with innate immune homeostasis in Drosophila melanogaster. Applied Environmental Microbiology. 74, 6171-6177.

Roy,A., Varshney,U., Pal,D., (2014). Avoiding acidic region streaking in two-dimensional gel electrophoresis: Case study with two bacterial whole cell protein extracts. J. Biosci. 39(4), 631–642.

Ricci, I., Valzano, M., Ulissi, U., Epis, S., Cappelli, A and Favia, G., (2012). Symbiotic control of mosquito borne disease. Pathog Glob Health 106: 380–385.

Sharon, G., Segal, D, Ringo, J., Hefez, A., Ziber-Rosenberg, I., Rosenberg, E., (2010). Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. Proceedings of the National Academy of Science USA 107:2051–2056.

Shin, S.C., Kin, SH, You H, Kim, B., Kim, A.C., Lee, K.A., (2011). *Drosophila microbiome* modulates host developmental and metabolic homeostasis via insulin signalling. Science 334:670–4.

Snihur HO, Budzanivs'ka IH, Polishchuk VP. 2005. Monitoring of cereal viruses in agrocenoses of Ukraine[. Mikrobiol Z;67: 88 –95.

Storelli G, Defaye A, Erkosar B, Hols P, Royet J, Leulier F. *Lactobacillus plantarum* promotes Drosophila systemic growth by modulating hormonal signals through TORdependent nutrient sensing. Cell Metabolism 2012; 14:403–14.

Seddas P., Boissinot S., Strub JM., Van Dorsselaer A., Van Regenmortel MH., Pattus F., (2004). Rack-1, GAPDH3, and actin: proteins of Myzus persicae potentially involved in the transcytosis of beet Western yellows virus particles in the aphid. Virology, 325: 399 – 412.

Verma, SC., and Miyashiro, T., (2013). Quorum Sensing in the Squid-Vibrio Symbiosis. International Journal of Molecular Sciences. 14, 16386-16401.

Valcu, C.M., and Schlink,K., 2006, Efficient extraction of proteins from woody plant samples for two-dimensional electrophoresis. *Proteomics 6,* 4166–4175.

Walsh, D.B., Bolda, M.P., Goodhue, R.E., Dreves, A.J., Lee, J., Bruck, D.J., Walton, V.M., O'Neal, S.D., Zalom, F.G., 2011. Drosophila suzukii (Diptera: Drosophilidae): Invasive Pest of Ripening Soft Fruit Expanding its Geographic Range and Damage Potential. Journal Integrity Pest Management. 2, 1– 7.

Yang, X., Thannhauser, T.W., Burrows, M., Cox -Foster D, Gildow, F.E., Gray, SM., (2008). Coupling genetics and proteomics to identify aphid proteins

associated with vector-specific transmission of polerovirus (luteoviridae). Journel of Virology 82, 291–299.

Zindel, R., Gottlieb, Y., Aebi, A., (2011). Arthropod symbioses: a neglected parameter in pest and disease control programmes. Journal of Applied Ecology, 48: 864-872.

Zwiener CM, Conley SP, Bailey WC, Sweets LE. 2005 Influence of aphid species and barley yellow dwarf virus on soft red winter wheat yield. J Econ Entomol;98:2013–2019.

Chapter III

Setting *Asaia* SF2.1 Growth Conditions in Bioreactor

Abstract

Acetic acid bacteria (AAB) known to be a strict aerobic bacteria living in various parts of insect for the establishment of endosymbiotic relationship. Our aim was to produce biomass for 2DE analysis under a condition simulating the hypoxic gut environment. In particular hypoxia and normal oxygen growths were set up in a bioreactor (BioFlo/CelliGen115) with real time monitoring of some parameters (e.g. oxygen level, Nitrogen level, agitation, aeration and foam/level etc.). The comparative proteomic approach requires a consistent amount of bacterial-specific protein, hypoxia was applied as a model condition in order to mimic the insect gut condition. Here we shown the setup of different growth conditions (normal 20% and low oxygen 6%) of *Asaia* SF2.1 strains in bioreactor and stabilized the conditions for bacterial growth strains followed by mass cultivation of *Asaia* SF2.1 growth. Our results shows the setup of different percentage of dissolved oxygen (6% and 20% oxygen) in bioreactor and evaluated the growth curve of *Asaia* SF2.1 in both conditions followed by mass cultivation of *Asaia* SF2.1 growth. Both conditions showed the higher biomass yield at late exponential phase. TCA-Acetone was the best method for protein extraction.

Introduction

Symbiotic bacteria has significant impact on evolution, diversity and it is present all over (Margulis L, et al., 1991 and Ruby E, et al., 2004). Some bacteria are harmful and referred to as parasite or pathogens, while other plays an important role in host system and are known as mutualists. There are diverse group of bacteria and its symbiotic associations with host system,

among which the most cohesive forms are found in so called endosymbiosis, where symbiotic bacterial live inside the host body which allows them to interact intimately with the partners (Yoshitomo Kikuchi,2009). Insects harboring endosymbiotic bacteria are vertically transmitted from mother to the offspring by an elaborate mechanism known as “transovarial transmission” where the symbiont directly infects the developing eggs or embryos within the maternal body (Buchner, 1965; Houk and Griffiths, 1980). The ability of this bacterium to invade different organs of its insect host suggests that *Asaia* can be transmitted by a variety of transmission routes, both vertical and/or horizontal (Damiani et al. 2008; Crotti et al. 2009; Gonella et al. 2012).

Asaia sp. strain SF2.1 is a Gram-negative member of the *Alphaproteobacteria,* family *Acetobacteraceae* (Yamada et al., 2000). Previous studies shows that annotation of genomes of *Asaia platycodi* (isolated from the malaria vector *Anopheles stephensi*) and *Saccharibacter sp* (isolated from honeybee *Apis mellifera*) has revealed a series of potential symbiotic traits that are secretion system (Sec-SRP and Tat for both genomes) of *Asaia platycodi* and *Saccharibacter* and motility and cell surface structures and (type IV and ABC transporters in the case of *A. platycodi*) that may have roles in the cross talk between the bacterium and the insect (Chouaia et al. 2014). Both genomes contain the operon for the production of acetoin and 2,3-butandiol. These molecules have been shown to play a role in insects’ pheromone signaling (Tolasch et al. 2003). 2,3-Butandiol has been implicated in the modulation of the innate immunity response of vertebrate hosts, facilitating tissue colonization by pathogenic bacteria (Bari et al. 2011). This trait was observed in other AAB symbionts (i.e., *Ac. tropicalis, C. intestini, Glucona. diazotrophicus, Glucona. europaeus, Glucona. oboediens, G. frateurii, G. morbifer, G. Oxydans, and G. thailandicus*).

AAB are known to survive in oxygen rich environments (Kersters et al. 2006). In contrast, many arthropod guts have varying oxygen level from aerobic to completely anoxic (pH of 5.8, 5.4) (Sudakaran et al. 2012). All the genes for operons of cytochrome bo3 (CyoA-D) and bd (CydAB) ubiquinol oxidase are

present in the genome of AAB, which have high affinity for oxygen. This indicate that AAB and both the *A. platycodi* and *Saccharibacter sp*., has the capacity to respire through an aerobic respiratory chain independent from the terminal cytochrome aa3 oxidase, an enzyme with low affinity for oxygen, that is absent in AAB. Even though AAB are commonly termed as strictly aerobic organisms thriving in normoxic environments, and it possess ubiquinol oxidases that should allow their survival under micro-oxic conditions, such as those existing in the insect gut (Sudakaran et al. 2012). Furthermore, phylogenetic comparisons shows the presence of terminal oxidase in the common ancestor of AAB and thus forms an ancestral character. *A. platycodi* and *Saccharibacter sp.* and all AAB have the potential to thrive at low oxygen concentrations (Chouaia et al. 2014). Recent technologies such as PCR, molecular phylogenetic analysis, sequencing, genomics, proteomics, in situ hybridization and metagenomics have enabled to directly observe endosymbiotic associations. Here, we shown the simulation of insect gut condition (hypoxia or 6% oxygen) and the normal environmental condition (20% oxygen) of *Asaia* SF2.1 growth in bioreactor, in order to find the novel genes role in endosymbiosis by using comparative proteomic approach (2D-PAGE).

Results and Discussion

Acetic acid bacterial (AAB) are aerobic organism and are known to live in oxygen rich environment. However, oxygen levels in insect gut regions of many arthropods might vary from aerobic to anoxia (2% to 7% O_2) (John T. Wertz and John A. Breznak, 2007; Sudakaran et al. 2012). Thus, in laboratory condition, we had setup hypoxia (6% O_2) and normal oxygen (20% O_2) condition in bioreactor to study the growth condition for further comparative proteomics using 2D-PAGE. It is already known from the genomic evolutionary analysis studies, the genes for the operons of cytochrome bo3 (CyoA-D) and bd (CydAB) ubiquinol oxidase is present in all AAB genome, which have high affinity for oxygen (Chouaia et al., 2014). This indicates that *Asaia* SF2.1 and other AAB have the capacity to respire in an aerobic condition and therefore, AAB have the potential to thrive at low oxygen concentrations like the enterobacteria colonizing animal guts (Chouaia et al., 2014).

However, AAB are usually described as strictly aerobic organisms thriving in normoxic environments, our results show that AAB *Asaia* SF2.1 grow and survive in hypoxia condition (6% oxygen) and also in normal condition (20% condition) survival under micro-oxic conditions, such as those existing in the insect gut (Sudakaran et al. 2012). In this work, the scaling up of biomass production of *Asaia* SF2.1 was carried out in two different conditions that is hypoxia (6% O_2) and normal (20% O_2) setup in the bioreactor (BioFlo/CelliGen115) with the controlled parameters such as pH, dissolved oxygen (DO), agitation, aeration foam/level and temperature, and stabilized the bacterial growth strains in order to evaluated the growth condition of dissolved oxygen of *Asaia* SF2.1. The setup of 6% and 20% oxygen conditions in bioreactor are discussed below:

Setup of 6% oxygen condition

For set up of hypoxia condition in bioreactor, oxygen must be limited to introduce in the media. During hypoxia condition, the organisms grows in low oxygen to survive (i.e., environment containing lower levels of oxygen 2-8%)

compared to oxygen in the atmosphere (i.e. <21% O_2). The dissolved oxygen 6% were setup and once the dissolved oxygen was stable at 6% according to the setup of present value (PV), then 45 ml of overnight culture of *Asaia* SF2.1 was inoculated into fresh two litres GLY medium in bioreactor. During *Asaia* SF2.1 growth, 6% of oxygen was completely maintained in bioreactor according to the setup and further monitored the growth based on OD. The hypoxia (6% oxygen) growth conditions of *Asaia* SF2.1 optimized in bioreactor, resulting in significantly increased cell biomass yield at late exponential phase (Fig. 3.1). Finally, as a result, insect gut condition was mimicked and three replicates growth condition (6% oxygen) of *Asaia* SF2.1 were successfully performed and mass cultivation of biomass were collected, when the bacterial culture reached 1 OD at 600nm at late exponential phase and saved our data through bio command programme.

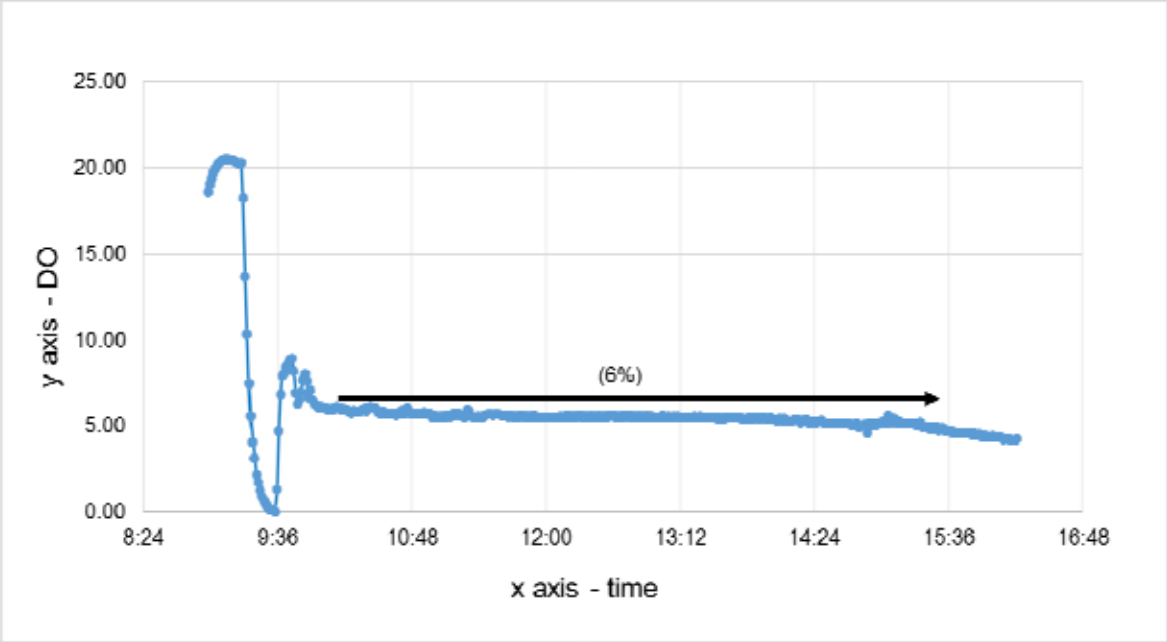


Figure 3.1 Setup of 6% dissolved oxygen growth condition of *Asaia* SF2.1 stabilized in bioreactor (BioFlo/CelliGen115).

Setup of 20% oxygen condition

Naturally in the atmosphere, 20-21% of saturated oxygen is present. The dissolved oxygen 20% were setup in bioreactor and once the current value

reached to stable condition, the setup steps were followed (See materials and methods section). The media was calibrated once the value reached 20% O_2, the stable condition was maintained. The dissolved oxygen stabilized at 20% and 45 ml of pre-inoculum culture of *Asaia* SF2.1 was inoculated into fresh two litres GLY medium in bioreactor and setup was controlled through bio command programme. According to our set up, the growth of 20% oxygen was maintained and further monitored the growth based on OD. Biomass was harvested, once the bacterial cultures reached 1 OD at 600nm at late exponential phase (Fig. 3.2). The same procedure were followed for three replicates of 20% oxygen growth. Finally, as a result, three replicates (growth conditions of *Asaia* SF2.1 20% oxygen) were performed and mass cultivation of biomass collected and saved our data through bio command programme. Both conditions that is normal (20% O_2) and microaerophilic (6% O_2), carried out on the same bioreactor, but the only difference between these two conditions is that the different percentage of oxygen introduced into the medium during growth setup.

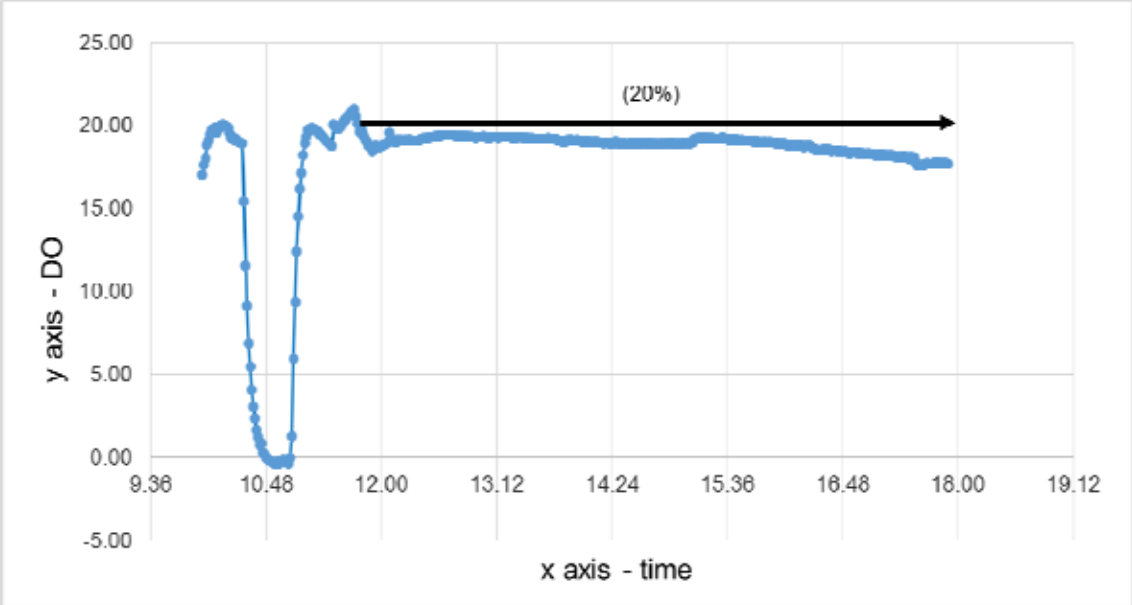


Figure 3.2 Setup of 20% dissolved oxygen growth condition of *Asaia* SF2.1 stabilized in bioreactor (BioFlo/CelliGen115).

Asaia SF2.1 biomass data

Initially, dissolved oxygen (20% O_2 and 6% O_2) were setup and investigated the cell growth. The evaluation of biomass during the time of growth (Fig. 3.3 & 3.4) in bioreactor under different oxygen (20% O_2 and 6% O_2) conditions were compared. In both condition (20% O_2 and 6% O_2) of *Asaia* SF 2.1 growth reached the OD 1 at 600nm in 7 hours. As a result, approximately 120 to 150 mg from 100 ml culture of *Asaia* SF2.1 biomass (mg) from both conditions (20% O_2 and 6% O_2) were obtained. From 1 ml of culture 5× 10^8 /ml number of cells were obtained by diluting the bacterial culture plating on a petri dish by serial dilution method in both condition. Therefore, it's clear from the exponential growth phase (See fig. 3.3 & 3.4) of *Asaia* SF2.1, which is approximately propositional to dissolved oxygen of *Asaia* SF2.1 (See fig 3.1 & 3.2).

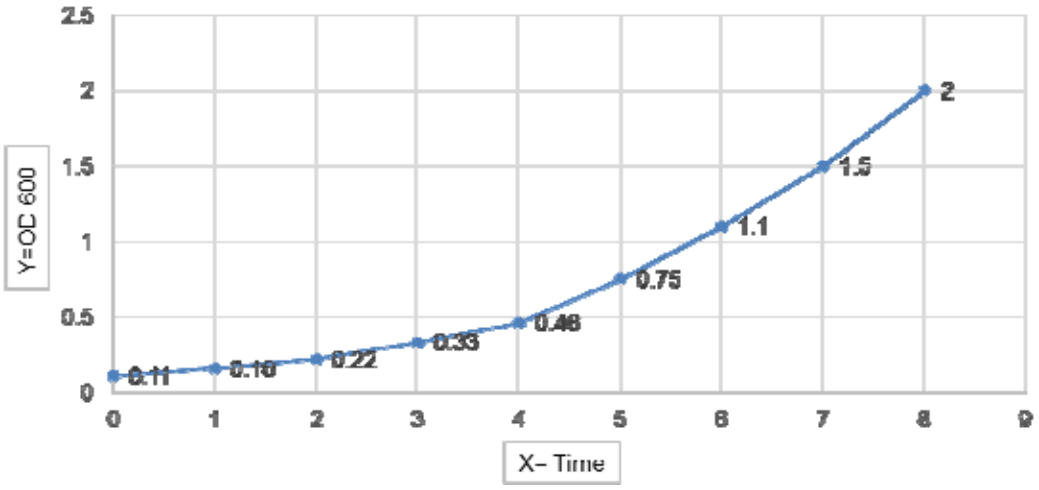


Figure 3.3 Shows the evaluation of *Asaia* SF2.1 biomass. Culture (*Asaia* SF2.1, 20% O_2 condition) measured for every 1 hour at 600 nm and biomass collected once reached to OD 1 at late exponential phase.

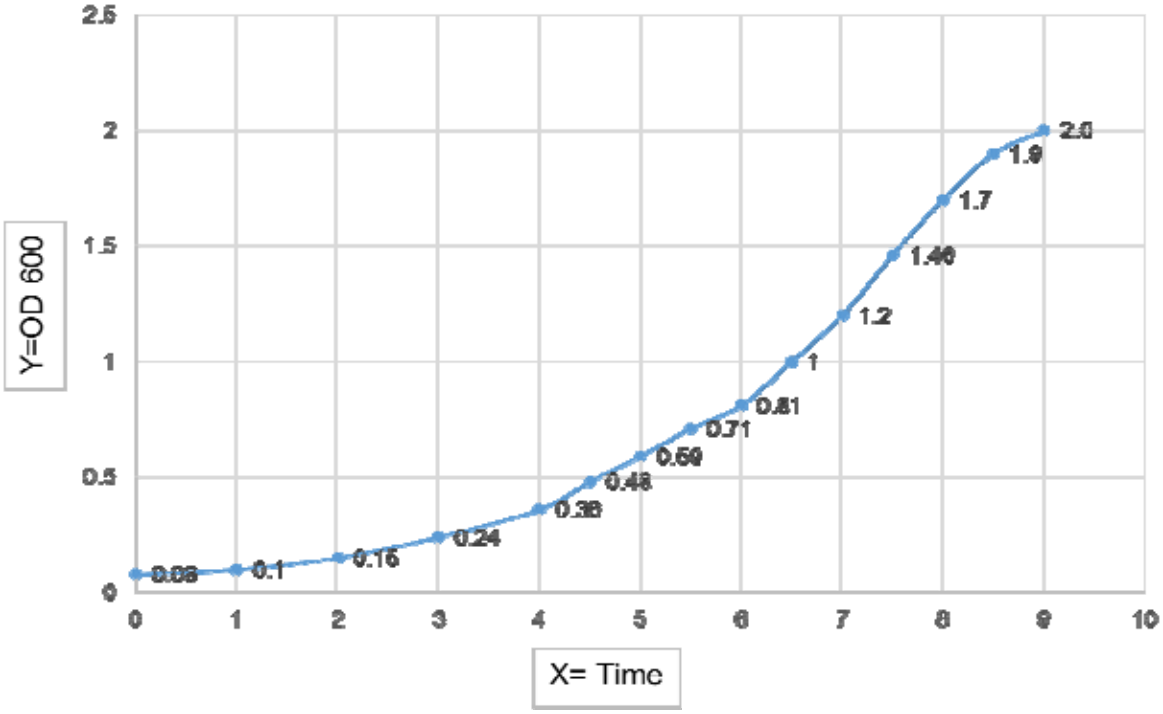


Figure 3.4 Shows the evaluation of *Asaia* SF2.1 biomass. Culture (*Asaia* SF2.1, 20% O_2 condition) measured for every 1 hour at 600 nm and biomass collected once reached to OD 1 at late exponential phase.

Comparative proteome analysis (2D-PAGE) of *Asaia* SF2.1 biomass

The biomass obtained from both conditions (20% O2 and 6% O2) were further applied for 2D-PAGE analysis. In particularly TCA-acetone sample preparation method were used for protein comparison between two conditions. Both conditions of *Asaia* SF2.1 cell biomass were extracted by TCA-acetone sample preparation method and further quantification of protein were performed by Bradford method to measure the amount of protein concentration of *Asaia* SF2.1. The approximate amount of protein were used for 2D-PAGE analysis of both conditions (ref. previous chapter). Finally our results (Fig. 3.3a, 3.3b) shows the clear spots of protein profile of *Asaia* SF2.1 in both conditions. Total five replicates of 2DE gels were performed for each condition (6% and 20% oxygen) of *Asaia* SF2.1 biomass and analyzed the comparison of protein profile

(or) spots at expression level by Image analysis (ImageMaster 2D Platinum 6.0).

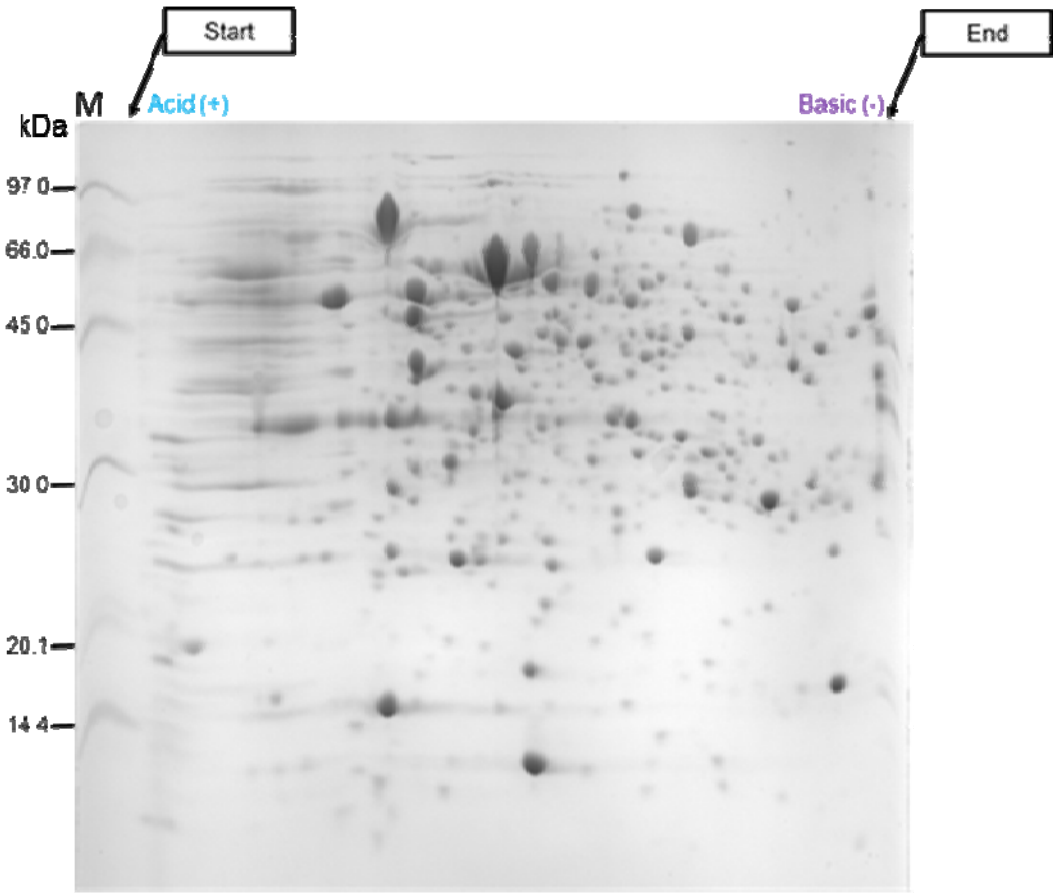


Figure 3.3a Shows optimization of 2DE protein profile of *Asaia* SF2.1, 6% dissolved oxygen condition: Protein extracted by grinding with pestle followed by TCA-Acetone precipitation solution and 400ug of protein loaded in gel. Soluble protein separated by first dimension (pH 4-7-NL IPG) and second dimension (12% SDS-PAGE).

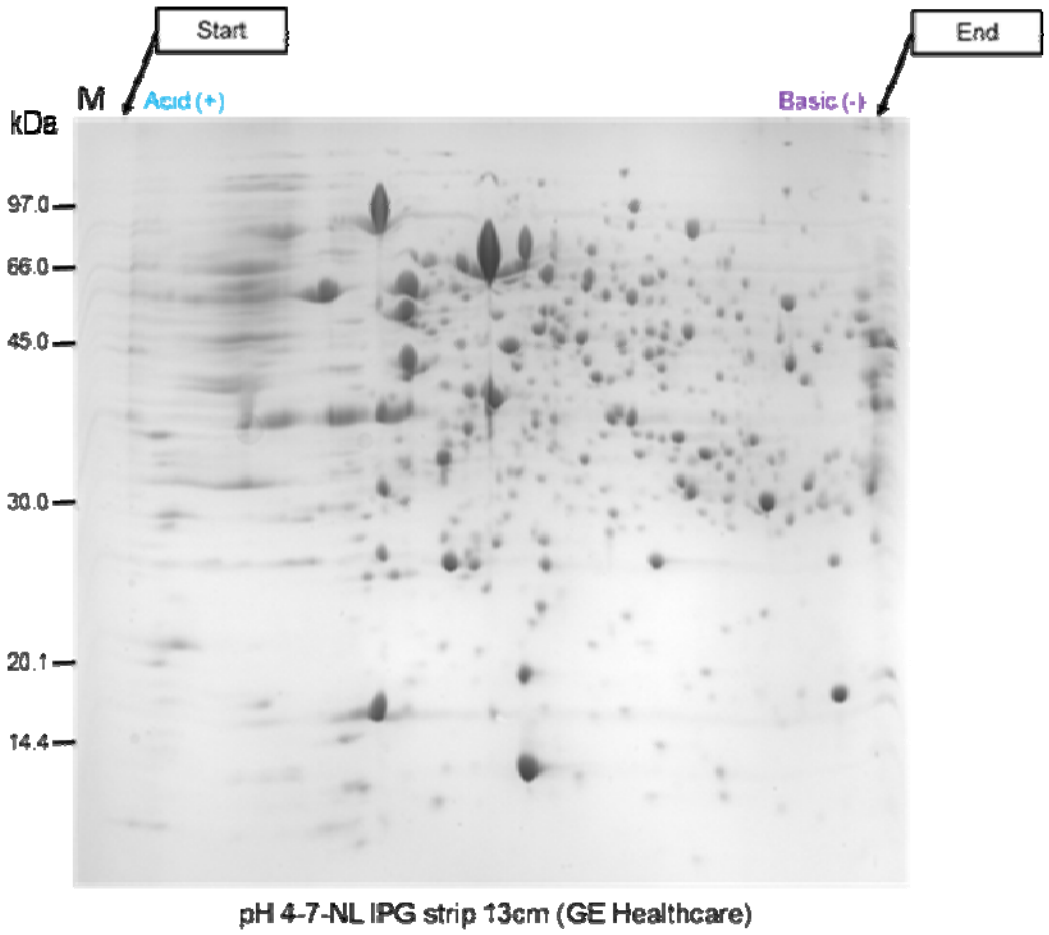


Figure 3.3b Shows optimization of 2DE protein profile of *Asaia* SF2.1, 20% dissolved oxygen condition: Protein extracted by grinding with pestle followed by TCA-Acetone precipitation solution and 400ug of protein loaded in gel. Soluble protein separated by first dimension (pH 4-7-NL IPG) and second dimension (12% SDS-PAGE).

Conclusion

Our results shows the setup of different percentage of dissolved oxygen (6% and 20% oxygen) in bioreactor and evaluated the growth condition of *Asaia* SF2.1 followed by mass cultivation of *Asaia* SF2.1 growth (i.e.) three replicates of each growth conditions (6% and 20% oxygen) in bioreactor were done. Both conditions showed the higher biomass yield at late exponential phase. TCA-Acetone was the best method for protein extraction and proteins were extracted. Five replicates of 2DE gels were obtained for each condition (6% and 20% oxygen) of *Asaia* SF2.1.

Materials and method

In laboratory, different growth condition (20% and 6% oxygen) of *Asaia* SF2.1 were setup in bioreactor (BioFlo/CelliGen115). The bioreactor equipped with temperature, pH, dissolved oxygen (DO), agitation speed, control unit, connected to a computer (Bio command software - centralized control) and automatic data process recording.

Before inoculating the culture, all valves were connected such as bioreactor valve connected with machine valve, bioreactor (sparge) - machine valve (sparge), bioreactor (return) - machine valve (Exh-cond-return), bioreactor (return-in) - machine valve (return-in), bioreactor (pH probe) - machine valve (pH only), bioreactor (O_2) - machine valve (O_2 only), pH and DO probes was calibrated. For *Asaia* SF2.1 growth in bioreactor, a pre-mixed gas that is air and nitrogen was introduced into the medium through the ring sparger (or) optional microsparger. The flow rate was controlled manually by rota meters (air flux ball) and (N_2 flux ball) .The (TMFC) thermal mass flow is regulated automatically according to values set via the control station of touch screen and controlled all the parameters such as dissolved oxygen (DO), agitation, aeration and foam/level. Each equipment have different functions that are discussed below.

Agitation system: A removable agitation motor located on its top of bioreactor and connected to the agitation shaft with a magnetic coupling. This motor provides cascade that means a speed range from 50 to 1200 rpm in-order to control the set point that is one or more parameters to influence such as Dissolved Oxygen (DO) to Agitation (AGIT) and foam control. So the agitation speed varies between the user-specified minimum and maximum set points in order to maintain the set percentage of DO. The whole process controlled by software ensures agitation speed control, throughout the speed range.

DO control: Dissolved oxygen (DO) was controlled by the range of 0-20%. The control was maintained by PID (Proportional-integral-derivative) controller

by changing the speed of agitation, and the thermal mass flow controller regulated flow rate and/or the percentage of oxygen in aeration. Before sterilization, the oxygen probe connected to the operating oxygen amplifier or polarizing module and after sterilization, kept overnight for polarization prior to calibrating the probe.

pH control: The pH was measured by range of 4-7 by PID (Proportional-integral-derivative) cascade controller, which operates peristaltic pumps, assigned to perform acid of HCl (0.1M) or base NaOH (0.1M) addition or which controls the use of gases for this purpose. The pH sensed by a gel-filled pH probe. The electrode was calibrated before sterilization and checked afterwards during sampling.

Temperature control: The media temperature sensed by a Resistance Temperature Detector (RTD) submerged in the thermowell and controlled at 30°C. The thermostat system is an open pressure free system. It includes a heater blanket and a valve for cooling water supply. Circulation pump delivers water of the pre-adjusted temperature to the culture vessel. The digital measurement and software ensures a precise and constant temperature control.

Culture cultivation in the bioreactor: The general procedure (Kadere et al. 2008) were followed, the Gly agar (glycerol 2.5%, yeast extract 0.5%, agar 2%, pH 5) plates were used for *Asaia* SF2.1 growth. The inoculum always contributed as small pinch from the glycerol stock culture and streaked in plates. Plates were incubated at 30^{o}C for 2 days in order to isolate the pure strain. Then isolated pure strain was inoculated in 500 ml flasks containing 100 ml of Gly liquid medium and incubated at 30°C overnight. The fermentation were carried out in a 10 litre bioreactor (New Brunswick BioFlo/CelliGen 115) with a working volume of 2 litres Gly medium. Then 45 ml of overnight culture was transferred into the bioreactor by using 50 ml syringe and controlled all the parameters and monitored the growth based on OD 600 nm (Jenway spectrophotometer 6705) for every 1 hour. Once reached the optical density at late exponential phase, immediately growth were arrested and cells harvested

by centrifugation at 3000 rpm for 15 min at 4^{o}C and washed the cell pellet 3 times with 1X PBS buffer and quantified the wet pellet. Further protein profiles from both conditions were evaluated by 2D-PAGE analysis in particularly using TCA-Acetone method.

Setup of 6% oxygen condition in bioreactor: First step, the dissolved oxygen (DO) 20% (Open air flux-3.3) were setup and once the current value reached the stable condition, pressed SET SPAN. Second step, the dissolved oxygen 0% were calibrated and opened the N2 flux -3.3 LMH (Gas-1) and closed the air (Gas-2), once the current value was stable, pressed SET ZERO. Third step, the medium was calibrated once the set value reached 6% O_2, and both the valves were opened i.e., N_2 (flux-2.3 LMH) & air (flux-1.2 LMH). Fourth step, once the dissolved oxygen was stable at 6% according to our set up of present value (PV), then 45 ml of overnight culture inoculated into fresh two litres GLY medium in bioreactor. During growth, 6% of oxygen was completely maintained in bioreactor according to our setup by adjusting N_2 flux-2.3 LMH & air flux-1.2 LMH (Liter/m2/h) the volume of gas and further monitored the growth based on OD. The hypoxia (6% oxygen) growth conditions optimized in bioreactor.

Setup of 20% oxygen condition: The steps were followed for the setup of 20% oxygen condition are: First step, the dissolved oxygen 20% (Open air flux-3.3) were setup and once the current value reached the stable condition, pressed SET SPAN. Second step, the dissolved oxygen 0% were calibrated, which indicates the absence of oxygen in the media. Third step, the air (Gas-2) was closed, and the valve N_2 flux-3.3 LMH (Gas-1) was opened and once the current value was stable, pressed SET ZERO. Fourth step, the media was calibrated once the set value reached 20% O2 and the valve was opened (i.e., air flux-2.6 to 3.1 LMH maximum level) and maintained the stable condition. Fifth step, the dissolved oxygen stabilized at 20% according to our set point of present value (PV) then 45 ml of pre-inoculum culture was inoculated into fresh two litres GLY medium in bioreactor and setup was controlled through bio command programme. The 20% oxygen condition was maintained and further monitored the growth based on OD.

References

Buchner, P. (1965). Endosymbiosis of Animals with Plant Microorganisms. New York: Interscience.

Bari W, Song YJ, Yoon SS. 2011. Suppressed induction of proinflammatory cytokines by a unique metabolite produced by Vibrio cholerae O1 El Tor biotype in cultured host cells. Infect Immun. 79:3149–3158.

Chouaia B, Gaiarsa S, Crotti E, Comandatore F, Esposti MD, Ricci I, Alma A, Favia G, Bandi C, and Daffonchio D, (2014). Acetic Acid Bacteria Genomes Reveal Functional Traits for Adaptation to Life in Insect Guts. Genome Biol Evol. 2014 Apr; 6(4): 912–920.

Crotti E, et al. 2009. Asaia, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. Environ Microbiol. 11:3252–3264.

Damiani C, et al. 2008. Paternal transmission of symbiotic bacteria in malaria vectors. Curr Biol. 18:R1087–R1088.

Gonella E., 2012. Horizontal transmission of the symbiotic bacterium Asaia sp. in the leafhopper Scaphoideus titanus Ball (Hemiptera: Cicadellidae). BMC Microbiol. 12 (Suppl 1): S4.

Houk, E.J., and Griffiths, G.W. (1980). Intracellular symbiotes of the Homoptera. Annu Rev Entomol 25: 161–187.

John T. Wertz and John A. Breznak, 2007. Stenoxybacter acetivorans gen. nov., sp. nov., an Acetate-Oxidizing Obligate Microaerophile among Diverse O2-Consuming Bacteria from Termite Guts. Appl. Environ. Microbiol.6819-6828

Kersters K, Lisdiyanti P, Komagata K, Swings J. 2006. The family Acetobacteraceae: the genera Acetobacter, Acidomonas, Asaia, Gluconacetobacter, Gluconobacter, and Kozakia. In: Dworkin M, Falkow S,

Rosenberg E, Schleifer K-H, Stackebrandt E, editors. The prokaryotes. Vol. 5, 3rd ed. New York (NY): Springer. p. 163–200.

Kadere,T.T., Miyamoto,T., R. K. Oniang`o.R.K., Kutima, P.M., Njoroge,S.M., (2008). Isolation and identification of the genera Acetobacter and Gluconobacter in coconut toddy (mnazi). African Journal of Biotechnology Vol. 7, pp. 2963-2971.

Margulis L, and Fester, R. 1991. Symbiosis as a source of evolutionary innovation. MIT Press, Cambridge, MA, USA

Sudakaran S, Salem H, Kost C, Kaltenpoth M. 2012. Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, Pyrrhocoris apterus (Hemiptera, Pyrrhocoridae). Mol Ecol. 21: 6134–6151.

Ruby E, Henderson B, McFall-Ngai. M, 2004. We Get By with a Little Help from Our (Little) Friends.Science 27: Vol. 303 no. 5662 pp. 1305-1307.

Tolasch T, Iter S, Toth M, Ruther J, Francke W. 2003. (R)-acetoin-female sex pheromone of the summer chafer Amphimallon solstitiale . J Chem Ecol. 29:1045–1050.

Yamada Y, Katsura K, Kawasaki H, Widyastuti Y, Saono S, Seki T, Uchimura T, Komagata K. 2000. Asaia bogorensis gen. nov., sp. nov., an unusual acetic acid bacterium in the alpha-Proteobacteria. Int J Syst Evol Microbiol; 50 Pt 2():823-9.

Yoshitomo Kikuchi, (2009). Endosymbiont Tolerance and Control within Insect Hosts Endosymbiont Tolerance and Control within Insect Hosts. Microbes and environments Vol24, No.3, 195 -204.

Chapter IV

Comparative 2DE gel analysis of *Asaia* SF2.1

Abstract

AAB are usually described as strictly aerobic organisms thriving in normoxic environments. In contrast, in many arthropods, the oxygen level may vary from aerobic to completely anoxic. AAB *Asaia* SF2.1 were found in insect gut, where the oxygen level is very low. Thus, in order to mimic the insect gut condition (hypoxia condition) growth of *Asaia* SF2.1. The aim was to setup different growth condition (i.e., hypoxia 6% and normal 20%) of *Asaia* SF2.1 and to perform the comparative proteomic analysis by 2D-PAGE followed by mass spectrometry analysis. Therefore, 2D-PAGE were performed to obtain the protein profile followed by data analysis of protein gel images using Image analysis software. The 2D-protein gels from each conditions were optimized based on the spot detection parameter and data analysis of intra class report were analysed. According to the data analysis and visual observations, 12 spots were selected in which some spots were more expressed while others less (or) not. After comparison between the two conditions (6% and 20% oxygen), the protein spots were picked for mass spectrometer analysis. MS analysis identified different proteins from the selected spots and functional categorization of proteins was carried out according to the gene ontology (GO) rules, protein spots were mostly identified as being involved in transcription, cellular respiratory function, cell wall biogenesis, protein biosynthesis, pentose-phosphate shunt etc. which pave the way to confirm in the role of endosymbiosis.

Introduction

Overview of protein detection and image analysis

Proteomics, one of the most important areas of research in the post-genomic era, is not new in terms of its experimental foundations. Proteomics enables understanding the structure, function and interactions of the entire protein content in a specific organism (Mishra et al., 2011). The term "proteomics" is a large-scale comprehensive study of a specific proteome, including information on protein abundances, their variations and modifications, along with their interacting partners and networks, in order to understand cellular processes (Pandey and Mann, 2000). This proteomic approach was created by combining 2-DE technology with mass spectrometry and bioinformatics. The rapid advancement of this technique in combination with other methods used in proteomics results in an increasing number of high-throughputs. This leads to an increasing amount of data that needs to be archived and analyzed (Wilke et al., 2003). It is a natural consequence of the huge advances in genome sequencing, bioinformatics and the development of robust, sensitive, reliable and reproducible analytical techniques (Mao et al., 2010).

In the current proteomic landscape, 2DE gel-based proteomics is the only setup in which there is a display of the output before the mass spectrometry stage, and this output is precisely the on-gel detection of proteins (Rabilloud et al., 2011). The important steps involved behind, that is quantitative analysis of 2DE steps, and it allows to perform a spot selection and this based on difference in expression level once the gel images were submitted to automatic spot detection program according to the manufacturer recommendations. The differential expression values must be accurate and reproducible that is the relative quantities of the same protein spots in different gels are correctly evaluated followed by spot filtering, spot editing, background correction, gel matching, normalisation, comparison, quantification and reporting and exporting of data and also spots densitometry (%V spot / V total spots) was calculated by using the 2DE software (Image Master 2D Platinum)

to obtained by expressing the intensity of each protein spot in a gel as a proportion of the total protein intensity detected for the entire gel. First of all, this means that only a very limited portion of the proteins present in the samples with replicates will need to be analyzed by comparative studies (Natale et al., 2011). The quantitation of protein expression in a proteome provides the first clue into how the cell responds to changes in its surrounding environments. The resulting over (or) under expressed proteins are deemed to play important roles in the precise regulation of cellular activities that are directly related to a given exogenous stimulant (Manso et al 2005; Eckerskorn et al 1997). However, the quantitative analysis of 2DE gels that will lead to better prediction of protein function of molecule and cell signaling, host defense, as well as protein interactions studies of endo-symbionts. In quantitative analysis, one of the parameter is a data analysis. It's refers to study the variations in protein expression among a series of gels, the gels were matched together. Initially, the gel images were stained and further analysed. Some gels has fewer proteins present in a simple pattern are compared by overlaying the gels and manually inspecting the profiles for proteins with any electrophoretic mobilities. And if the number of proteins present in the gel increases, the computer programs with specific software to match the profile becomes necessary. However, image analysis process in 2DE gel-based proteomics must be stressed, because it's usually not the simple comparison of two gels, but the multiple comparison of several gel images, since the very early days of the technique (Mao et al., 2011).

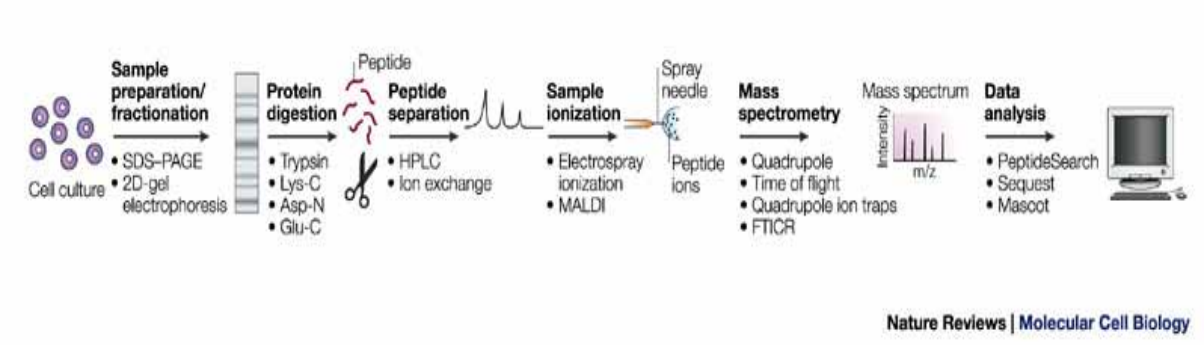
Mass spectrometric analysis of protein spots

Mass spectrometry (MS) at the beginning period of time dated back to last century was a susceptible technique. Mass spectrometry is a powerful technique in analytical chemistry that was originally designed to determine the composition of small molecules in terms of their constituent elements with very high sensitivity. Hence, it is used in the wide range of applications in different fields. One such field is proteomics, where in large-scale analysis of the function of genes studied and is becoming a central field in functional genomics

(Pandey and Mann, 2000). Currently MS is used in the molecular weight of a protein identification, detect and characterize the post translational modifications and potentially can identify any covalent modification that alters the mass of a protein.

Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry have evolved over the last few years as very sensitive and versatile techniques for the structural analysis of proteins and other biomolecules. The steps included in the MS pipeline were shown in (Fig 4.1), includes the digestion of the proteins by proteolytic enzymes (i.e., trypsin, chymotrypsin and elastase) to yield shorter pieces (peptides) that are subsequently sequenced. Even if the MS measure the mass of intact proteins, the sensitivity of the mass spectrometer for proteins is much lower than for peptides (Steen and Mann, 2004). Mass spectrometry is efficient at

Figure 4.1 General Scheme of 2DE gel-based proteomics and Mass Spectrometry



Pipeline. A protein population is prepared from a biological source such as a cell culture. The proteins are first separated from a mixed sample by means of an SDS gel. After detection of the proteins in the gels, the resulting images are quantitatively analyzed to determine the spots of interest. Those spots are then excised and submitted to in-gel digestion (generally with trypsin). The resulting peptides are then eluted and analyzed by mass spectrometry, the resulting peptides are loaded onto an HPLC column coupled to a mass spectrometry, leading to protein identification and characterization. The peptides are ionized before entering the mass spectrometer. The sequence of the protein determined using data analysis software (Steen and Mann, 2004).

obtaining sequence information from peptides that are up to 20 residues long. Digesting the whole protein into a set of peptides and separated on the HPLC column, using a solvent gradient of increasing organic content, so that the

peptides emerge in order of their hydrophobicity and it is assumed during the analysis, only a single peptide species enters the mass spectrometer during each cycle. When a peptide species arrives at the end of the column, it flows through a needle. At the needle tip, the liquid is vaporized and the peptide is subsequently ionized by the action of a strong electric potential.

The MS might make two runs for every peptide that arrives at the end of the HPLC columns. During the second run, gas phase peptide ions undergo collision-induced dissociation (CID) with molecules of an inert gas such as helium or argon and get broken into pieces. The result of this second run is a spectrum - that is, the masses of a large number of smaller peptide fragments. At low collision energies CID (<100eV), fragmentation occurs mainly along the peptide backbone bonds, i.e., the N-C bond between amino acids. At higher energies, fragments generated by breaking internal C-C bonds are also observed (Sadygov et al., 2004). Based on the charge retained on the 'N' and 'C'-terminal side of the fragmented peptide, the ions are denoted as a; b; b and x; y; z. The HPCL runs throughout the cycle though a sequence that consists of obtaining a mass-to-charge ratio of the species eluted from the HPLC column followed by obtaining tandem mass spectra of the most abundant species of peptides. The concentration of the inert gas involved in the collision is calibrated with the expectation that each molecule of a given peptide will break in a single place. Therefore, peptides are expected to dissociate into nested sets of fragments and subsequently successive peaks for different amino acids based on the molecular mass were detected. Thus the amino acid peaks detected were subjected to data analysis for further characterization of proteins, posttranslational modifications other macromolecules. Protein identifications were obtained with the embedded ion accounting algorithm (Sequest HT) of the software Proteome Discoverer (version 1.4, Thermo Fisher Scientific) after searching a UniProtKB/TrEMBL Protein Knowledgebase (taxonomical restriction: *Asaia platycodi* SF2.1, 3273 sequence entries).

Data management

Extracted Protein are separated from biological sample. Further proteins are detected by experimental conditions. Gel image visualization using Image Master 2D Platinum, further set up and optimizing the spot detection parameter, after gel matching comparison with a reference gel and spots were annotated. Finally comparison of data analysis to study the expression and identification of spot picking for MS analysis. Those spots interested are then excised and submitted to in-gel digestion (generally with trypsin). The resulting peptides are then eluted and analyzed by mass spectrometry, leading to protein identification and characterization (Fig 4.2). The aim of work was to pick protein spots of 2DE gel from both conditions (i.e., hypoxia or low oxygen 6% and normal oxygen 20%) for mass spectrometer analysis.

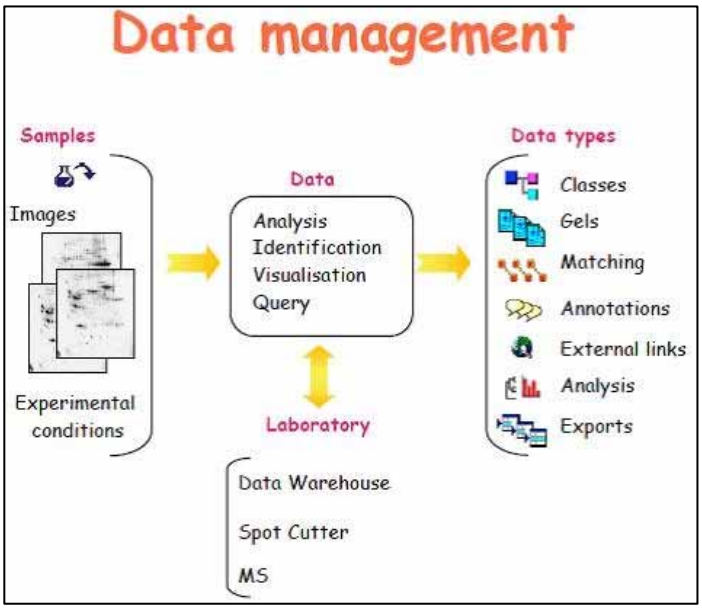


Figure 4.2 Overview of data management of 2DE protein image

Results and Discussion

Gel image analysis using Image Master 2D Platinum 6.0

The gel images with proteins spots from both (hypoxia and normal) conditions were evaluated by image analysis software. According to the data analysis and visual comparison of multiple gels i.e., each spot were compared 16 times with same spot of other replicates and verified protein

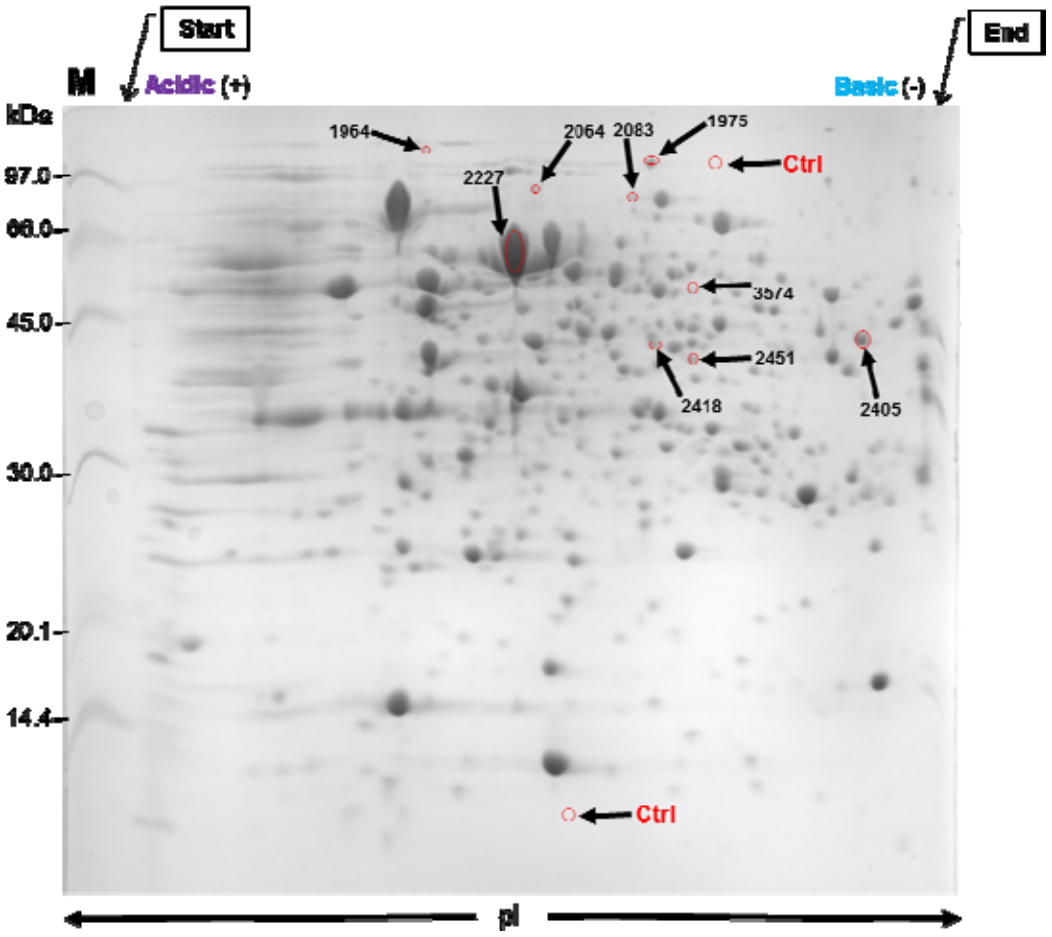


Figure. 4.3a. A proteome reference map of *Asaia* SF2.1 under hypoxia (6% O_2) condition. Separation of proteins by 2D-PAGE (400 ug on IPG Strip 13cm, pI 4-7 linear (GE healthcare, Code no: 17-6001-13), M-Marker (Code no: 17044601, GE Healthcare) and 12% SDS–PAGE) and then visualized by coomassie staining. The highlighted spots (arrow mark symbol) picked based on the expression differences by image analysis.

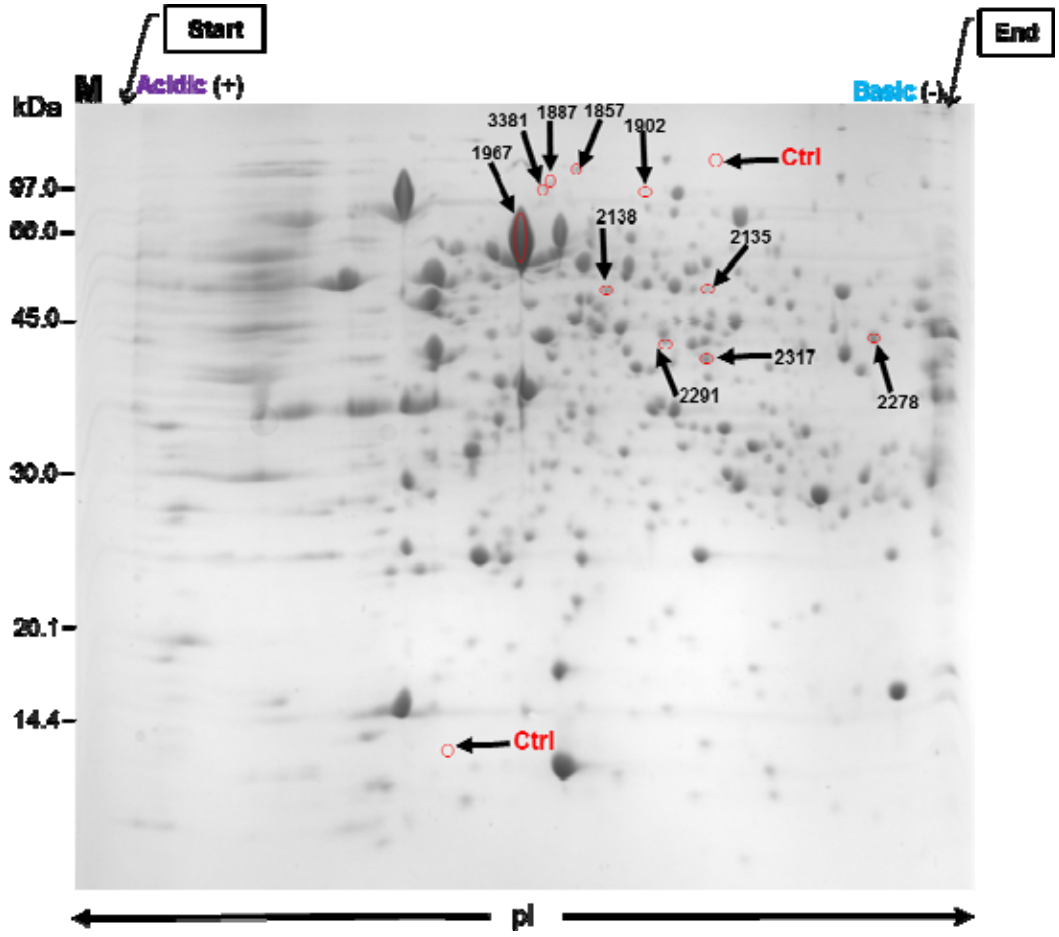


Figure. 4.3b. A proteome reference map of *Asaia* SF2.1 under normal (20% O_2) condition. Separation of proteins by 2D-PAGE (400 ug on IPG Strip 13cm, pI 4-7 linear (GE healthcare, Code no: 17-6001-13), M-Marker (Code no: 17044601, GE Healthcare) and 12% SDS–PAGE) and then visualized by coomassie staining. The highlighted spots (arrow mark symbol) picked based on the expression differences by image analysis.

expression between two conditions (6% and 20% oxygen). The results from data analysis indicates same spots with different expression levels from different replicates (6% and 20% oxygen) of 2DE gel and 12 spots were selected from different replicates of two different conditions (Fig 4.3a, 4.3b) for mass spectrometer analysis (MS). As a result, MS analysis (Table 1) identified different proteins, involved in several cellular function such as, Carboxypeptidase-related protein, ATP synthase subunit alpha, Serine-tRNA ligase, Broad-specificity glycerol dehydrogenase, Elongation-factor G, Transaldolase, Aconitate-hydratase, Various polyols ABC transporter, periplasmic substrate-binding-protein, Gamma-glutamyltranspeptidase, 6-phospho gluconate dehydrogenase decarboxylating, Biotin carboxylase of

acetyl-CoA carboxylase, Uroporphyrinogen decarboxylase, and UDP-N-acetylglucosamine-1-carboxyvinyl transferase.

# Spot	# Gel	# ID	Accession	Description GN= gene name - [Entry name]	Score	Coverage	# Unique Peptides	# PSMs	# AAs	MW [kDa]	calc. pI	Conformity in MW gel
1	E	1973	[illegible]	Carboxypeptidase-related protein GN=ASAP_3100 - [[illegible]_SPROT]	148.49	47.49	19	65	499	53.5	6.38	[illegible]
2	B	1987	[illegible]	60 kDa chaperonin GN=groL - [[illegible]_SPROT]	1717.36	86.30	32	369	547	58.0	5.34	ok
			[illegible]4	ATP synthase subunit alpha GN=atpA1 - [[illegible]_SPROT]	187.49	32.42	23	34	511	55.1	5.43	ok
3	D	2136	[illegible]	Serine--tRNA ligase GN=serS - [[illegible]_SPROT]	88.27	34.76	13	35	430	46.8	5.55	ok
4	F	2083	[illegible]	Broad-specificity glycerol dehydrogenase, subunit SldA GN=ASAP_1834 - [[illegible]_SPROT]	80.32	31.51	18	32	735	79.8	6.24	ok
5	F	2004	[illegible]	Uncharacterized protein GN=ASAP_3131 - [[illegible]_SPROT]	125.41	23.02	17	46	745	75.4	5.47	[illegible]
6	D	1087	[illegible]	Elongation factor G GN=fusA1 - [[illegible]_SPROT]	214.35	51.76	31	67	701	77.0	5.41	[illegible]
7	H	2021	[illegible]	Transketolase GN=ASAP_1876 - [[illegible]_SPROT]	337.11	66.49	62	166	935	102.5	5.22	ok
			[illegible]	Aconitate hydratase GN=ASAP_3309 - [[illegible]_SPROT]	45.84	28.95	18	22	888	95.7	5.33	[illegible]
8	F	2480	[illegible]	Various polyols ABC transporter, periplasmic substrate-binding protein GN=ASAP_1116 - [[illegible]_SPROT]	281.05	44.39	19	94	437	47.3	7.52	ok
			[illegible]	Gamma-glutamyltranspeptidase GN=ASAP_0401 - [[illegible]_SPROT]	50.13	24.01	11	19	691	67.4	6.49	[illegible]
9	B	2517	[illegible]	6-phosphogluconate dehydrogenase, decarboxylating GN=ASAP_1875 - [[illegible]_SPROT]	574.25	58.69	28	189	331	36.2	6.00	ok
10	H	3304	[illegible]	Biotin carboxylase of acetyl-CoA carboxylase GN=ASAP_3205 - [[illegible]_SPROT]	136.71	38.82	24	61	445	48.8	5.96	?
11	F	2415	[illegible]	Uroporphyrinogen decarboxylase GN=hemE - [[illegible]_SPROT]	73.05	33.04	13	29	351	38.1	5.97	ok
			[illegible]	UDP-N-acetylglucosamine 1-carboxyvinyltransferase GN=murA - [[illegible]_SPROT]	51.14	44.19	13	19	430	45.6	5.92	ok
12	F	1964	[illegible]	Uncharacterized protein GN=ASAP_1977 - [[illegible]_SPROT]	47.89	25.25	21	32	888	100.2	5.48	ok ?
Spots from replicates (A, B, C D) from 20% O_2 & replicates (E, F, G, H) from 6% O_2												

Table 1. Shows identified proteins after mass spectrometry analysis. In the table are listed, for each gel band spots (A, B,C,D from 20% 2DE replicates, and E,F,G,H from 6% 2DE replicates), the following parameters: alphanumeric unique protein sequence identifier provided by UniProtKB/TrEMBL protein knowledgebase; protein name, gene and protein characters with a naming convention [Entry name] identifiers; protein identification's SEQUEST HT Score; percentage of protein sequence covered by identified peptides; number of matching peptide sequences, unique to the protein; total number of identified peptide sequences (peptide spectrum matches) (# PSMs); number of Amino Acids; proteins theoretically calculated Molecular Weight and isoelectric point (pI).

Functional categorization of proteins was obtained from (GO) gene ontology (Table 2), protein spots were mostly identified as being involved in cellular respiratory function, pentose-phosphate pathway, protein biosynthesis, cellular respiratory function, transcription etc. Generally the metabolism of carbohydrates requires the activity of enzymes of the central carbon metabolic network that include the glycolytic pathway, oxidative pentose phosphate (PP) pathway and the tricarboxylic acid (TCA) cycle. These three basic pathways provide the reduced compounds needed to fuel the electron transport chain and catalyze adenosine triphosphate (ATP) formation via oxidative phosphorylation. The central carbon metabolic plays an essential roles in the cell, providing energy metabolism and precursors for many biosynthetic reactions. One of the Aconitate hydratase is a key regulator of the TCA cycle is

involved in cell respiration, producing NADH and FADH2 for the electron transport chain., with eight reactions that process incoming molecules of acetyl CoA that leave the cycle in the form of carbon dioxide. The gene ontology analysis showed interesting putative candidates, which might be involved in the role of endosymbiosis of *Asaia* SF2.1. Interestingly candidates such as 6-phosphogluconate dehydrogenase, Transaldolase, and Aconitate hydratase were selected. In particular, 6-phosphogluconate dehydrogenase was selected for further confirmation, which is an important enzyme of oxidative phase of pentose phosphate pathway (PPP) (Rauch et al., 2010 and Minic et al., 2015). The PPP is the major pathway for the recycling of NADP+ to NADPH and for the production of ribose-5-phosphate and erythrose-4-phosphate used in the synthesis of nucleotides, nucleic acids and aromatic amino acids. PP pathway also involved in the protection of cells against oxidants. According to Thompson S.N et al., 1999, pentose pathway plays a unique role in carbohydrate oxidation by simultaneously allocating substrate for growth and energy production. Also PP pathway (trehalose sugar) plays the role in dietary selection and feeding behavior, and is therefore important in regulating insect growth and development. Interestingly, it's known that *Candidatus* Evansia muelleri Xc1 from Xenophyes cascus, which is the only obligate endosymbiont present in the association and has the role in fulfilling nutritional requirements by pentose phosphate pathway (Garcia et al., 2014). TCA cycle and respiratory machinery which are involved in amino acids production. Evansia involved in pentose phosphate pathway produce different metabolites, which are used as input for other pathways and reducing power (NADPH) (Garcia et al., 2014). Evansia also possesses a reduced TCA cycle, able to produce all necessary metabolites, with HisC producing a bypass from oxalacetate to 2-oxoglutarate (Garcia et al., 2014). Thus we can say that, these pathways are mainly involved in endosymbiosis of host development. Moreover, we speculate, the presence of 6-phosphogluconate dehydrogenase enzyme in *Asaia* may have similar role in meeting the nutritional requirement by acting through pp pathways and TCA pathways in hypoxia condition.

Table 2. Gene ontology (GO) annotation analysis showed interesting putative candidates after MS results

Query				Result (Blast)							
Spot Id	Gene from Arabi SF 2.1	Query seq (from MS)	Accession (Reviewd)	Gene annotation score (5)		Species	Identity (%)	Pathway	E-Value	Hit Score -12	Manual spot Observation
2317 (20%)	putative 6-PGD	ADAJ00QB34								7	20% Over expressed
			G3EED7	4	Exp. Evi. at protein level	Gluconobacter oxydans	78.3%	PPP : The glucose-6-phosphate catabolic process in which, coupled to NADPH synthesis, glucose-6-P is oxidized with the formation of carbon dioxide (CO2) and ribulose 6-phosphate. ribulose 6-P then enters a series of reactions interconverting sugar phosphates	0		
1967 (20%)	putative (60 kDa chaperonin)	ADAJ00QB34								8	20% Over expressed
			P35471	2	Exp. Evi. at transcript level	Rhizobium meliloti	78.8%	BioCyc collection of pathway : Interacting selectively and non-covalently with ATP, adenosine 5'-triphosphate, a universally important coenzyme and regulator.	0		
			P33891	2	Exp. Evi. at transcript level	Bradyrhizobium diazoefficiens	77.3%	BioCyc collection of pathway	0		
			P35489	2	Exp. evid. at transcript level	Rhizobium meliloti	78.8%	BioCyc collection of pathway	0		
			B2S624	2	Exp. evid. at transcript level	Brucella abortus (strain S19)	77.90%	BioCyc collection of pathway	0		
			P33809	2	Exp. evi at transcript level	R. leguminosarum bv. viciae	77.10%	ATP binding	0		
			P35470	2	Exp. evid at transcript level	Rhizobium meliloti	74.30%	ATP binding	0		
			P77629	2	Exp. evi at transcript level	Bradyrhizobium diazoefficiens	76.00%	ATP binding	0		
1967 (20%)	putative (ATP synthase subunit a	ADAJ00QB34								8	20% Over expressed
			P0B036	4	Exp. Evi. at protein level	Rhodospirillum rubrum	73%	Catalytic activity: The transport of protons against an electrochemical gradient, using energy from ATP hydrolysis; plasma membrane ATP synthesis coupled	0		
			P33381	5	Exp. evi. at protein level	Drosophila melanogaster	67.40%	ATP binding	0		
			Q8WXK1	4	Exp. evid at protein level	Caenorhabditis elegans	67.90%	Mitochondrial membrane ATP synthase	0		
			P72345	4	Exp. evid at protein level	Rhodobacter capsulatus	68.70%	ATP binding .	0		
2083 (6%)	Putative (Broad-specificity glycero	ADAJ00QD54								5	6% Over Expressed
			Q3XLL1	3	(Exp. evid. at protein level	Gluconobacter thailandicus	73.3%	BioCyc collection of pathway:	0		
			Q7JJN9	2	(Protein inferred from homo	Glucanobacter oxydans	72.9%	BioCyc collection of pathway	0		
2021(6%)	Putative (Transaldolase)	ADAJ00QE46								3	6% Less Expressed
			P9WG53	3	Exp. evid. at protein level	M. tuberculosis	43.6%	ppp : Transaldolase is important for the balance of metabolites in the pentose-phosphate pathway.	0		
			Q11BF4	2	ALL sp (Protein inferred from	-	-	PPP pathway			

	Query		Result (Blast)									
Spot Id	Gene from Asaia SF 2.1	Query asaia (from MS)	Accession (Reviewd)	annotation score (S)	Gene	Species	Identity (%)	Pathway	E-Value	Hit Score -12	Manual spot Observation
2001 (6%)	Putative (Aconitate hydratase)	A0A060QFC8								7	6% Less expressed
			P37032	6	Exp. evi. at protein level	Legionella pneumophila subsp	63.30%	TCA-Pathway, propanoate degradationPathway(which is part of Organic acid metabolism)	0		
			Q93F6	4	Protein inferred from homology	*Pseudomonas aeruginosa*	80.40%	TCA and propanoate degradationPath	0		
			P70920	6	Exp. evi. at transcript level	*Bradyrhizobium diazoefficiens*	61%	TCA and propanoate degradationPath	0		
2405 (6%)	Putative (Gamma-glutamyltranspe	A0A060QED3								4	6% over expressed
			P39287	4	Exp. evi. at protein level	*Pseudomonas sp. (strain A14)*	53.1%	Glutathione metabolismPathway:, (The protein is involved in the pathway glutathione metabolism, which is part of Sulfur metabolism)	0		
2418 (6%)	Putative (Uroporphyrinogen decar	A0A060QJ51								3	6% over expressed
			P32620	3	Exp. Evi. at protein level	*Rhodobacter sphaeroides*	46.30%	Pathway: protoporphyrin-IX biosynthesis	0		
3574 (6%)	Putative (Biotin carboxylase of acc	A0A060QKC4								3	6% less expressed
			P43873	3	Exp. Evi. at protein level	Haemophilus influenzae	67.7%	Pathway: peptidoglycan biosynthesis I			
2418 (6%)	UDP-N-acetylglucosamine 1-carbox	A0A060QJT0		2	Protein inferred from homology	All species	-	BioCyc collection of pathway:	-	3	6% over expressed
1887 (20%)	Putative (Elongation factor)	A0A060CLU2								7	20% Over expressed
			Q9SI75	6	Exp. evid. at protein level	*Arabidopsis thaliana*	57.50%	Pathway: polypeptide chain elongation.	0		
1984 (6%)	Putative (Uncharacterized prote	A0A060QL42								5	6% over expressed
			B3EM80	4	Deoxyribonuclease CdiA-c 1.1	Citrobacter rodentium (strain ICC168)	25.30%	BioCyc collection of pathway:	0		
2064 (6%)	Putative (Uncharacterized protein	A0A060QB39								7	6% Over expressed
			Q8XD84	4	Adhesin/invasin TibA autotransporter (Exp. evidence at protein level)	Escherichia coli O157:H7 (strain EC988)	26.7%	Toxic component of a toxin-immunity protein module, which functions as a cellular contact-dependent growth inhibition (CDI) system.	0		
			Q03155	6	AIDA-I autotransporter (Experin	*Escherichia coli O78:H11*	29.50%	BioCyc collection of pathway:	0		
			D2TV89	2	Probable autotransporter ROD_p	*Escherichia coli*	27.60%	Cell adhesion, Virulence	0		
1975 (6%)	Carboxypeptidase-related protein	A0A060QJY9	-		(Protein inferred from homology	All species	-	Pathway: selenocysteinyl-tRNA(Sec) biosynthesis		3	6% Over expressed
2138 (20%)	Serine--tRNA ligase	A0A060QFN7	-	-	(Protein inferred from homology)	All species	-	Pathway: selenocysteinyl-tRNA(Sec) biosynthesis		3	20% Over expressed
2405 (6%)	Various polyols ABC transporter, peripl	A0A060CIV3	-	-	-	-	-	-		2	6% over expressed
			Gene ontology (GO) annotation analysis showed interesting putative candidates after MS results.								

Conclusion

Successfully optimized the spot detection parameters match and intra class report were analysed from both conditions (6% and 20% oxygen). Protein expressions between the two groups were analysed by statistical data analysis and visual comparison. 12 spots were selected and picked from different replicates (6% and 20% oxygen) of 2DE gel for further MS analysis. MS identified different proteins from both conditions are involved in several cellular function. Among all proteins, majority of the proteins were over expressed in hypoxia (6% oxygen) conditions compared to the normal (20% oxygen) condition. Overall data obtained from the comparative proteomic approach indicate that adaptation in the hypoxia environment might affect important sugar-related pathways (pentose phosphate pathway and TCA cycle). In particular, 6-phosphogluconate dehydrogenase mainly involved in pentose phosphate pathway for confirmation based on the gene ontology. A common trait of *Asaia* hosts is the fact they feed on sugar-based diets, and thus we can suggest that sugar-related metabolic pathways are particularly active in endosymbiosis bacterium *Asaia* SF 2.1.

Materials and methods

Chemicals

All reagents were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise stated. Hypergrade for LC-MS Water and Acetonitrile (ACN), and formic acid (FA) were purchased from Merck KGaA (Darmstadt, Germany). Mass grade Trypsin was obtained by Promega (Madison, WI, USA).

In gel trypsin digestion

Spots of interest were cut and destained twice with 50% ACN in 25 mM ammonium bicarbonate pH 8 (NH_4HCO_3). After dehydration with 100% ACN samples were reduced with 10 mM DTT in 100 mM NH_4HCO_3 at 56 °C for 45 min and alkylated with 55 mM Iodoacetamide in 100 mM NH_4HCO_3 for 30 min in the dark at r.t.. After complete dehydration with 100% ACN, a solution of 0.01 µg / µL Trypsin in 100 mM NH_4HCO_3 was added, and proteins were digested at 37 °C for 16 h. The reaction was stopped by adding TFA at final concentration of 0.1 %. The supernatants containing tryptic peptides were collected along with peptide fragments extracted from gel slices with first 1 % TFA, then 60% ACN, 0.1% TFA in water, and finally 100 % ACN. Tryptic peptides were then lyophilized and resuspended with 40 µL of 3 % ACN, 0.1 % FA before High-resolution Liquid Chromatography - ElectroSpray Ionization-tandem Mass spectrometry (HPLC-ESI-MS/MS) analysis.

Gel image analysis using Image Master 2D Platinum 6.0

The comparative image analysis of the protein spots from both conditions (hypoxia and normal) were performed by using Image Master 6.0 2-D platinum (GE Healthcare) software. The software program were useful to detect the quantification changes in protein expression profile between same species in different conditions. Although, visual analysis is necessary to understand the quantitative and qualitative changes of protein. Initially, protein gels were converted into digital data using scanner and further analysed. For proper

evaluation using the image analysis software (Mao et al., 2010), The grey-scale TIFF format image with adequate resolution and 16 bit intensity were obtained using Adobe Image Ready 7.0. The image resolution is part of the parameters that can be adapted in the imaging software. The gel image with proteins spots were evaluated by image analysis software.

Set up and optimization of spot detection parameter

Generally, spots represent the proteins on the gel. Once gels were added to the workspace, the spots were automatically detected by Image master followed by image analysis. The Image analysis steps were as follows: aligning all gel images, computing image fusion, creating match sets, propagating the consensus spot pattern to all gel images for quantification, and verify the statistical analysis of protein expression from matched spots of both groups. Initially, workspace was created and all gels were imported and directly converted into image master format (.mel). The alignment step is performed prior to the spot detection to make sure the resulting spot boundaries identical on all gel images. Initially very small region were focussed on the gel using region tool to setup the spot parameter optimization and based on the setup, the parameters were fine-tuned. Changes in one of the spot detection parameters were immediately reflected in the selected region. Once setup the spot detection parameters (Smooth-1, Min area-11, Saliency-3), the maximum number of proteins were detected, while minimizing the number of artifacts incorrectly detected as spots and especially reducing the number of undetected proteins. This requires finding good compromise between the sensitivity and the specificity of the spot detection algorithm. During optimizing the setup with parameters, automatic spot detection carried out. Importantly the spot detection procedure is initial step for matching process and expression profile extraction. The spot detection and protein expression profiles extraction occurred in a separated and independent step.

Gel matching comparison with a reference gel

After spot detection was over, further created matching steps of each groups. The match is the basic element for searching and investigating protein expression changes across gels, through the use of reports, histograms, and statistical methods. A match represents the relationship between corresponding spots in different gels. Before to start with matching step, clear and round spots were selected as land marks means that relevant information that is separated into label categories in both groups of reference gel. Therefore best quality of spots were completely present in reference gel as always preferable. During matching step, all images were aligned and matched by using the common spots present in gels as landmarks, to detect potential differentially expressed proteins. Each group of all replicate gels were compared with reference gel and matched together. Once matching was over from each groups, the gel populations of one group were compared with other groups by through the use of intra-class report in order to investigate protein expression changes across gels. Finally, in order to pick the spots for mass spectrometer (MS) analysis, data analysis were carried out using student t-test, where $P < 0.05$ was considered as significant threshold. The selected spots were cut precisely and placed in micro centrifuge tubes, and subjected to in-gel trypsin digestion followed by mass spectrometer analysis for the identification of proteins with the expression altered by the “interaction” condition

Mass spectrometry analysis

HPLC-ESI-MS/MS experiments were performed on an Dionex UltiMate 3000 RSLC nanoSystem (Thermo Fisher Scientific, Waltham, MA, USA) directly coupled to a Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer (Thermo Fisher Scientific) with ESI source. Reverse-phase chromatography was performed on a Zorbax 300SB-C18 (3.5 µm particle diameter; column dimension 150 x 1.0 mm) (Agilent Technologies, Santa Clara, CA, USA) a with injection volume set to 20 µL, at a flow rate of 80 µL/min, using an aqueous solution of 0.1% FA as eluent A and ACN/water (80:20, v/v) with 0.1 % FA as

eluent B, and with chromatographic conditions as follows: linear gradient from 5 to 70% B in 40 min and total LC-run of 65 min. Mass spectra were collected by the mass spectrometer (resolution of 60000) operating in positive ion mode and data dependent scan mode in which each full mass spectrometric scan (mass range 200-2000 m/z) was followed by data dependent fragmentation experiments (MS/MS) of the five most abundant multiple-charged precursor ions via collision-induced dissociation (CID). Protein identifications were obtained with the embedded ion accounting algorithm (Sequest HT) of the software Proteome Discoverer (version 1.4, Thermo Fisher Scientific) after searching a UniProtKB/TrEMBL Protein Knowledgebase (release 2015_09 of 16-Sept-15; taxonomical restriction*: Asaia platycodi* SF2.1, 3273 sequence entries). The search parameters were 5 ppm tolerance for precursor ions and 0.02 Da for product ions, one missed cleavage, carbamydomethylation of cysteine as fixed modification, oxidation of methionine as variable modification, minimum two peptides matched per protein, minimum protein score of 40, and false discovery rate under 0.01 (i.e.: the expected fraction of incorrect peptide spectrum match in the entire data set is less than 1%, calculated on a decoy database).

References

Eckerskorn, C., Strupat,k., Schleuder,D., Hochstrasser,D., Sanchez, J.c., Lottspeich, F., Hillenkamp, F., (1997). Analysis of Proteins by Direct-Scanning Infrared MALDI Mass Spectrometry after 2D-PAGE Separation and Electroblotting. Analytical Chemistry, Vol. 69, 2888-2892.

Garcia., D.S., Latorre., A., Moya., A., Gibbs., G., Hartung., V., Dettner., K., Stefan.,M.K., Silva., J .F. (2014). Small but Powerful, the Primary Endosymbiont of Moss Bugs, Candidatus Evansia muelleri, Holds a Reduced Genome with Large Biosynthetic Capabilities. Genome Biol. Evol. 6(7):1875–1893.

Mao,S., Yuanming Luo,Y., Zhang., T., Li, J.,Guanhui Chen,Z.,⊥ Zhang, Y., Yin Li,Yanhe Ma., (2010). Proteome Reference Map and Comparative Proteomic Analysis between a Wild Type Clostridium acetobutylicum DSM 1731 and its Mutant with Enhanced Butanol Tolerance and Butanol Yield. Journal of Proteome Research Vol. 9, *3046–3061*

Mao,S., Yuanming Luo,Y., Zhang., T., Li, J.,Guanhui Natale., M., Maresca,B., Abrescia, p., Bucci, E.M., (2011). Image analysis workflow for 2D-GE based on Image J. Proteomics Insights: 4 37-49.

Manso, M.A., Leonil,J., Jan, G., Gagnaire.V., (2005). Application of proteomics to the characterisation of milk and dairy products. International Dairy Journal 15, 845–855.

Nawin C. Mishra, Günter Blobel, 2000. Introduction to Proteomics: Principles and Applications. ISBN: 978-0-471-75402-2

Natale., M., Maresca,B., Abrescia, p., Bucci, E.M.,(2011). Image analysis workflow for 2D-GE based on ImageJ. Proteomics Insights:4 37-49.

Pandey A, Mann M. (2000). *Nature* 405:837– 46

Rabilloud, T., Lelong, C., (2011). Two-dimensional gel electrophoresis in proteomics: A tutorial. Jounal of proteomics 74, 1829 – 1841

Rauch B., Pahlke, J., Deppenmeier, U (2010). Characterization of enzymes involved in the central metabolism of *Gluconobacter oxydans.* Vol 88, pp 711-718

Steen, H, Mann. M. (2004). The ABC's (and XYZ's) of peptide sequencing. Nature Reviews Molecular Cell Biology, 5:699-711

Sadygov, R.G. Cociorva, D. Yates, J.R. 2004. Large-scale database searching using tandem mass spectra: Looking up the answer in the back of the book. Nature Methods, 1(3):195, 202

Thompson SN. 1999. Blood sugar formation from dietary carbohydrate is facilitated by the pentose phosphate pathway in an insect Manduca sexta Linnaeus. Biochim Biophys Acta. ;1472(3):565-75.

Wilke A, Rückert C, Bartels D, Dondrup M, Goesmann A, Hüser AT, Kespohl S, Linke B, Mahne M, McHardy A, Pühler A, Meyer F. Bioinformatics support for high-throughput proteomics. 2003. J Biotechnol. 19;106 (2-3):147-56.

Zoran Minic., (2015). Proteomic Studies of the Effects of Different Stress Conditions on Central Carbon Metabolism in Microorganisms. J. Proteomics Bioinformatics2015,8:5

Acknowledgement

I wish to sincerely thank Dr. Fabio Forlani and Professor Daniele Daffonchio for welcoming me in their lab and supervising my PhD work; Dr. Elena Crotti for teaching me the wonderful art of working in a microbiology lab. I recall with gratitude Dr. Alberto Vitali from Institute of Chemistry of Molecular Recognition-UOS Rome, CNR, Roma, Italy and Dr. Valeria Marzano from Institute of Biochemistry and Clinical Biochemistry, Catholic University of Rome, Roma, Italy, for their scientific collaboration with us. All the lab members from Microbiology and Biochemistry, Aristodemo Carpen, Stefano De Benedetti, Mauro Marengo, Jessica Capraro, Cristina Catto and Microbiology lab Violetta Vacchini, Emanuela Lombardi, Matteo Callegari, Erica Prosdocimi, Francesca Mapelli, Maurizio, Lorenzo Vergani, Marta barbato, Zahraa Zeaiter, Michela Gambino, not only for scientific discussion, but also for congeniality and sincere friendship. I also thank all the friends which shared these years with me. Last but not least, I wish to thank my family for the support they provided during all these years. In particular, I cannot help but thanking my friend David for his invaluable help throughout my doctoral studies. Thanks to *Asaia*, the ongoing journey with you is awesome!