Insights on fungal solid-state fermentation for waste valorization: conidia and chitinase production in different reactor configurations.

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Abstract

Several reactor configurations are used to produce fungal biopesticides by solid-state fermentation. Agro-industrial residues of different biodegradability can be used to produce fungal conidia. This work presents a comparison between packed-bed and tray reactor configurations to produce *Beauveria bassiana* (BB) and *Trichoderma harzianum* (TH) conidia using rice husk or beer draff as substrates complemented with wood chips, focusing on the feasibility of using substrates with different biodegradability. Conidia production, mean temperature and respiration indexes have been analysed with all presented reactor configurations. Higher conidia productions were obtained for both strains when using beer draff and wood chips mixture as substrate. Higher conidia production was coupled with higher respiration indexes, however, no significant differences in mean temperature were shown between any of the studied reactors. Chitinase activity was also analysed in beer draff tray bioreactors, obtaining similar chitinase production profiles with both fungal strains and higher values using TH. Maximum chitinase production was achieved between 2-3 days after maximum conidia production depending on the strain. As a result of our work, successful scaling of both packed bed and tray configurations using beer draff and wood chips to produce BB or TH conidia would be advisable. More in-depth studies should be performed in order to find an optimal conidia production time which maximizes both conidia production and chitinase activity, as it would maximize the biopesticide effect of the final product.

Keywords: fungal solid-state fermentation, agro-industrial residues, substrate biodegradability, reactor configurations, chitinase production

1. Introduction

The traditional use of chemical pesticides for pest management has led to numerous problems as they are harmful both for human health (due to their toxicity and mutagenic capabilities) and the environment (due to their toxicological effect). Biocontrol agents are considered an environmentally friendly alternative due to their harmless nature, being a promising replacement due to their effectiveness on more than 1000 species while presenting no harm to humans or to the ecosystem [1-3].

Among biocontrol agents, fungal biopesticides represent one of the most interesting options. Solid state fermentation (SSF) (defined as a process that occurs in the absence or near absence of free water) remains as the most used production method as it is the only way to obtain aerial conidia, the most infective fungal propagule [2,5]. In addition, lower costs mainly related to the use of agro-industrial wastes as substrates (serving both as nutrient and support for fungi) also make SSF preferred over SmF [6]. Obtained products present an added value due to residue valorization [7]. A complete list of tested substrates was presented by Sala et al. [4]. Among all used strains, *Beauveria* spp. has become one of the most-studied and used fungal entomopathogens worldwide due to its pathogenicity on more than 700 host species [2]. On the other hand, *Trichoderma* spp. is also a widely recognised biocontrol agent due to its antagonistic properties, being especially effective against soil-borne diseases [8,9].

Different reactor configurations have been used to produce fungal biocontrol agents by SSF. Packed bed and tray reactors are some of the most studied designs, presenting different advantages and drawbacks. Packed bed reactors facilitate oxygen availability via continuous forced aeration, as well as

maintaining constant moisture when supplying saturated air. Their main disadvantage lies in heat removal, causing important difficulties in process scale-up. Tray reactors have been traditionally used for SSF conidia production due to their simplicity. They can also allow aeration specially when the bottom of the trays is perforated, and moisture control when placed in closed chambers. Bed height is often between 5 and 15 cm. However, tray reactors also present mass transfer and heat limitations, leading to internal temperature gradients and large gas concentrations with substrate depth more than 40 mm [10]. Substrates with high potential biodegradability are the ones which can cause more difficulties in terms of heat removal. Use of high porosity substrates to allow maximum heat transfer is a possible path to reduce heat removal effects on fungal growth and conidiation [11,12]. In addition, starchy substrates (which are preferred for fungal growth) combine adequate porosity levels with moderate biodegradability [13].

Mycoparasitism, which is closely related to fungal biocontrol agents' insecticidal activity, depends on the presence of various enzymes. Conidia formation occurs without overall protein synthesis, but with the formation of wall. Consequently, chitin content at the end of growth is considered as a good indicator of conidia formation in fungi [14]. Chitinases partially degrade various insects, nematodes or fungi cell wall [15], presenting high relevance in pests' control due to their high abundance in insects, arthropods and fungi [16]. Recent attention has been given to the capacity presented by fungal biocontrol agents of producing chitinase and other hydrolytic enzymes. In the case of *Trichoderma*, chitinase has been stated as the enzyme responsible of its biocontrol capabilities [17]. Despite fungal growth and enzyme production close relation with fermentation conditions, there is still a lack of studies on both of them [18], specially related to SSF systems.

The aims of this work are: i) to compare the suitability of two substrates with different biodegradability (rice husk and beer draff) for fungal conidia production by SSF using both *Beauveria bassiana* (BB) or *Trichoderma harzianum* (TH) as inoculum. ii) to compare and assess the feasibility of packed-bed and tray reactor configurations for fungal conidia production. iii) to provide a first approach on conidia and chitinase simultaneous production.

2. Materials and methods

2.1. *Fungal strains*

Tests were carried out using two different strains, *Beauveria bassiana* (BB) (CECT 20374) and *Trichoderma harzianum* (TH) (CECT 2929). The original strain was preserved at -80ºC in sterile cryovials containing 10% glycerol. Fungal strains were cultured in potato dextrose agar (PDA) (BB) or in malt extract agar (MEA) (TH) at 30ºC for 6-8 days before use.

2.2. *Raw materials*

Rice husk (Husk Ventures S.L., Barcelona) and beer draff (Cervesa del Montseny S.L., Sant Miquel de Balenyà) were used as substrates for fungal conidia production. Rice husk was stored at room temperature (20 – 25ºC) and beer draff was stored frozen. Moisture was adjusted (50-55%) before inoculation by adding the necessary volume of water when using rice husk and the necessary quantity of wood chips (Acalora, Ivars d'Urgell) when using beer draff: 70 beer draff/30 wood chips (w/w) when working with 1.5 L volume reactors and 40 beer draff/60 wood chips (w/w) when working at 22L, according to previous works with the substrate. Wood chips added to beer draff ensured a proper air filled porosity

(AFP_R, 70-80%) in beer draff fermentations while providing area for fungal growth and conidiation [11]. Raw materials characterization for all substrates used in all presented fermentations (expressed as mean values for all supplies of the same substrate) is presented in Table 1. All substrates were autoclaved (121ºC for 30 min) prior to inoculation.

2.3. *Solid-state fermentation*

Four different reactor configurations (1.5L packed bed, 22L packed bed, 2 tray and 3 tray) were used. 300 g of non-inoculated material were charged in 1.5L reactors, 3000 g of rice husk or 4000 g of beer draff mixture with wood chips were charged in 22L reactors and a total of 900 g (450 g per tray) of rice husk and a total of 2250 g (750 g per tray) were charged in tray bioreactors. Inoculation was always performed in laminar flow chamber. Conidia production time and initial fermentation conditions were previously determined by Sala et al. [19]. Previous to all tests, reactors / trays were cleaned with water and bleach to prevent possible contamination, as they could not be autoclaved. Temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) were used to obtain accurate temperature profiles at different reactor positions depending on the reactor configuration. In all reactor configurations constant aeration was continuously provided by means of a mass flowmeter (Mass-Stream D-6311, Bronkhorst, NL). The oxygen percentage in the output gases was measured by an electrochemical O_2-A_2 oxygen sensor (Alphasense, UK). Data analysis was performed by a non-commercial tailor-made software Arduino® based that calculates the respiration rates. Specific oxygen uptake rate (sOUR) was calculated according to Puyuelo et al. [20], expressed as 1h average value (sOUR) and recorded on-line in order to provide an indicator of the biological activity.

2.4. *Conidia counting*

To determine fungal conidia, a Neubauer chamber (Brand™ 717805) was used. 10 g of sample (conidiated substrate) were mixed with 50 mL of Tween 80 0.1%, shaken for 20 minutes at 150 rpm and appropriately diluted before counting. All cell counts were performed per triplicate and related to the dry matter present in the reactor at the counting time, following Equation 1:

$$\text{Concentration} = \frac{\text{Nº of conidia}}{\text{CV} \cdot \text{DF}} \cdot \frac{EV}{SWW} \cdot \frac{SWW}{SDM} \quad (1)$$

Where: Concentration is the conidia concentration in the initial tube (conidia g^{-1}dm); nº of conidia, the counted conidia in the Neubauer chamber at a known dilution; CV, Neubauer chamber counting volume (mL); DF, dilution factor of the counting tube; EV, extraction volume (mL); SWW, sample wet weight (g ww); SDM, sample dry matter (g dm).

2.5. *Chitinase activity assay*

2.5.1. Reagent preparation

Reagent (colloidal chitin) and DNS preparations were performed according to Berna et al. [21] and Miller [22]. Phosphate buffer was prepared by adding 3.0 g NaPO2 to 400 mL distilled water in agitation. When dissolved, pH was measured and adjusted to 6.0 by adding NaOH. Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 mL sulphuric acid 95% at close to 0ºC temperature.

2.5.2. Activity determination

To perform chitinase activity assay, 10 g of sample were incubated in 30mL phosphate buffer pH 6.0 at ambient temperature for 2.5 h without agitation. 50 mL of the liquid extracted were mixed with 450 ml

phosphate buffer 50 mM and 500 mg colloidal chitin 1% w/v. Sample was incubated for 30 min at 37ºC, 750 mL DNS were added, incubated for 10 min at 100ºC and centrifuged.

Supernatant absorbance was measured at 540 nm. Using Equation 2, enzymatic concentration is estimated using a previously prepared calibration curve.

$$Chitinase\ activity\ \left(\frac{U}{gdm}\right) = \frac{(m\Delta ABS + h) \cdot DF \cdot B}{gdm} \quad (2)$$

Where: m: calibration curve slope; Δabs: sample Absorbance – controls Absorbance; DF: extract dilution factor; B: total extract volume; gdm: grams of dry matter of the initial sample.

Controls' absorbances have to be diminished from sample absorbance. In extract control, colloidal chitin is replaced with phosphate buffer. In chitin control, liquid extract is replaced with phosphate buffer. In the blank, all the process is performed using phosphate buffer.

2.6. *Total sugar content analysis*

Total sugar content was estimated using the Anthrone method [23]. Total glucose content was expressed as gram of glucose equivalent per gram of dry matter according to Equation 3:

$$Total\ sugar\ content = \frac{C}{P} \cdot V \quad (3)$$

Where: Total sugar content (g g^{-1}dm); C, concentration of glucose equivalents (g L^{-1}); P, weight of the dry sample (g); V, total volume of the supernatant (L).

2.7 Analytical methods

Moisture (%), dry matter (%), organic matter (%) and pH have been determined for initial and final samples using standardized methods [24]. C/N analysis was performed by means of chemical elemental analysis. AFP_R was calculated according to Equation 5 as presented by Richard et al. [25]:

$$AFP_R = 1 - BD_t \left(\left(\frac{1 - DM}{D_W}\right) + \frac{DM * OM}{PD_{OM}} + \left(\frac{DM(1 - OM)}{PD_{ash}}\right)\right) \quad (4)$$

Where: AFP_R, air-filled porosity (%); BD_t, total bulk density on a wet basis (kg m^{-3}); dm, dry matter on a wet basis (%); OM, organic matter on a dry basis (%); D_w, water density (1000 kg m^{-3}); PD_{OM}, organic fraction particle density (1600 kg m^{-3}) and PD_{ash}, ash particle density (2500 kg m^{-3}).

2.8 Statistical analysis

One-way ANOVA ($p < 0.05$ confidence) with the Tukey test was used to compare conidia production between different reactor configurations.

3. Results and Discussion

3.1. *Fungal conidia production using different reactor configurations*

Conidia production, mean temperature and maximum sOUR obtained in 1.5L packed bed, 22L packed bed and tray bioreactors are shown in Figure 1. All possible fermentations using BB or TH and rice husk or beer draff are shown, with the only exception of BB rice husk in tray bioreactor.

When comparing between substrates, conidia productions on rice husk were always lower than those on beer draff using the same reactor configuration and fungal strain. This behaviour was coupled with much lower sOUR values presented by rice husk in comparison to beer draff in packed bed reactors, where rice husk never surpassed 0.75 gO_2 kg^{-1}dm at both tested scales while sOUR beer draff varied between 2.70 to a maximum of 4.45 gO_2 kg^{-1}dm in tray reactor. As sOUR is related to substrate

biodegradability, rice husk and beer draff can be considered as different biodegradability substrates [11]. As shown in Table 2, parameters at the start of the fermentation were similar between substrates (with the only exception of AFP_R in beer draff fermentations), meaning differences in conidia production might have been due to differences in the intrinsic substrate properties just as biodegradability. Fungal growth and conidiation using bulking agents as sole substrate have been previously demonstrated [12,19]. Consequently, the use of both a moderately biodegradable substrate (beer draft) and a bulking agent should be preferable due to them being complementary: beer draff provides carbon and nitrogen while bulking agent provides growth and conidiation area. Other works on fungal growth using bulking agents also supplemented with a nutrient solution in order to obtain higher growth and sporulation can be found [12,27].

Comparing between strains, BB and TH present different behaviours. No significant differences were observed between reactor configurations in terms of conidia production with both of the tested strains. However, and as shown in Figure 1, maximum mean conidia production achieved using BB and beer draff was obtained in 22L reactor fermentations, while both tray and 1.5L fermentations obtained more similar values. This result is remarkable, as most of reported BB aerial conidia production is not performed using packed-bed bioreactors as fermenters but by superficial production, which ranges from polypropylene bags and tray bioreactors to environmentally-prepared chambers for fungal growth and conidiation [28]. The use of a different substrate mixture between 22L beer draff bioreactors and the rest of the tested conformations (resulting in significant differences in AFP_R values, as presented in Table 2) for both fungi could possibly have affected conidia production, improving 22L packed-bed performance in comparison to 1.5L or tray performances. This might be highly relevant, considering the importance of AFP_R when working with organic wastes [29]. In contrast, TH conidia production was significantly similar between different reactor configurations when looking at the same substrate. In comparison, TH conidia production has overall been superior to BB's. This behaviour might be attributed to the superior enzymatic production capabilities of TH [8] in comparison to BB, which is mostly related to its entomopathogenic properties [2]. The genera *Trichoderma* has been previously used to produce several enzymes, most of them being lignocellulosic enzymes such as cellulases, xylanases and endoglucanases [30-33], whereas there are no reports on the use of BB for the production of similar enzymes.

Achieved mean temperature were similar between most of the reactors, presenting higher deviations in tray bioreactor than in packed bed and in beer draff than in rice husk. Taking into account the bed height of the 22L packed bed reactor in comparison to one tray bed's height, obtaining similar mean temperatures in both designs with both strains opens scaling-up possibilities for packed-beds, while suggesting higher bed heights should also be tested for tray configurations, as demonstrated by several authors [34-36].

Globally, our results suggest a relation between conidia production and substrate biodegradability, as higher productions were obtained in beer draff reactors for both strains. In addition, no correlation between conidia production and mean temperature has been observed, as no significant differences in mean temperatures were found between different reactor configurations.

3.2. Chitinase production analysis

Figure 2 shows chitinase production in both beer draff tray fermentations, figure 2a) shows BB fermentation results and figure 2b) shows TH fermentation results. Analyses were conducted separately for each tray. Similar chitinase activity profiles were achieved using both strains, with only differences observed in the time of maximum chitinase production, which was located at 9 days for BB and at 8 days for TH. Activities were not significantly different between trays in most of the analysed samples. Highest values were achieved by TH in trays 1 and 2 (465-510 U g^{-1}dm), while maximum BB values in all trays were located near 300 U g^{-1}dm. In both cases, maximum chitinase activity was achieved some days after achieving maximum conidia production (more than 1 day for BB and more than 2 days for TH), suggesting maximum chitinase production is achieved after maximum conidiation, as suggested by Desfarges et al. [14], implying a link between chitinase production, conidia production and decrease in metabolic activity, as shown with both sOUR and temperature profiles. TH profiles were similar to the ones obtained by Sandhya et al. [37] using SmF. Airflow role in chitinase production varied between strains: while it seemed independent due to not presenting significant differences between trays in BB, it was different in TH at the point of maximum production, where the lowest chitinase concentration was achieved in the tray located further from the air inlet (tray 3), being significant in comparison to the rest. Airflow influence in comparison to non-aerated cultures when producing chitinases had been previously demonstrated by other authors using various *Trichoderma* strains [38]. Further research should be performed in order to improve chitinase values while at the same time maximizing conidia production.

4. Conclusions

Successful conidia production has been achieved using BB or TH in both packed bed and tray reactors. Higher productions with both strains were obtained when fermenting beer draff complemented with wood chips, which yielded higher conidia production in comparison to rice husk due to the use of a substrate mixture while also obtaining higher respiration profiles. While in the case of BB significant differences in terms of conidia production were shown between 22L packed bed reactor and the other reactor configurations, these differences were not observed when working with TH, suggesting TH as a most versatile strain in SSF than BB. No significant differences in terms of mean temperature were observed between all performed fermentations, indicating an overall correct heat transfer even when working with 22L packed bed reactor. Chitinase analysis in tray bioreactors revealed different optimal production times for conidia and for chitinase production. Activity profiles were similar between strains, with higher values using TH.

Future work should focus on scaling-up and improving packed-bed and tray reactors with both substrates, but specially with beer draff. Scale up to pilot scale can be considered for packed bed reactors while the use of more trays has to be studied for tray reactors as well as enhancing bed height, as packed-bed reactors have demonstrated the feasibility of using larger bed heights to produce fungal conidia with both tested strains. Additionally, more analyses on chitinase production should be performed in order to find the most optimal production time in terms of both conidia and chitinase activity, especially in packed-bed conformation.

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Figure captions

Fig. 1. Conidia production (bars), mean temperature (dots) and maximum sOUR (triangles) obtained in all fermentations. Conidia production for packed bed reactors corresponds to maximum conidia production time. In tray fermentations, conidia values correspond to mean values obtained from all trays at maximum conidia production time. Standard deviations are shown for conidia production and mean temperature and statistical analysis are shown for conidia production with same strains and substrates

Fig. 2. Conidia production, mean temperature, sOUR and chitinase production profiles in beer draff tray bioreactors. a) *Beauveria bassiana* and b) *Trichoderma harzianum*

Figure 1

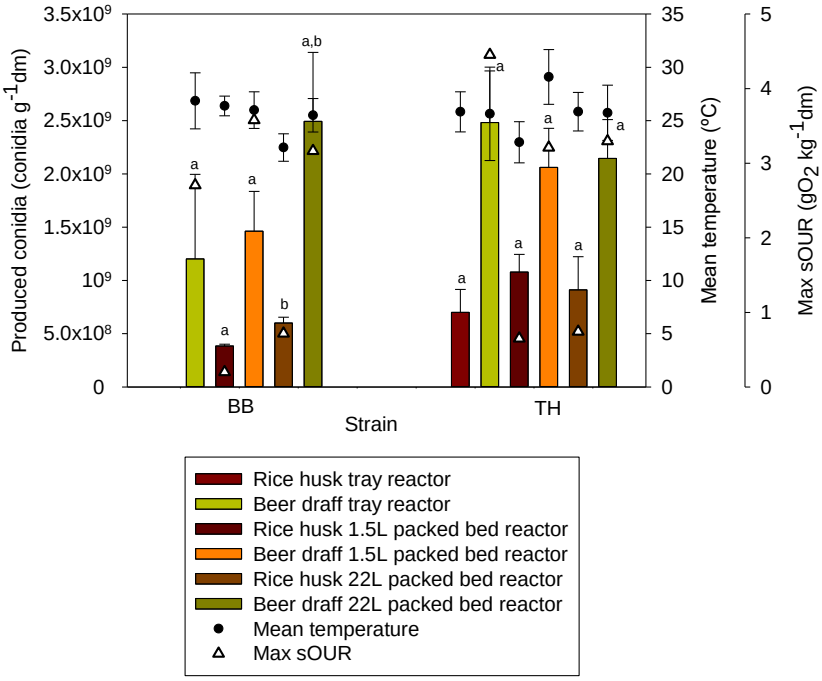
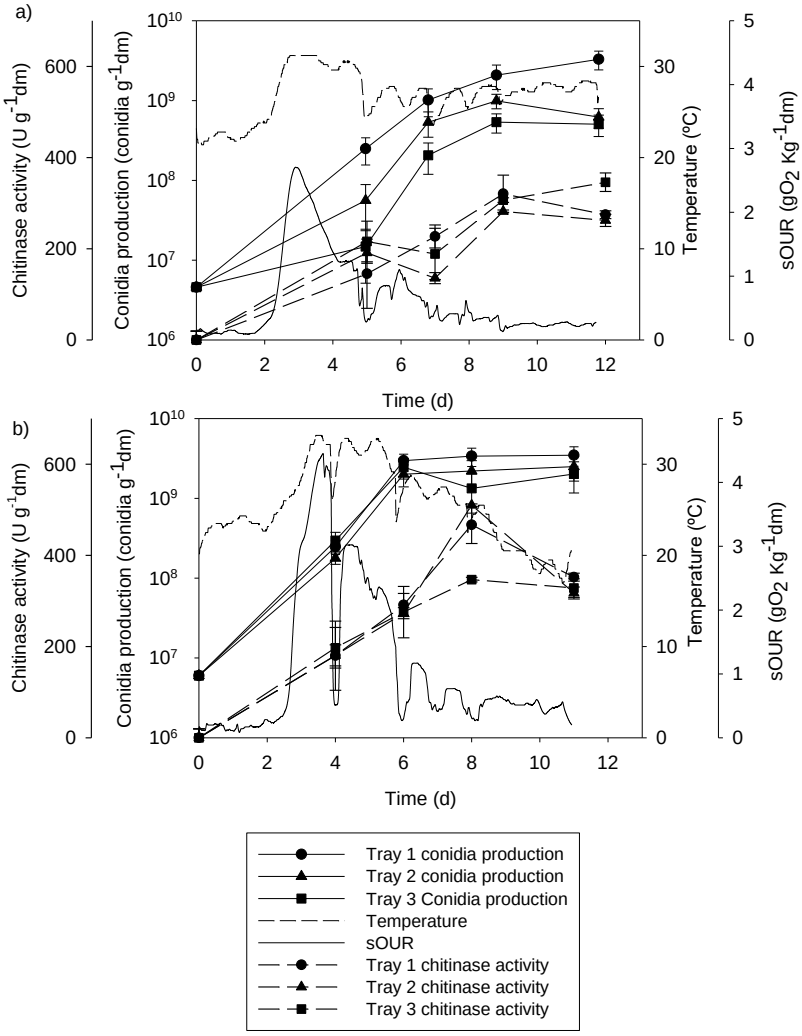


Figure 2



Tables.

Table 1: Characterization of raw residues used in the different SSF tests (rice husk, beer draff and wood chips). Presented values correspond to mean values of 3 batches used for each substrate.

Parameter	Rice husk	Beer draff	Wood chips
Moisture (%)	10.3 ± 0.5	79.7 ± 3.7	9.7 ± 0.3
Organic matter (%)	82.6 ± 4.1	95.9 ± 1.4	99.1 ± 0.7
pH	5.9 ± 0.3	6.0 ± 0.4	5.1 ± 0.2
Carbon (%)	40.3 ± 0.3	48.7 ± 0.5	-
Nitrogen (%)	0.4 ± 0.1	4.6 ± 1.2	-
C/N ratio	94.0 ± 17.9	10.6 ± 2.5	-
Total sugar content (mg g^{-1}dm)	17.7 ± 0.4	122.7 ± 8.3	13.1 ± 0.4
AFP_R (%)	89.7 ± 0.7	63.8 ± 2.5	95.3 ± 0.5

Values are the average of independent samples and its standard deviation

Table 2: Initial substrate characterization for all presented fermentations.

Fermentation/Parameter	**Moisture (%)**	**pH**	**Total sugar content (mg g^{-1}dm)**	**Air filled porosity (%)**	**Mixture (substrate/wood chips) (%/%)**
1.5L rice husk	64.2±1.0	6.1±0.1	12.4±0.8	83.8±0.4	100/0
22L rice husk	58.7±0.3	6.6±0.1	13.6±0.8	86.2±0.4	100/0
Tray rice husk	58.5±0.1	6.8±0.1	13.9±0.3	87.4±0.6	100/0
1.5L beer draff	63.8±5.1	5.7±0.2	91.1±7.3	69.6±0.7	70/30
22L beer draff	55.2±4.8	5.1±0.8	67.9±5.3	80.1±0.9	40/60
Tray beer draff	63.4 ± 3.6	4.9±0.3	103.5 ± 13.5	78.9 ± 2.5	70/30